ECOLOGY, EVOLUTION, AND BEHAVIOR OF VIVIPAROUS FISHES

EDITED BY: J. Jaime Zúñiga-Vega, Bart Pollux, Jerald B. Johnson and Andrea S. Aspbury

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ECOLOGY, EVOLUTION, AND BEHAVIOR OF VIVIPAROUS FISHES

Topic Editors:
J. Jaime Zúñiga-Vega, National Autonomous University of Mexico, Mexico
Bart Pollux, Wageningen University and Research, Netherlands
Jerald B. Johnson, Brigham Young University, United States
Andrea S. Aspbury, Texas State University, United States

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Editorial: Ecology, Evolution, and Behavior of Viviparous Fishes

J. Jaime Zúñiga-Vega[1]*, *Andrea S. Aspbury*[2], *Jerald B. Johnson*[3] *and Bart J. A. Pollux*[4]

[1] Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de Mexico, Ciudad de Mexico, Mexico, [2] Population and Conservation Biology Group, Department of Biology, Texas State University, San Marcos, TX, United States, [3] Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT, United States, [4] Experimental Zoology Group, Department of Animal Sciences, Wageningen University, Wageningen, Netherlands

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Editorial on the Research Topic

Ecology, Evolution, and Behavior of Viviparous Fishes

Edited and reviewed by:
Elise Huchard,
UMR5554 Institut des Sciences de l'Evolution de Montpellier (ISEM), France

****Correspondence:***
J. Jaime Zúñiga-Vega
jzuniga@ciencias.unam.mx

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INTRODUCTION

Few organisms have contributed more to our understanding of ecology, evolution, and behavior than freshwater viviparous fishes (Meffe and Snelson, 1989; Uribe and Grier, 2005, 2010; Evans et al., 2011). These encompass freshwater species from several families, including Poeciliidae, Goodeidae, Anablepidae, and Zenarchopteridae. The poeciliids, a clade of almost 300 species (at last count), have been particularly important as research targets; and there is growing interest in other groups, including the zenarchopterids, the freshwater viviparous halfbeaks (Farhana et al., 2018). The former are primarily distributed throughout the Americas and the Caribbean, occurring in a variety of selective environments and ecological conditions, with the latter found in tropical Asia and New Guinea. Species in both groups tend to be integral to the ecological communities that they occupy. They are also strikingly important as models for understanding both micro- and macro-evolutionary processes. Indeed, they have proven to be critical to our understanding of pre- and post-copulatory sexual selection, complex social behavior, learning and cognition, the origin of unisexual species, genetic variation in natural populations, assembly of freshwater communities, and even the formation of tumors (Vrijenhoek, 1994; Evans and Pilastro, 2011; Krause et al., 2011; Rios-Cardenas and Morris, 2011; Lu et al., 2018). Further, their unique form of viviparous reproduction has made them the target of numerous studies focused on the anatomy and physiology related to internal fertilization and live birth (Uribe and Grier, 2005, 2010). At last count, almost 9,000 manuscripts have been published on just the poeciliid livebearing fishes.

One of the strengths of studying livebearing fishes is that they exhibit such a variety of phenotypic adaptations. This is true of evolutionary diversification among species, but is also prominently seen in evolutionary divergence within species. This is due in part to their ability to occupy such a wide range of habitats (Johnson and Bagley, 2011). Although most livebearing fishes are small stream specialists, they are known to tolerate extreme differences in water temperature, salinity, stream flow rate, water chemistry, and a variety of other abiotic factors (Torres-Dowdall et al., 2013; Martínez et al., 2016; Riesch et al., 2016). This range of tolerance makes them remarkably effective at invading new habitats, which has occurred historically with some species introduced globally to control mosquitos far outside their natural range, and some species introduced outside their ranges as a result of the aquarium trade (Maddern et al., 2011; Santi et al., 2020).

With so much already known, it may seem that there is little still to learn from these fishes. Yet, in many ways, we are only now beginning to uncover the potential of these fishes to inform our understanding of several fundamental concepts in biology. Some of this potential is driven by technological advances that allow us to answer questions in new ways. For example, the ability to peer into the genomes of species, or to integrate long-term field studies with controlled laboratory experiments, offer the promise of new insights. Some potential lies in the fact that more work is being done on species that previously have received little or no attention, providing understanding that uniquely comes from comparative work (e.g., Furness et al., 2019, 2021). And some potential lies in knowing some systems so well that we can now begin to integrate knowledge across disciplines that previously were considered independently. Here, we introduce a set of papers published in a Research Topic of *Frontiers in Ecology and Evolution* focused on recent work in viviparous fishes. These articles can be organized into five broad categories: Behavior, Ecology, Life-History Evolution, Morphology, and Sexual Selection. Some articles used invasive species as model systems and, thus, our Research Topic also contributes to our understanding of how viviparity may be related to the invasive potential of some fish species. Further, we provide a general overview of the significant contributions contained in each article and suggest questions for future investigations.

BEHAVIOR

Several articles explore behavior in relation to environmental change. For example, Lukas, Kalinkat et al. examined behavior and invasion success by testing whether dispersing individual guppies (*Poecilia reticulata*) exhibit behavioral types that differ from those remaining in the source population. They found that guppies exhibit a stable behavioral syndrome composed of boldness and activity, even in the presence of strong seasonal changes in abiotic environmental factors, indicating that individuals with high activity levels are also bolder. Their work showed that when individuals occupy unpredictable and fluctuating environments, having consistent behavioral expressions could help guppies successfully invade, and adapt to disturbed habitats. Santiago-Arellano et al. also examined behavior in invasive guppies asking if heterospecifics (both native and other invasive viviparous fish) affect guppies' refuge emergence latency. When guppies were with other guppies, other invaders, and native goodeids, they had a shorter emergence latency than when alone. Latency reduction was highest when with conspecifics or the invader *Poeciliopsis gracilis*. Their work shows that established invaders might provide benefits to new invaders.

In an invasive livebearing poeciliid, *Gambusia affinis*, Miner et al. investigated if they were behaviorally or physiologically tolerant to the environmental perturbation of artificial light at night (ALAN). They found that exposure to ALAN lowered glucose levels in the brain and decreased swimming activity, but did not significantly affect cortisol release rates, reproduction, survival, or growth. They suggest that *G. affinis* may behaviorally adjust to ALAN rather than modulate their endocrine stress response, which might be one mechanism of their tolerance to disturbed habitats. Devigili et al. found that variation in canopy cover and water flow influenced sexual interactions and locomotor behaviors in the pygmy halfbeak *Dermogenys collettei* (family Zenarchopteridae). Their work supports the argument that gathering basic ecological and behavioral information from wild populations can lead to a better understanding of the interaction between natural and sexual selection in viviparous fishes.

Two studies addressed how lateralization or handedness could affect behaviors. Johnson et al. showed that variation in male *Xenophallus umbratilis* gonopodial corkscrew morphology (either left- or right-handed) was linked to decision making behaviors, including behaviors not associated with mating. Interestingly, right and left morph males responded with opposite directional behaviors when presented with the same stimuli. Connelly and Ryan compared visual lateralization in the asexual, gynogenetic Amazon molly (*Poecilia formosa*), and the sexually reproducing, and sexually parasitized Sailfin molly (*P. latipinna*). Because Amazon mollies are hybrids with *P. latipinna* as one parental species, these two species may have similar lateralization. Alternatively, variability or plasticity in this trait might aid in the evolutionary maintenance of the asexual species. In their experiments they failed to detect a significant eye bias in either species, and the degree of variation in visual lateralization was similar between the asexual and sexual species.

ECOLOGY

Lukas, Auer et al. examined how changes in dissolved oxygen that occur throughout the day in a sulfidic spring affect predator-prey interactions. They documented that as dissolved oxygen decreases from morning to afternoon, *Poecilia sulphuraria* increase their rates of aquatic surface respiration, making themselves more vulnerable to bird predators. This interesting study demonstrates that abiotic factors that affect prey species may have strong indirect effects on their predators. Langerhans et al. conducted a thorough study of the consumption of hard-shelled prey (durophagy), which are difficult to digest and hence are a costly type of prey. They found that durophagy is quite common among teleost fishes and is widespread across populations of the viviparous fish *Gambusia holbrooki*. According to their findings, durophagy (in particular molluscivory) is common under conditions of food limitation. This study greatly contributes to our understanding of the benefits of consuming costly prey, the ecological conditions that elicit its occurrence, and the mechanisms by which predators alleviate its costs.

LIFE-HISTORY EVOLUTION

By focusing on an adaptive radiation of *Gambusia hubbsi* inhabiting blue holes in Bahamas, Hulthén et al. disentangled the effects of predation and food availability on the evolution of life-history traits. They observed a complex interaction between these two selective factors shaping fecundity, offspring size, body growth, and age at maturity. One of the greatest strengths of this study is that it dissociates the effects of predation and food availability, which usually covary across natural populations. In contrast, Golden et al. also examined effects of predation intensity on life history diversification in *Alfaro cultratus* and found no differences in size at maturity, fecundity or offspring size between low- and high-predation environments. Apparently, morphological restrictions limit the selective effects of predation because females from this species have a narrow body and do not develop a distended abdomen during pregnancy. This study thus stands out because it offers a different perspective on the potential effects of predators on life-history evolution, which may not always be as strong as observed in other viviparous species.

A simultaneous examination of multiple selective agents potentially causing intraspecific life-history variation in *Poeciliopsis prolifica* was conducted by Roth-Monzón et al. Their results revealed that both population density and interspecific competition are strong selective agents for most life-history traits. This important contribution demonstrates that other ecological factors, in addition to the well-known effect of predation, interact in shaping life-history diversification. Saleh-Subaie et al. examined the Trexler-DeAngelis model (Trexler and DeAngelis, 2003, 2010), which is one of the most prominent hypotheses that attempts to explain the causes and consequences of the evolution of matrotrophy (post-fertilization embryonic nutrition) from the ancestral lecithotrophy (pre-fertilization embryonic nutrition). They observed in five species of the genus *Poeciliopsis* that females with greater degrees of matrotrophy had poorer body condition, which is consistent with one of the predicted consequences of matrotrophy according to the Trexler-DeAngelis model. This finding reveals that post-fertilization embryonic nutrition entails severe energetic costs for females of viviparous fishes.

MORPHOLOGY

Viviparous fishes are excellent models for examining questions about the origin of, and importance of morphological variation. Safian et al. examined how poeciliids could be used as model systems to test hypotheses about fish fin regeneration. They reviewed the current knowledge about the mechanisms behind fin regeneration in fish and noted that the Poeciliidae are a model family for comparative studies of fin regeneration because these fishes live in very different habitat types, have a well-defined phylogeny, and exhibit an incredible amount of variation in morphology and reproductive traits. Langerhans and Rosa-Molinar extended this perspective by investigating the basis of morphological innovation in the gonopodial structure of *Gambusia* fishes. They document changes in the ano-urogenital vertebral region that have facilitated divergence in swimming abilities and body shape between habitats that vary in predation regimes. This study suggests that the structure of this body region, that originally evolved in the sperm transfer context, has been co-opted to alter swimming performance, and therefore whole-organism survival, facilitating phenotypic diversification when predation regimes vary. Zerulla and Stoddard reviewed the general biology of the polymorphic melanic side-spotting patterns formed by macromelanophores, that are present in some species of *Gambusia*, *Limia*, *Phalloceros*, *Poecilia*, and *Xiphophorus*. The side spot patterns formed by macromelanophores are influenced by the presence of dominant genes, acting in a polygenic fashion, that can be either sex-linked or autosomal. Despite the influence of dominant genes, there is often a low frequency of the side-spotting morph in populations, suggesting that there must be selective pressures influencing these traits. However, while side-spotting morphology had wide variation across taxa they did not find that it correlates with sexual selection.

SEXUAL SELECTION

Much of our knowledge of patterns and processes of sexual selection comes from studies of viviparous fishes (Basolo, 1995; Evans and Pilastro, 2011; Rios-Cardenas and Morris, 2011). Several articles in our Research Topic add to this knowledge. Although theory predicts strong species recognition as a first step in mate choice, Magellan and García-Berthou discuss how selection for accurate species recognition may not be as strong in invasive species with coercive mating systems, such as mosquitofish (*Gambusia holbrooki*). The pairing of rapid learning of species identity with an innate predisposition for sex recognition over species recognition, could be a factor in the invasive success of mosquitofish. Dekker et al. examined multiple paternity in *Poecilia gillii* and found that over 70% of reproductive females carry embryos from different fathers. They documented that some broods were sired by up to nine different males and in most cases these males did not contribute with a similar number of offspring, thus revealing interesting reproductive skew. This is a noteworthy contribution to our knowledge of the processes of post-copulatory sexual selection that occur in viviparous fishes. Reznick et al. summarized the consequences that the evolution of matrotrophy from the ancestral lecithotrophy has brought on the evolution of male traits associated with sexual selection. They emphasize that males from species with matrotrophy and placentas are less likely to have elaborate ornaments and courtship behavior, are smaller relative to females, and have longer gonopodia. This association indicates that the evolution of matrotrophy led to a shift from pre-copulatory to post-copulatory sexual selection. They also document an accelerated speciation rate in species that exhibit clear signs of pre-copulatory mate choice.

FUTURE DIRECTIONS

Convergent Evolution

Convergent evolution of similar phenotypes in distinct evolutionary lineages is an important phenomenon that has shaped the natural history on earth. Viviparous fish families such as the Poeciliidae, Anablepidae, Goodeidae (all three in Order Cyprinodontiformes), and Zenarchopteridae (Order Beloniformes) are excellent systems to study convergent evolution. They form groups of closely related species that occur in different habitats characterized by a tremendous variation in biotic and abiotic conditions. This has produced a stunning diversity in morphology, physiology, behavior, life-history, and reproductive adaptations. Many of these features evolved repeatedly across the family tree and vary both within and among species. Family-wide phylogenetic comparative approaches that assess how phenotypic features co-evolve across species, or evolve in association with ecological factors, will allow us to address important questions. For example, is evolution repeatable within and among families? Is phenotypic convergence the result of common solutions to similar selective pressures? To what extent does convergent evolution produce similar (but not necessarily always identical) phenotypes? Finally, and equally interesting, do similar selective pressures always lead to similar convergent adaptations or can they also lead to different (non-convergent) phenotypic adaptations? Answers to these captivating questions from live-bearing fishes may significantly further our general understanding of the evolution of phenotypic convergence at both micro- and macro-evolutionary levels (Losos, 2011; Wake et al., 2011).

Genomics of Adaptation

Another promising avenue of future research is one that focusses on uncovering the molecular pathways underlying the convergent evolution of complex adaptations. Recent advances in sequencing technologies, declines in sequencing costs, and innovative computational approaches mark the beginning of a genomics revolution (Giani et al., 2020), with more and more fish genomes becoming rapidly available. The field of comparative genomics is bringing this full genome information together with research on ecological selective factors and phenotypic adaptations, giving rise to new opportunities for studying the genomic basis of complex adaptations. We foresee that future studies will focus on two timely topics. The first centers on uncovering the genomic basis of complex trait evolution. The genetic possibilities are countless and may include changes in protein coding genes, gene duplications/losses, or changes in non-coding regulatory elements that result in adjustments of the spatiotemporal expression patterns of key genes. Moreover, these changes may range from relatively minor (epi)genetic changes in only a few, or even single, key genes, to more elaborate modifications at multiple locations in different developmental pathways (Wagner and Altenberg, 1996; Carroll, 2008). The second topic focuses on the extent to which convergent evolution in independent lineages is associated with parallel or alternative changes at the genomic level. Comparing the genomes of populations or species that independently evolved new phenotypic traits allows the identification of genomic differences associated with these phenotypes (Elmer and Meyer, 2011; Stern, 2013; van Kruistum et al., 2021). It is noteworthy to point out that such comparative approaches become statistically more powerful in identifying phenotype-related genes as the number of independent origins included in the study increases.

Population Dynamics and Conservation

Another important aspect that deserves attention is the population status of most species of viviparous fishes. To date, we know little about their demographic trends or conservation needs. A few studies have estimated sex- or stage-specific mortality rates, but the main purpose of these studies has been to identify the sources of extrinsic mortality and its micro-evolutionary consequences, rather than to know if populations are growing, stable, or declining (Reznick and Bryant, 2007; Johnson and Zúñiga-Vega, 2009; Zúñiga-Vega et al., 2012). In the light of the current global situation, with numerous water bodies drying up or increasing in pollution (Kingsford, 2011; Wen et al., 2017), we must make additional efforts to estimate demographic parameters such as mortality, recruitment, and population growth rates. Furthermore, temperature is increasing in many water bodies, mainly near human settlements, a phenomenon called "urban hot-tubs" (Brans et al., 2018). We still do not understand how such warmer temperatures are affecting the populations of aquatic organisms.

Certainly, many species of viviparous fishes are widespread and others (e.g., some poeciliid species) are considered invasive species, with numerous populations growing in areas outside their native geographic ranges (Albornoz-Garzón and Villa-Navarro, 2017; Santi et al., 2020). However, many other species have restricted distributions (e.g., most species in the family Goodeidae) and inhabit regions with high rates of habitat degradation (Meffe et al., 1983; Domínguez-Domínguez et al., 2006). Some studies have made significant efforts to propose conservation status of particular species, aiming to call the attention of managers and government environmental agencies (Tobler and Plath, 2009; Tobler and Schlupp, 2009; Echelle et al., 2013). Still, demographic characteristics of most viviparous species remain entirely unknown and quantitative information is urgently needed to guide future conservation strategies.

CONCLUDING REMARKS

The breadth of research on livebearing fishes—that which has been completed, is underway, or is being planned—is truly astounding. The articles contained in this Research Topic of *Frontiers in Ecology and Evolution* capture some of this breadth with clever studies that expand the boundaries of our understanding. But they also reveal biases in this larger body of work. Some species are much more extensively studied than others; some biological disciplines are over-represented; and some important questions have yet to be asked. Perhaps the most exciting thing that will come from work on livebearing fishes is the synthesis of biological knowledge that can occur. Rather than viewing organisms as a collection of traits independently evolving, several species of livebearing fishes are poised to be understood as an integrated expression of genes interacting with a complex environment—this approach offers a way to more fully understand the fundamental bauplan of fishes, and maybe of all vertebrates (Gould, 2002). Similarly, as we come to examine more and more species of livebearing fishes, our understanding of the contraints and flexibility of evolution will also become clearer. Examples of parallel evolution already excite the possibility of understanding the predictability of evolutionary change (Reznick et al., 1996; Johnson, 2001; Ingley et al., 2014; Tobler et al., 2015). And of course, the applied use of this group of fishes continues to grow, including their use to understand disease, development, and conservation, to name but a few. Clearly, future work studying freshwater viviparous fishes holds much promise.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this Editorial article and to the organization of the Research Topic.

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High Degree of Multiple Paternity and Reproductive Skew in the Highly Fecund Live-Bearing Fish *Poecilia gillii* (Family Poeciliidae)

Myrthe L. Dekker[1], Andres Hagmayer[1], Karen M. Leon-Kloosterziel[1], Andrew I. Furness[2] and Bart J. A. Pollux[1]*

[1] Experimental Zoology Group, Wageningen University & Research, Wageningen, Netherlands, [2] Department of Biological and Marine Sciences, University of Hull, Hull, United Kingdom

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Claus Wedekind,
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Estación Biológica de Doñana (EBD),
Spain

***Correspondence:**
Bart J. A. Pollux
bart.pollux@wur.nl

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Multiple paternity is a common phenomenon within the live-bearing fish family Poeciliidae. There is a great variety in brood sizes of at least two orders-of-magnitude across the family. However, little is known about the ramifications of this remarkable variation for the incidence and degree of multiple paternity and reproductive skew. Mollies (subgenus *Mollienesia*, genus *Poecilia*) produce some of the largest broods in the family Poeciliidae, making them an excellent model to study the effects of intra-specific variation in brood size on patterns of multiple paternity. We collected samples of the live-bearing fish *Poecilia gillii* from 9 locations in Costa Rica. We measured body size of 159 adult females, of which 72 were pregnant. These samples had a mean brood size of 47.2 ± 3.0 embryos, ranging from 4 to 130 embryos. We genotyped 196 field-collected specimens with 5 microsatellite markers to obtain location-specific allele frequencies. In addition, we randomly selected 31 pregnant females, genotyped all their embryos ($N = 1346$) and calculated two different parameters of multiple paternity: i.e., the minimum number of sires per litter using an exclusion-based method (GERUD) and the estimated number of sires per litter using a maximum likelihood approach (COLONY). Based on these two approaches, multiple paternity was detected in 22 and 27 (out of the 31) females, respectively, with the minimum number of sires ranging from 1 to 4 (mean ± SE: 2.1 ± 0.16 sires per female) and the estimated number of fathers ranging from 1 to 9 (mean ± SE: 4.2 ± 0.35 sires per female). The number of fathers per brood was positively correlated with brood size, but not with female size. Next, we calculated the reproductive skew per brood using the estimated number of sires, and found that in 21 out of the 27 multiply sired broods sires did not contribute equally to the offspring. Skew was not correlated with either female size, brood size or the number of sires per brood. Finally, we discuss several biological mechanisms that may influence multiple paternity and reproductive skew in *P. gillii* as well as in the Poeciliidae in general.

Keywords: polyandry, viviparity, microsatellites, brood size, female size, COLONY, GERUD

INTRODUCTION

Female multiple mating, or polyandry, often leads to offspring being sired by multiple fathers, a phenomenon which is referred to as multiple paternity. Polyandry is prevalent in the animal kingdom, but it remains debated why it evolved in in most animal taxa (Jennions and Petrie, 2000; Zeh and Zeh, 2001; Simmons, 2005). Nonetheless, there are several hypotheses concerning how polyandry and multiple paternity can increase female fitness (Keller and Reeve, 1995; Zeh and Zeh, 1996, 2001; Jennions and Petrie, 2000; Stockley, 2003; Simmons, 2005).

First, females might select multiple mates in order to receive direct benefits, such as nuptial gifts (Thornhill, 1976; Kondoh, 2001), or fertilization assurance (Sheldon, 1994). Second, polyandry can also arise because of indirect genetic benefits (Yasui, 1998; Jennions and Petrie, 2000), in three ways: (i) Females can mate with multiple males to improve the genetic quality of the offspring by 'trading-up' (Pitcher et al., 2003). This hypothesis predicts that a female that mates with a 'genetically inferior' male can increase her fitness by mating with another male of higher genetic quality (Jennions and Petrie, 2000; Simmons, 2001; Pitcher et al., 2003), (ii) Multiple paternity can also increase a female's fitness by increasing the genetic diversity of offspring (Yasui, 1998; Fox and Rauter, 2003). If the environment is unpredictable, the genetic diversity of the offspring could increase a female's reproductive success by ensuring that some of her offspring will survive when the environment changes, which is a form of bet-hedging (Loman et al., 1988; Yasui, 1998; Fox and Rauter, 2003; Garcia-Gonzalez et al., 2015). However, bet-hedging strategies only seem to outweigh the fitness costs of multiple mating in certain situations (Yasui, 1998; Fox and Rauter, 2003; Yasui and Garcia-Gonzalez, 2016), (iii) Multiple mating can further arise because it can promote post-copulatory sexual selection (e.g., by enhancing sperm competition; Parker, 1970). The enhanced sperm competition results in an increased fertilization chance by genetically fit males (Zeh and Zeh, 1997), thereby increasing the fitness of a females' offspring. Third, multiple paternity can also arise without female choice, as males force mating upon the females, i.e., coercive mating (Clutton-Brock and Parker, 1995). By doing so, males can increase their reproductive output. When more males mate coercively with the female, this can lead to polyandry and multiple paternity. In this way, multiple paternity can arise without increasing the fitness of a female.

In the live-bearing fish family Poeciliidae, both female mate choice (often in association with male courtship behavior) and coercive mating occur (Magurran, 2011; Rios-Cardenas and Morris, 2011). Moreover, multiple paternity is widespread in this family; multiple sired broods are found, for instance, in the genera *Gambusia* (Greene and Brown, 1991; Zane et al., 1999), *Heterandria* (Soucy and Travis, 2003), *Poecilia* (Travis et al., 1990; Kelly et al., 1999; Hain and Neff, 2007; Neff et al., 2008; Girndt et al., 2012), and *Xiphophorus* (Luo et al., 2005; Simmons et al., 2008; Tatarenkov et al., 2008; Paczolt et al., 2015). In multiple sired broods, it is common that sires do not contribute equally to the offspring, a phenomenon that is referred to as reproductive skew. Previous studies have shown that in natural populations of Poeciliidae often pronounced male reproductive skew is observed, this is for instance the case for swordtails (Luo et al., 2005; Simmons et al., 2008; Tatarenkov et al., 2008), guppies (Hain and Neff, 2007; Neff et al., 2008), and mollies (Girndt et al., 2012). There are several factors that can cause and enhance male reproductive skew in natural populations. For instance, when females mate with multiple males this will intensify sperm competition, which often results in one or a few male(s) being more prominent in siring offspring (Parker, 1970; Constantz, 1984; Evans, 2010). An increase in the number of sires may hereby lead to an increased chance of skew. Next to sperm competition, cryptic female choice can also cause paternity skew. This refers to a post-copulatory sexual selection process in which the female biases sperm use, thereby altering the paternal contribution (Eberhard, 1996; Firman et al., 2017).

In the Poeciliidae, there is a remarkable variation in brood sizes of at least two orders-of-magnitude, both within and among species. Most earlier multiple paternity studies have focused on species with small broods, such as *Poecilia reticulata* (Kelly et al., 1999; Hain and Neff, 2007; Neff et al., 2008), *Heterandria formosa*, (Kelly et al., 1999; Soucy and Travis, 2003), *Xiphophorus multilineatus* (Luo et al., 2005), *X. nigrensis* (Smith, 2014), and *X. birchmanni* (Paczolt et al., 2015). Only a few earlier studies focus on species with large broods, i.e., *Poecilia latipinna* and *Gambusia affinis*, but most of these only analyze a small part of the actual brood (Travis et al., 1990; Greene and Brown, 1991; Girndt et al., 2012), except for a study by Gao et al. (2019) that studied multiple paternity in whole broods of *Gambusia affinis* and *G. holbrooki*. Therefore, little is currently known about how the large variation in brood size affects multiple paternity, the number of sires per brood and male reproductive skew within species.

Prior studies on poeciliid fishes have found a positive relationship between multiple paternity and female traits such as brood size and female size (Neff et al., 2008; Girndt et al., 2012). Several hypotheses have been proposed to explain why multiple paternity may be correlated with brood size and female size. The number of offspring a female can carry is restricted by both the limited space available in the body cavity (physical limitation; Reznick and Miles, 1989; Pires et al., 2011) and the exacerbated negative consequences of an increase in reproductive allocation on a female's swimming ability (physiological limitation; Fleuren et al., 2019; Quicazan-Rubio et al., 2019). These factors explain (at least partly) why female size is often positively correlated with brood size (Cheong et al., 1984; Reznick et al., 1992, 1993; Neff et al., 2008; Schrader and Travis, 2009; Hagmayer et al., 2018, 2020): larger females can physically carry larger broods than smaller females. Simple mathematics dictate that the larger a brood, the larger the absolute number of potential sires that can contribute to that brood (Avise and Liu, 2011): i.e., a brood of two offspring can be sired by a maximum of two fathers, while a brood of 30 offspring can be sired by a maximum of 30 different males. Several studies have furthermore shown that males prefer to mate with larger females (Bisazza et al., 1989; Ptacek and Travis, 1997; Dosen and Montgomerie, 2004; Herdman et al., 2004; Hoysak and Godin, 2007), presumably because they increase their chance of producing more offspring as these females generally carry more eggs (Schlupp, 2018). These above processes may explain the observed positive associations between female size, brood size and multiple paternity.

In this study, we investigate the degree of multiple paternity and reproductive skew in the live-bearing fish *Poecilia gillii* (Poeciliidae) across its South-Western range in Costa Rica. Members of the subgenus *Mollienesia* (genus *Poecilia*) are an excellent model to study the effects of brood size variation on patterns of multiple paternity at an inter-specific level, because they produce some of the largest broods in the family (Reznick and Miles, 1989; Winemiller, 1993; Johnson and Bagley, 2011). Specifically, we will test to what extent female size and brood size within *P. gillii* correlate with the (i) degree of multiple paternity (i.e., the number of sires within a brood) and (ii) male reproductive skew.

MATERIALS AND METHODS

Animal Ethics

The study was assessed by the institutional Animal Welfare Body (AWB) of Wageningen University (the Netherlands). The AWB judged that the study complied with the Dutch Act on Animal Experiments (AAE), which complies to European Directive 2010/63/EU. All fish were collected and exported following the regulations of the Costa Rican government under permit number SINAC-CUS-PI-R-005-2017, SINAC-PNI-ACLAP-004-2020, and SINAC-ACOSA-DT-PI-INV-003-2020.

Field Collection

We collected individuals of the live-bearing fish species *Poecilia gillii* (family Poeciliidae) in Costa Rica using seine and cast nets during the dry season from 27 February to 12 March 2017. To minimize the potentially confounding effect of location on multiple paternity, we randomly sampled 9 different locations in the Rio Terraba General and the Rio Coto drainages in the Province of Puntarenas, Costa Rica (**Table 1**). To obtain sufficient samples to estimate population allele frequencies per location, additional samples were collected from two of these locations (specifically; N = 9 individuals from Pedegroso, and N = 8 from Tigre) from 18 February to 16 March 2020, which resulted in a total of 196 specimens for molecular analysis (range of specimens per location: 15–26). The collected specimens were euthanized in the field with an overdose of MS-222 (Tricaine methanesulfonate) and preserved in 96% ethanol. The fish were subsequently transported to the Pollux lab at Wageningen University (the Netherlands) and stored in a freezer at −20°C until further processing.

Female Life History Traits

We measured the total length (TL; i.e., length from snout to the distal end of tail), standard length (SL; i.e., total length excluding length of caudal fin) and total wet mass of 159 females, of which 72 were pregnant (**Supplementary Table S1**). For all pregnant females, we measured the wet mass of the reproductive tissue (ovary with embryos) and counted and staged their developing embryos, following Haynes (1995) staging classification for poeciliid fishes (**Supplementary Table S1**). Based on these measurements, we calculated the reproductive allotment (RA) by dividing wet mass of the ovary by the total wet mass of the female (**Supplementary Table S1**). For molecular paternity analysis, we randomly selected 32 *Poecilia gillii* females that were pregnant with broods that were at developmental stage 20 or later (early eyed; Haynes, 1995) to ensure that sufficient DNA could be extracted from the embryos (resulting in a total of 1371 embryos for further analysis).

TABLE 1 | Overview of sampling sites for *Poecilia gillii.*

Drainage	River	Latitude	Longitude
Coto	Coloradito	8.604993	−82.900390
	Incendio	8.443007	−82.995610
	Palma Norte	8.985969	−83.519638
	Pedegroso	9.356514	−83.719630
	Secco	8.744335	−82.943788
	Tigre	8.547105	−83.333584
	Vacca	8.433390	−82.966493
Terraba	Ceibo	9.216445	−83.315422
	Sucio	8.809893	−82.910482

DNA Extraction

DNA was extracted from the 32 gravid females of *Poecilia gillii* and all their embryos (N = 1371) for multiple paternity analysis, and an additional samples (males, females and juveniles) to estimate population allele frequencies. For the DNA extraction, we used the following adapted protocol from the Wizard Genomic DNA purification kit (Promega). Entire embryos and tailfin clips from adults were taken and dried for 10 min at room temperature (20–25°C) and stored at −20°C before DNA extraction. Lysis was performed using 300 μL Nuclei Lysis Solution/EDTA mix (by preparing a total of 310 μL mix: 60 μL of 0.5 M EDTA and 250 μL of Nuclei Lysis Solution) and 9 μL of Proteinase K (20 mg ml^{-1}) and by incubating at 55°C for 2–2.5 h (vortexed every 30 min). Protein precipitation was performed by adding 100 μL Protein Precipitation solution, after which the samples were vortexed for 20 s, cooled on ice for 5 min, and finally centrifuged for 4 min at 16000 × *g*. DNA was precipitated from the supernatant by adding 300 μL isopropanol, mixing, and leaving the samples at room temperature (20–25°C) for 30–60 min, then centrifuging at 16000 × *g* for 1 min to form a pellet. Next, the supernatant was decanted and the pellet (DNA) was washed with 300 μL 70% cooled ethanol (0 °C), mixed, centrifuged at 16000 × *g* for 2 min and dried at room temperature for 20–30 min. Finally, the pellet was dissolved in 25 μl Tris (10 mM) for embryonic samples and in 50 μl Tris (10 mM) for maternal samples. The stock DNA was diluted to 20 ng/μL for further analyses.

Microsatellite Analysis

All obtained DNA samples (N = 1567) were analyzed using 5 polymorphic microsatellite loci (**Table 2**). The microsatellites were obtained from previous studies on different *Poecilia* species (**Supplementary Table S2**): Locus AB-195 was originally isolated

TABLE 2 | Microsatellite loci characteristics for *Poecilia gillii* at the 9 different sampling sites, obtained with GenAlex v. 6.5.

	Site	Ceibo	Coloradito	Incendio*	Palma norte	Pedegroso	Secco	Sucio	Tigre	Vacca	Mean	Total
Locus	N_{mother}	4	5	1	3	1	2	9	3	4	3.88	32
	N_{embryo}	115	110	25	179	43	97	414	127	261	168.25	1371
	N_{extra}	22	20	18	22	17	23	16	15	11	18.22	164
	N_{total}	141	135	44	204	61	122	439	145	276	174.11	1567
AB-195	N_A	4	4	3	8	8	4	4	3	5	4.78	17
	range	258–294	273–303	273–294	258–322	267–294	276–355	276–285	258–291	273–303		258–355
	H_O	0.59	0.73	0.68	0.87	0.87	0.23	0.18	0.48	0.57	0.58	
	H_E	0.51	0.64	0.63	0.71	0.74	0.32	0.17	0.38	0.62	0.52	
	E_1	0.366	0.315	0.219	0.550	0.356	0.323	0.117	0.151	0.418	0.313	
GA-III28	N_A	5	3	1	10	8	1	7	2	3	4.44	21
	range	200–240	206–236	224	196–262	212–254	220	190–256	190–224	206–236		190–262
	H_O	0.82	0.68	–	0.16	0.73	–	0.57	0.26	0.44	0.53	
	H_E	0.76	0.63	–	0.15	0.67	–	0.53	0.27	0.44	0.49	
	E_1	0.479	0.346	NA	0.240	0.475	NA	0.283	0.181	0.222	0.318	
GA-IV42	N_A	5	2	1	8	4	4	6	2	3	3.89	14
	range	178–244	178–212	178	176–246	194–214	194–244	194–216	176–178	176–212		176–246
	H_O	0.72	0.27	–	0.71	0.15	0.67	0.50	0.43	0.21	0.46	
	H_E	0.71	0.32	–	0.63	0.18	0.60	0.53	0.34	0.19	0.44	
	E_1	0.409	0.149	NA	0.317	0.279	0.477	0.253	0.132	0.093	0.264	
PoecTTA	N_A	10	5	3	13	5	5	6	5	5	6.33	17
	range	100–145	103–133	118–133	112–155	118–136	100–130	115–139	127–139	112–133		100–155
	H_O	0.63	0.61	0.12	0.80	0.57	0.77	0.71	0.62	0.70	0.61	
	H_E	0.69	0.57	0.15	0.78	0.62	0.71	0.63	0.74	0.58	0.61	
	E_1	0.522	0.352	0.158	0.736	0.489	0.489	0.349	0.448	0.457	0.444	
Pvm16	N_A	4	4	2	10	9	3	8	2	3	5.00	23
	range	241–273	249–309	249–263	249–283	225–309	261–295	233–273	243–249	249–287		233–309
	H_O	0.67	0.60	0.45	0.87	0.70	0.38	0.62	0.25	0.47	0.56	
	H_E	0.66	0.63	0.47	0.76	0.65	0.38	0.63	0.22	0.56	0.55	
	E_1	0.357	0.458	0.187	0.721	0.539	0.232	0.551	0.050	0.259	0.373	
Mean	N_A	5.60	3.60	2.00	9.80	6.80	3.40	6.20	2.80	3.80	4.89	
	H_O	0.69	0.58	0.25	0.68	0.61	0.41	0.52	0.41	0.48	0.51	
	H_E	0.67	0.56	0.25	0.61	0.57	0.40	0.50	0.39	0.48	0.49	
All loci	E_1	0.940	0.866	0.466	0.983	0.943	0.861	0.862	0.684	0.835	0.827	

*N_{mother} = number of mothers; N_{embryo} = number of embryos; N_{extra} = extra samples for the calculation location-specific allele frequencies, N_{total} = total number of samples. We show the number of alleles (N_A), the allelic range, the observed (H_O) and expected heterozygosity (H_E), and the exclusion probability (E_1) per locus and for the 5 loci combined. *This location was excluded from the multiple paternity analyses, due to the low exclusion probability.*

in *P. sulphuraria* and *P. mexicana* (Slattery et al., 2012), GA-III28 and GA-IV42 in *P. formosa* (Tiedemann et al., 2005; also polymorphic in *P. latipinna*, Girndt et al., 2012), PoecTTA in *Poecilia* sp. (J. S. Taylor, unpublished data; also polymorphic in *P. reticulata*, Kelly et al., 1999) and Pvm16 in *P. vivipara* (Tonhatti et al., 2014). We amplified each microsatellite locus by a polymerase chain reaction in 15 μL total volume, using 2 μL (40 ng) genomic DNA (for PoecTTA 4 μL DNA to improve PCR quality), 0.6 μL dNTPs (20 mM), 3 μL 5x Promega Taq buffer (7.5 mM MgCl2), 0.2 μL (10 μM) forward primer (labeled), 0.2 μL (10 μM) reverse primer and 0.09 μL Taq polymerase (Promega GO-Taq G2). For amplification we used the following thermal cycling conditions: initial denaturation at 95°C for 3 min, followed by 30–35 cycles with a denaturation step of 30 s at 95°C, annealing at 50–60°C for 30 s and an elongation step of 30 s at 72°C (see **Supplementary Table S2** for primer specific cycles), followed by a final elongation step at 72°C for 5 min. We checked the PCR products by gel electrophoresis and diluted samples based on band thickness. We analyzed the diluted PCR samples with capillary gel electrophoresis using an ABI3730 Sequencer (Applied Biosystems). For the analysis, we used a total volume of 10 μL per sample containing 1 μL of diluted PCR samples (all markers combined) and 9 μL ladder mix (0.5% (v/v) Liz500 size ladder in formamide). We genotyped the samples using GeneMapper software version 4.0. We scored peaks with automatic binning in GeneMapper but checked all samples manually and rescored when automatic scoring errors occurred. Allele count, range and observed and expected heterozygosity were calculated per sampling site (**Table 2**), using GenAlex version 6.5 (Peakall and Smouse, 2006, 2012). Exclusion probabilities were furthermore calculated per sampling site, using GERUD 2.0 (Dodds et al., 1996; Jones, 2005). As exclusion probabilities are determined by both the number and frequency of alleles in a population (Dodds et al., 1996), we included location-specific allele frequencies in our analyses that were based on the 196 field-collected individuals. The exclusion probability represents the probability of a marker to exclude a random unrelated male from paternity for a mother-offspring pair (Dodds et al., 1996; Wang, 2007) and provides a comparative measure of marker information. However, it has been argued that the exclusion probability should not be used as a measure of confidence in parentage analyses, because: (i) exclusion probability calculations do not account for marker errors, and (ii) the probabilities are derived for single-offspring parentage tests and therefore do not correct for experiment-wide error that arises from the hundreds of comparisons in a typical parentage study (Jones et al., 2010). Nevertheless, the low exclusion probability in the Incendio (<0,5; **Table 2**), prompted the exclusion of the single female from this location from our subsequent paternity analyses.

Paternity Analysis

Multiple paternity was quantified for the remaining 31 pregnant females and their 1346 embryos (using two software programs: GERUD version 2.0 (Jones, 2005) and COLONY version 2.0 (Jones and Wang, 2010). GERUD is an exclusion-based method that calculates the minimum number of sires for multi-locus data using co-dominant markers, such as microsatellites (Jones, 2005). It excludes maternal alleles in the offspring to calculate the number of alleles that offspring received from their fathers (Jones, 2005). Based on this data, it reconstructs the paternal genotypes to calculate the minimum number of sires (Jones, 2005). As missing data is not accepted in GERUD, we excluded samples that lacked data for one or more markers (see **Table 3**). COLONY calculates the estimated number of sires based on maximum likelihood methods, using simulated annealing to search for a global optimum (Jones and Wang, 2010). The program uses multi-locus data and considers all mothers and embryos jointly for paternity assignments (Jones and Wang, 2010). When marker diversity is limited (e.g., low expected heterozygosities and/or few microsatellite markers with a limited number of alleles), COLONY tends to overestimate the true number of sires (Sefc and Koblmüller, 2009). We performed a full-likelihood analysis in COLONY per sampling location (i.e., population), using the maximum run length and the highest precision. Population allele frequencies were estimated with GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012), utilizing the extra samples for each location (**Table 2**). Allele frequencies for each locus and sampling location were then applied to run the COLONY analyses. The expected genotyping error rate was set at 0.025, as suggested by Wang (2004). Mating systems for males and females were set on polygamous, and, as we do not have any information on sibship size in *Poecilia gillii,* no prior was set for sibship size. COLONY can run with missing data, but with limited genetic info paternity estimates become less accurate. Therefore, we excluded samples from the analysis that had missing genotypes for more than 2 out of the 5 loci (see **Table 3**). Since location-specific allele frequencies are used in these analyses to determine the number of compatible fathers, we calculated the number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) using GenAlex version 6.5 (Peakall and Smouse, 2006, 2012), and the exclusion probabilities (E_1) using GERUD 2.0 (Dodds et al., 1996; Jones, 2005) per locus per sampling location (**Table 2**).

Reproductive Skew

We calculated the male reproductive skew per mother using the estimated number of sires obtained from COLONY. The effective number (N_{eff}) of sires was estimated by $N_{eff} = 1/\Sigma(O_i/BS)^2$, in which BS is the total brood size of a female, and O_i is the number of offspring assigned to each sire i. We then calculated the reproductive skew (R_{skew}) by dividing the effective number of sires by the actual number of sires, and subtracting the outcome from 1 (Neff et al., 2008).

Data Analysis

All the analyses were carried out in R v 3.5 (R Core Team, 2019). Mixed models were fitted using the lme4 package (Bates et al., 2015), and significance tests for the fixed effects were performed with lmerTest (Kuznetsova et al., 2017). Poisson models were checked for over- and under-dispersion using functions implemented in the in the DHARMa package (Hartig, 2018). In the case of data dispersion, we switched to a Conway–Maxwell–Poisson (CMP) distribution, which is a viable count distribution that generalizes the Poisson distribution in light of data dispersion (Shmueli et al., 2005). CMP models can be fitted using the glmmTMB package (Brooks et al., 2017). Model equations of the analyses discussed below can be found in the Supporting methods.

To evaluate the effect of maternal standard length on the probability of being pregnant and brood size, we fitted two separate generalized linear mixed effect models that each included maternal standard length as an explanatory variable and sampling location as a random effect (a categorical variable with 9 levels). In the first model (model 1), the probability of being pregnant (a dichotomous variable: 1 = pregnant, 0 = not pregnant; $N = 159$ females) was fitted using a binomial response distribution and a logit link function. In the second model (model 2), brood size (a discrete variable based on the embryo counts in a brood) was analyzed for pregnant females only ($N = 72$) using a Poisson distribution and a log link function.

The potential effects of brood size and female size on multiple paternity were assessed by fitting the (i) minimum number of sires inferred from GERUD (model 3), and (ii) estimated number of sires inferred from COLONY (model 4) in generalized linear mixed effect models with a log link for the Conway–Maxwell–Poisson-distributed response to handle the under-dispersed data in (i), and the Poisson-distributed response in (ii). These models included female standard length, brood size and their interaction term as fixed effects and sampling location as a random effect (the latter to account for between-location variation in multiple paternity that is not accounted for by the fixed effects). The correspondence of the number of sires obtained with the two different programs (GERUD and COLONY) was evaluated by calculating the Spearman rank correlation coefficient between the minimum (GERUD) and estimated number of sires (COLONY).

To study the paternity skew in broods, we first tested whether the observed contribution of sires in a brood significantly deviates from the expected contribution if there was no skew (i.e., equal

TABLE 3 | Life history traits and multiple paternity for 31 studied females.

Sampling info			Life history traits					Paternity analyses				
								GERUD		***COLONY***		
Drainage	**River**	**Female**	**Length (mm)**	**Mass (g)**	**Brood size (#)**	**RA (%)**	**Stage**	**Embryos analyzed**	**Sires**	**Embryos analyzed**	**Sires**	**Skew**
Coto	Coloradito	Pgi52	46.4	1.72	28	11.8	35	27	1	27	1	-
		Pgi63	45.2	1.61	27	11.8	47.5	27	1	27	5	0.24
		Pgi64	50.7	2.74	28	12.2	35	28	2	28	3	0.19
		Pgi65	46.0	1.56	11	15.8	32.5	11	2	11	3	0.42
		Pgi66	47.3	1.62	17	16.8	35	17	2	17	4	0.11
	Palma Norte	Pgi92	78.3	8.12	71	7.8	22.5	63	2	69	5	0.37
		Pgi118	50.5	2.27	30	9.6	25	26	1	30	1	-
		Pgi122	74.1	7.29	80	14.6	30	77	4	78	4	0.23
	Pedegroso	Pgi75	44.5	1.72	43	6.6	25	42	1	43	5	0.10
	Secco	Pgi138	67.3	5.99	88	9.3	30	84	3	87	8	0.29
		Pgi149	58.4	4.71	9	6.3	35	8	2	9	4	0.35
	Tigre	Pgi61	52.3	2.70	57	20.3	45	57	2	57	5	0.49
		Pgi72	52.7	2.38	17	12.7	45	17	1	17	2	0.08
		Pgi73	57.5	3.29	53	4.5	25	53	2	53	5	0.16
	Vacca	Pgi49	65.8	4.46	74	15.5	35	71	3	74	6	0.26
		Pgi50	55.6	3.34	61	11.5	22.5	58	2	61	4	0.13
		Pgi51	56.2	3.11	76	21.6	47.5	76	2	76	5	0.47
		Pgi69	52.3	2.80	50	22.7	45	49	2	50	5	0.47
Terraba	Ceibo	Pgi39	50.4	2.17	51	21.2	35	50	1	50	1	-
		Pgi40	53.2	1.52	16	9.5	45	16	1	16	2	0.20
		Pgi48	57.0	3.55	30	17.8	45	30	2	30	4	0.45
		Pgi76	44.8	1.64	19	11.5	35	17	1	17	1	-
	Sucio	Pgi7	60.8	4.96	37	14.6	42.5	36	3	37	6	0.53
		Pgi30	59.4	3.55	27	13.5	40	26	1	27	3	0.28
		Pgi34	59.9	4.68	60	15.6	40	58	3	60	7	0.35
		Pgi37	58.2	3.62	33	7.6	42.5	33	3	33	6	0.46
		Pgi47	59.4	3.15	27	10.6	35	27	2	27	4	0.24
		Pgi77	74.0	6.23	63	14.3	45	55	3	62	5	0.31
		Pgi78	54.8	2.62	31	14.5	35	29	2	30	4	0.13
		Pgi81	61.8	4.53	54	19.6	37.5	53	3	54	4	0.34
		Pgi83	65.0	5.92	82	14.6	25	79	4	82	9	0.19

For each female the standard length, total wet mass, reproductive allotment (RA) and brood size is given. For each brood the developmental stage (according to Haynes, 1995) is given. The minimum number of sires was estimated with GERUD, the estimated number of sires and reproductive skew were calculated in COLONY. The embryos analysed in these programs can be less than the actual brood size, as some samples were omitted because of lacking genotype data for one or more markers (for details see "Materials and Methods").

contribution) by means of χ^2-tests for goodness of fit (Yue and Chang, 2010; Green et al., 2017). To identify potential sources of variation in paternity skew, we then fitted a series of generalized linear models with a logit link function for the quasibinomial-distributed response, as paternity skew values range between 0 and 1. To prevent model over-fitting, we did not include a random sampling location effect. The full model included brood size, female standard length, and the estimated number of sires inferred from COLONY as fixed effects. In addition, we tested all the two-way interactions by adding them to the model case-by-case. Because none of the interaction terms were significant (brood size × female standard length: $T_{22} = 0.174$, $p = 0.165$; brood size × number of sires: $T_{22} = -1.730$, $p = 0.098$, female standard length × number of sires: $T_{22} = -1.327$, $p = 0.198$), these were excluded from the final model (model 5).

RESULTS

Variation in Female Life History Traits

A total of 159 adult *Poecilia gillii* females collected from 9 locations were studied to determine their life history traits (**Supplementary Table S1**). Female standard length (SL) ranged from 32.0 to 84.8 mm and female wet mass from 0.46 to 8.65 g. Of these 159 studied females, 72 females were pregnant with a mean (± SE) brood size of 47.2 ± 3.0 (range: 4–130) embryos and a reproductive allocation (RA) of 13.2 ± 0.57% (range: 3.1–30.8%). Female SL was positively associated with the probability of being pregnant (**Figure 1A**; model 1: $Z_{156} = 2.53$, $p = 0.011$) and with brood size (i.e., the number of embryos within a brood) (**Figure 1B**; model 2: $Z_{69} = 16.32$, $p < 0.001$).

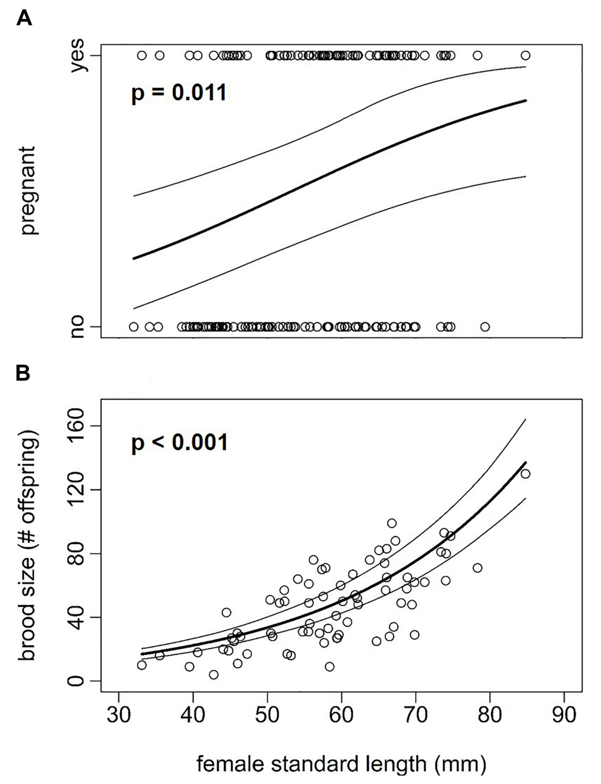

FIGURE 1 | Association of female standard length (mm) with **(A)** the probability of being pregnant (±95% CI, *n* = 159 females) and **(B)** brood size (±95% CI, *n* = 72 females) in *Poecilia gillii* calculated in model 1 and 2, respectively. *P*-values are given at the top.

Multiple Paternity

Molecular characteristics of the 5 microsatellite loci used to detect multiple paternity in *Poecilia gillii* are summarized in **Table 2**. Loci were on average 93.33% (SE = 4.71%) polymorphic. Marker diversity was high with the total number of alleles per marker ranging from 14 (for GA-IV42) to 23 (Pvm16) (**Table 2**, **Supplementary Table S2**).

The minimum number of sires in the broods of the 31 females was determined with GERUD (**Table 3**). Multiple paternity was observed in 22 of the 31 studied females, with the minimum number of sires ranging from 1 to 4 (mean ± SE: 2.1 ± 0.16 sires per female). There was a significant positive correlation between brood size and the minimum number of sires (**Figure 2B**; model 3: $Z_{25} = 2.393$, $p = 0.017$), but not between female SL and the minimum number of sires (**Figure 2A**; model 3: $Z_{25} = 1.937$, $p = 0.053$). In addition, there was no significant interaction effect between SL and brood size (model 3: $Z_{25} = 0.375$, $p = 0.708$). Next, the estimated number of sires contributing to the litters of the of the 31 *P. gillii* females was calculated with COLONY (**Table 3**). Multiple paternity was observed in 27 of the 31 females, with the estimated number of sires ranging from 1 to 9 (mean ± SE: 4.2 ± 0.35 sires per female). The estimated number of sires was positively associated with brood size (**Figure 2D**; model 4: $Z_{26} = 2.198$, $p = 0.028$), but not with female standard length (**Figure 2C**; model 4: $Z_{26} = 0.936$, $p = 0.349$). There was no significant interaction between brood size and female standard length on the estimated number of sires ($Z_{26} = -0.951$, $p = 0.342$). We found a strong positive correlation between the minimum number of sires calculated in GERUD and the estimated number of sires calculated in COLONY (**Figure 3**; Spearman's rank correlation: r = 0.698 $p < 0.001$).

Paternity Skew

Broods with multiple paternity (determined with COLONY) were associated with considerable paternity skew, ranging from 0.08 to 0.53 (**Table 3**) with a mean (± SE) of 0.27 ± 0.03. A value of zero implies that all sires contribute equally to the brood (no skew), and a value of one implies sires differ maximally in their contribution to the brood (maximal skew). In most broods (21 out of 27 multiply sired broods), the observed paternal contribution among offspring (i.e., skew) significantly deviated from the expected contribution (**Figure 4A**; goodness-of-fit χ^2-tests $p < 0.05$). There was no significant correlation between skew and the number of sires inferred from COLONY (**Figure 4B**; model 5: $T_{23} = 1.116$, $p = 0.276$), SL (**Figure 4C**; model 5: $T_{23} = 0.761$, $p = 0.455$), or brood size (**Figure 4D**; model 5: $T_{23} = -0.674$, $p = 0.507$).

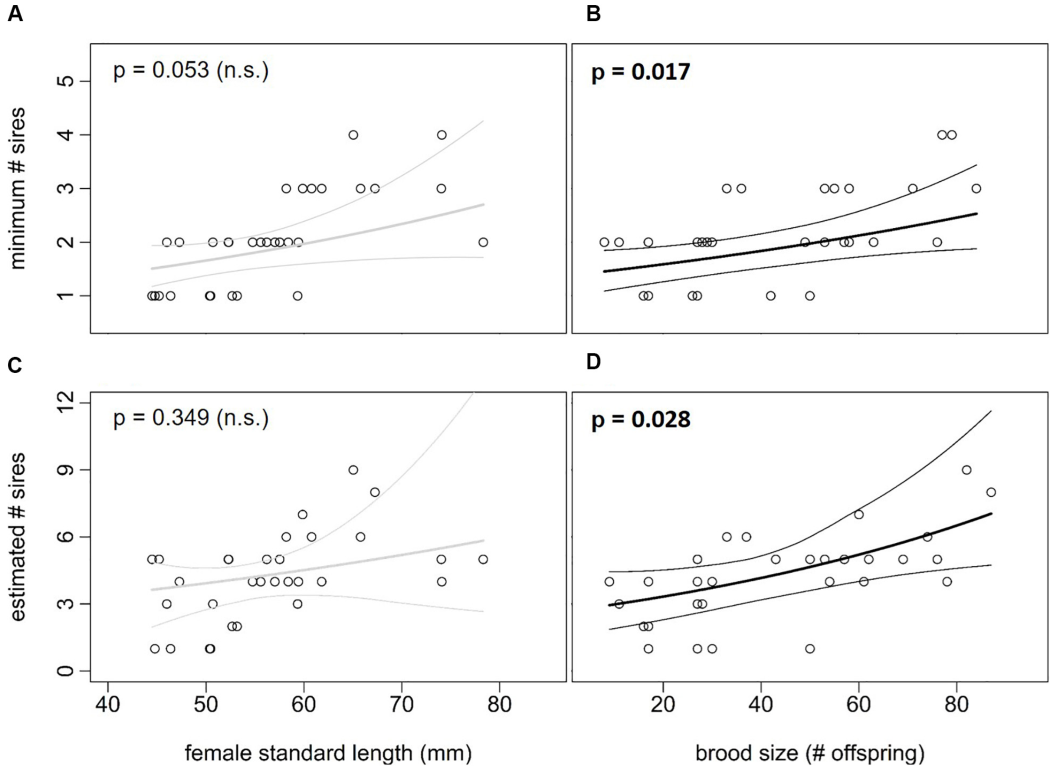

FIGURE 2 | Relationships between female traits and number of sires (±95% CI) in *Poecilia gillii*, calculated in model 3 and 4, respectively (n = 31 females). **(A)** Correlation between female standard length (SL) and minimum number of sires (calculated with GERUD). **(B)** Correlation between brood size (number of offspring per female) and minimum number of sires. **(C)** Correlation between SL and number of sires (calculated with COLONY). **(D)** Correlation between brood size and number of sires. *P*-values are given at the top.

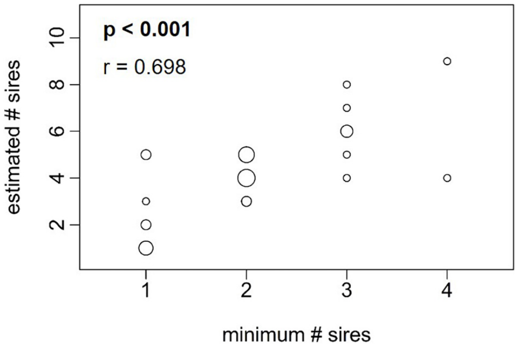

FIGURE 3 | Correlation between the minimum number of sires (calculated with GERUD) and the estimated number of sires (calculated with COLONY) in *Poecilia gillii*. Dot size represents number of overlapping data points, varying between 1–6 (n = 31 females). r- and *p*-values are given at the top.

DISCUSSION

Multiple Paternity

Local environmental conditions (e.g., light intensity, predation risk, food availability) might play an important role in determining (the incidence of) multiple paternity within species (Kelly et al., 1999; Soucy and Travis, 2003). Moreover, local 'logistic constraints' (sensu Avise and Liu, 2011; e.g., population density, sex ratio, mating strategy) are also likely to influence geographic patterns of multiple paternity. To minimize the potentially confounding effect of local conditions on multiple paternity, we randomly sampled 31 pregnant females from 8 different locations to characterize multiple paternity in *P. gillii* across its South-Western range in Costa Rica. Moreover, many studies on multiple paternity in species with large broods have analyzed only a fraction, or a set number of embryos, per brood (Travis et al., 1990; Greene and Brown, 1991; Girndt et al., 2012 but see Gao et al., 2019), potentially underestimating the absolute number of sires that may have contributed to the broods. To avoid this, we genotyped and analyzed all embryos (N = 1346) to quantify multiple paternity in our 31 *P. gillii* females.

We report a high incidence of multiple paternity in *P. gillii*; ranging from 22 to 27 out of 31 broods (i.e., 71%–87%, based on estimates of the minimum and estimated number of fathers per litter, respectively). Such high incidences of multiple paternity are common within this genus, e.g., in *Poecilia reticulata* multiple paternity was observed in 95% of the pregnant females (Neff et al., 2008). However, in other genera, lower rates of multiple paternity are also observed, sometimes as low as 23% in *Poeciliopsis monacha* (Lesie and Vrijenhoek, 1977) or 46% in *Heterandria formosa* (Soucy and Travis, 2003). In some genera, there is a

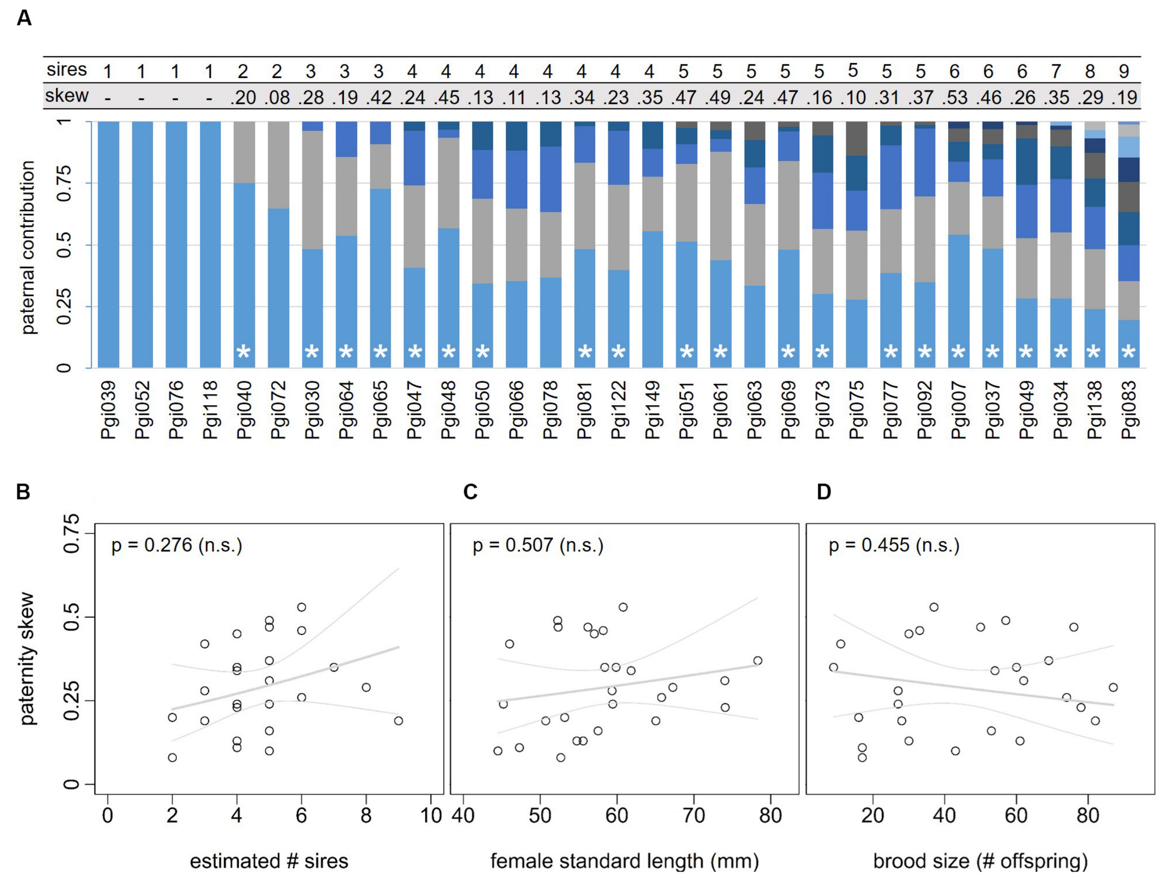

FIGURE 4 | A Paternity skew in 31 female *Poecilia gillii*. The bar graph shows the relative contribution in percentages of sires to the broods of 31 females (different shades represent different sires within each brood). Asterisks indicate that observed paternal contribution among offspring differed significantly from the expected (equal) contribution (goodness-of-fit χ^2-tests, $p < 0.05$), meaning that paternity was significantly skewed for those broods. The paternity skew and number of sires that contribute to each brood are shown above the graph. Panels **(B–D)** show the association of paternity skew (±95% CI) with: **(B)** the estimated number of sires (inferred by COLONY), **(C)** female standard length (SL), and **(D)** brood size (number of offspring), estimated in model 5 (based on $n = 27$ females). *P*-values are given at the top.

considerable variation in the incidence of multiple paternity, ranging from 28% in *Xiphophorus multilineatus* (Luo et al., 2005) to 84% in *X. birchmanni* (Paczolt et al., 2015), possibly due to different mating strategies among the species. For instance, in the genus *Xiphophorus*, males of sword-carrying species show more dominant behavior and females seem to have much lower rates of multiple paternity than species that do not carry swords (Luo et al., 2005; Paczolt et al., 2015). Environmental factors, like predation, can also affect mating strategies and influence the rate of multiple paternity. For example, in guppies, the number of sneak mating attempts and the degree of multiple paternity were significantly higher for high predation populations (Matthews et al., 1997; Kelly et al., 1999).

Our results show a strong positive correlation between the minimum number of sires and the estimated number of sires in *Poecilia gillii* ($r = 0.698$, $p < 0.001$). The mean minimum number of sires (± SE) for *P. gillii* calculated in GERUD was 2.1 ± 0.16 (range 1–4), which is comparable to the minimum number of sires reported in other poeciliids: e.g., 1.5 in *Heterandria formosa* and 2 in *Gambusia holbrooki* (Zane et al., 1999; Soucy and Travis, 2003; Simmons et al., 2008; Coleman and Jones, 2011; Girndt et al., 2012). The mean estimated number of sires for *P. gillii* (4.2 ± 0.35 SE) was slightly higher than those reported for other poeciliid species: e.g., 1.8 in *Xiphophorus hellerii*, 3 in *Poecilia latipinna*, and 3.5 in *Poecilia reticulata* (Hain and Neff, 2007; Neff et al., 2008; Tatarenkov et al., 2008; Girndt et al., 2012) likely related to the large brood sizes in *P. gillii* (mean ± SE brood size: 47.2 ± 3.0 embryos; range: 4–130; $N = 72$ females).

Effect of Female Traits on Multiple Paternity

We found a significant positive correlation between brood size and the number of sires in *Poecilia gillii* (GERUD; model 3: $Z_{25} = 2.393$, $p = 0.017$, COLONY; model 4: $Z_{26} = 2.198$, $p = 0.028$). This correlation can arise simply because the larger a brood, the larger the number of potential sires that can contribute to that brood (Avise and Liu, 2011), but it could also arise because females with larger broods mate with more males (e.g., possibly to ensure fertilization of all eggs, or to increase the genetic variability of her offspring in bigger broods). To date, correlations between brood size and multiple paternity have been studied in species from four genera of the family Poeciliidae: *Gambusia*, *Heterandria*, *Poecilia*, *Xiphophorus*. Similar positive relationships have been reported in the closely related *Poecilia latipinna* and *P. reticulata* (based on estimated number of sires: Travis et al., 1990; Greene and Brown, 1991; Neff et al., 2008; Girndt et al., 2012; Zeng et al., 2017), and the more distantly related *Xiphophorus birchmanni* (based on minimum number of sires: Paczolt et al., 2015) and *Gambusia holbrooki* (based on minimum and estimated number of sires: Zeng et al., 2017). Some studies did, however, not find a positive relationship between brood size and multiple paternity; i.e., no significant correlation was found in *Gambusia affinis*, *Heterandria formosa* and *Xiphophorus helleri* (Greene and Brown, 1991; Soucy and Travis, 2003; Tatarenkov et al., 2008).

We did not find a significant relationship between the number of sires and female size in *P. gillii*. Most previous studies in the Poeciliidae do not report a significant correlation between female size and multiple paternity (Trexler, 1997; Zane et al., 1999; Neff et al., 2008; Girndt et al., 2012; Paczolt et al., 2015; Zeng et al., 2017), although a significant relationship was found in *Gambusia affinis* and *Poecilia latipinna* (Travis et al., 1990; Greene and Brown, 1991). We expected to find a positive relationship between female size and multiple paternity, as many earlier studies have shown a male preference for larger females (including the closely related *P. reticulata* and *P. latipinna*; Bisazza et al., 1989; Ptacek and Travis, 1997; Dosen and Montgomerie, 2004; Herdman et al., 2004; Hoysak and Godin, 2007; Schlupp, 2018). But this preference, usually assessed using choice-experiments in the lab, might not reflect actual mating opportunities in natural situations. In mating systems that include sexual harassment, larger females may be better than small females at avoiding unwanted sexual copulation attempts; i.e., earlier studies have shown that larger females are more likely to swim away from approaching males by moving to deeper and faster flowing water to avoid sexual harassment by males (Brewster and Houde, 2003; Croft et al., 2006; Magellan and Magurran, 2006). Laboratory and field observations have shown that *Poecilia gillii* lacks courtship behavior and relies solely on coercive mating, with males relentlessly chasing females and females continuously fending of obtrusive and unwanted suitors (Ptacek, 2005; Goldberg et al., 2019; Furness, Hagmayer and Pollux, unpublished data). Female poeciliid fish seem to avoid these coercive mating attempts in two ways: by chasing away males or by moving to a habitat with a lower density of males (Croft et al., 2006; Magellan and Magurran, 2006; Magurran, 2011). In both strategies, larger females are likely to be more successful in avoiding unwanted mating attempts than smaller females. In other cases, when some males are dominant and deny female access to other males (e.g., in swordtails: Luo et al., 2005), larger females might actually have lower rates of multiple paternity. This may be the case in *Poecilia gillii*, as some males are dominant and guard a harem of females/a territory, continuously chasing away competitors (Goldberg et al., 2019; Furness, Hagmayer and Pollux, unpublished data). Further, male preferences could also be more complicated, as was for instance shown in *Brachyrhaphis rhabdophora*: large males of this species had a preference for large females, but small males had a preference for small females (Basolo, 2004). Because we do not have information on the body sizes of sires in this study, we cannot assess whether this was the case for *Poecilia gillii* as well.

Paternity Skew

Our study shows that paternity is strongly skewed in *Poecilia gillii*; skew was on average 0.27 (in which 0 represents no skew, and 1 represents the maximal skew), and in 21 out of the 27 multiple sired broods, sires did not contribute equally to the offspring. Substantial skew was also observed in natural populations of several other poeciliid species: *Gambusia holbrooki* (Zane et al., 1999), *Poecilia latipinna* (Girndt et al., 2012), *P. reticulata* (Neff et al., 2008), *Xiphophorus birchmanni* (Paczolt et al., 2015), *X. hellerii* (Simmons et al., 2008; Tatarenkov et al., 2008), *X. multilineatus* (Luo et al., 2005), and *X. nigrensis* (Smith, 2014). The high degree of paternal skew may be due to both pre- and post-copulatory mechanisms (discussed below).

Pre-copulatory Mechanisms

There are several pre-copulatory mechanisms that might cause male reproductive skew. First, female mating behavior can lead to reproductive skew. A compelling example can be found in the guppy (*P. reticulata*), where females can increase the duration of copulation when mating with an attractive male, thereby increasing the amount of sperm inseminated (Pilastro et al., 2004, 2007). Second, females may 'trade-up,' meaning that they mate with additional males if they encounter males of better genetic quality than those previously mated with. This strategy tends to increase reproductive skew, particularly if the female preferentially uses the sperm from the most recent copulation event to fertilize her eggs, a phenomenon that is referred to as last-male sperm precedence (Pitcher et al., 2003). Third, paternity skew can be caused when males in a population vary in their reproductive success. This might occur when there are different male mating strategies in the population. In *Xiphophorus nigrensis*, for example, different mating strategies exist within a single population: sneaky and displaying males. For this species, reproductive success was significantly lower for sneaky males than for the dominant displaying males (Zimmerer, 1989). In guppies, males also have these two mating strategies, and Pilastro and Bisazza (1999) showed that courting males delivered three times higher numbers of sperm to the females than the sneaky ones, which significantly increased their probability of insemination. This could also be the case in *Poecilia gillii*, as males show different mating strategies with large and colorful dominant males guarding a harem of females and continuously chasing away competitors, whereas small and dull males mimic females in appearance and sneak mate when the opportunity presents itself (Goldberg et al., 2019; Furness, Hagmayer and Pollux, unpublished data).

Post-copulatory Mechanisms

The high degree of skew could also be an indication that there is strong post-copulatory selection. For instance, sperm competition can lead to increased fertilization chances for fast-swimming sperm, leading to a higher contribution of males with fast sperm compared to males with slower swimming sperm (Constantz, 1984; Evans and Pilastro, 2011). Sperm competition is enhanced by polyandry (Constantz, 1984; Evans and Pilastro, 2011), therefore, the high degree of skew found in *P. gillii* could be caused by the high occurrence of multiple mating in *P. gillii.* Another factor that influences post-copulatory selection is sperm storage, which is common among poeciliids (Lopez-Sepulcre et al., 2013). When sperm is stored there is a prolonged time for sperm competition (Birkhead and Parker, 1997), and more opportunity for the females to influence paternity by cryptic female choice (Birkhead, 1998; Evans and Pilastro, 2011).

CONCLUSION AND FUTURE PERSPECTIVES

In this study, we assessed the influence of brood size and female length on the degree of multiple paternity and paternity skew in the live-bearing fish *Poecilia gillii*. Our results show that there is a high degree of multiple paternity and male reproductive skew in *P. gillli*. Since both the degree of multiple paternity and male mating strategies tend to vary greatly between populations/species of Poeciliidae, future studies should focus on elucidating the link between observed male mating strategies in natural populations (e.g., sneak mating, dominance and courtship), and the degree of multiple paternity and paternal skew. In addition, since many factors can potentially affect multiple paternity in the field, controlled laboratory studies should be performed to help identify the proximate causes of multiple paternity. Finally, the relative contribution of pre- (i.e., behavior) and post-copulatory sexual selection processes (i.e., sperm competition or cryptic female choice) to male reproductive skew can be assessed in controlled laboratory studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was assessed by the institutional Animal Welfare Body (AWB) of Wageningen University (the Netherlands). The AWB judged that the study complied with the Dutch Act on Animal Experiments (AAE), which complies to European Directive 2010/63/EU. All fish were collected and exported following the regulations of the Costa Rican government under permit number SINAC-CUS-PI-R-005-2017, SINAC-PNI-ACLAP-004-2020, and SINAC-ACOSA-DT-PI-INV-003-2020.

AUTHOR CONTRIBUTIONS

MD and BP conceived the project idea. MD, AH, AF, and BP planned the fieldwork and collected the samples. MD and KL carried out the dissections and performed the molecular analyses. MD analyzed the data and wrote the first draft of the manuscript supervised by BP. All authors critically reviewed the initial manuscript, provided helpful input and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.579105/full#supplementary-material

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Does Asymmetrical Gonopodium Morphology Predict Lateralized Behavior in the Fish *Xenophallus umbratilis*?

Erik S. Johnson[1]*, **Mary-Elise Nielsen**[1] **and Jerald B. Johnson**[1,2]

[1] Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT, United States, [2] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT, United States

Edited by:
Renoult P. Julien,
UMR5175 Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), France

Reviewed by:
Michel Raymond,
UMR5554 Institut des Sciences de l'Evolution de Montpellier (ISEM), France
Felix Ströckens,
Ruhr University Bochum, Germany

***Correspondence:**
Erik S. Johnson
smerikjohnson@gmail.com

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Why bilaterally symmetrical organisms express handedness remains an important question in evolutionary biology. In some species, anatomical asymmetries have evolved that accompany behavioral handedness, yet we know remarkably little about causal links between asymmetric morphological traits and behavior. Here, we explore if a dextral or sinistral orientation of the male intromittent organ predicts side preferences in male behaviors. Our study addresses this question in the Costa Rican livebearing fish, *Xenophallus umbratilis*. This fish has a bilaterally symmetrical body plan, with one exception—the male anal fin (gonopodium), used to inseminate females, terminates with a distinct left- or right-handed corkscrew morphology. We used a detour assay to test males for side biases in approach behavior when exposed to four different stimuli (predator, potential mate, novel object, empty tank control). We found that left morph males preferred using their right eye to view potential mates, predators, and the control, and that right morph males preferred to use their left eye to view potential mates and predators, and their right eye to view the control. Males of both morphs displayed no eye bias when approaching the novel object. Our results suggest that there is a strong link between behavior and gonopodium orientation, with right and left morph males responding with opposite directional behaviors when presented with the same stimuli. This presents the intriguing possibility that mating preferences—in this case constrained by gonopodial morphology—could be driving lateralized decision making in a variety of non-mating behaviors.

Keywords: detour test, eye-bias, laterality, livebearing fishes, mate choice, novel object, Poeciliidae, predator-mediated behavior

INTRODUCTION

Body plan can have an important effect on behavior. For example, radially symmetrical species move differently than bilaterally symmetrical species (Hollo and Novak, 2012; Wakita et al., 2020). For example, bilateral symmetry typically results in behaviors that occur in an anterior-posterior orientation, including foraging (Jumars et al., 2015; Kane et al., 2017), locomotion (Hollo and Novak, 2012), and mating (Koshio et al., 2007), whereas radial symmetry leads to no such orientation (Wakita et al., 2020). An important property of bilateral symmetry is that it also allows an individual to express handedness, defined here as the propensity to use one side of the body preferentially over the other (Palmer, 2006; Bryden, 2016); we use the term "handed" in its broad sense as defined in previous work (Hata et al., 2011; Buchanan et al., 2015). Handedness is typically understood in a morphological context (Bock and Marsh, 1991), such as differences in the properties of structures [e.g., claw size in crabs (Spani et al., 2020)], or the orientation of structures [e.g., shell torsion in snails (Kurita and Wada, 2011)]. However, handedness can also be expressed behaviorally (Wiper, 2017). This occurs through decision-making, expressed where an individual moves to one side preferentially over the other, a phenomenon we refer to as behavioral handedness.

There is a growing body of research focused on behavioral handedness. For example, we know that such handedness can be expressed differently among closely related species (Bisazza et al., 1997b). We also know that in some cases, males and females differ from each other (Fitch et al., 1993). Further, we know that behavioral handedness can vary depending on environmental context, including factors such as individual posture or task complexity in primates, or neonatal handling in rats (Fitch et al., 1993; Fu et al., 2019). However, in each of these cases, the focal species studied exhibited morphological symmetry—that is, the morphologies on the left and right sides of the body were essentially mirror images. What happens in species that are bilaterally asymmetrical in some aspect of their morphology? We are still learning about behavioral handedness in these cases (Vallortigara and Rogers, 2005; Gunturkun et al., 2020). Clearly, such information may be critical to understanding why such behavioral biases evolved in the first place.

To address this gap in our understanding requires a species that shows a morphological asymmetry for some functional trait, and that also has the potential to show behavioral handedness. Here we present such a system. *Xenophallus umbratilis* is a livebearing fish native to Costa Rica; it shows a morphological asymmetry in the male mating structure, the gonopodium. We posit that this forces males to approach potential mates by turning their body to the left or right, in order to evaluate a female and to successfully copulate with her. Previous work in livebearing fishes shows that in some species, males have an eye-bias wherein they use one eye to evaluate mates and the other eye to evaluate risk, such as predators (de Andrade and de Sousa, 2018). Hence, in *X. umbratilis*, it may be that such eye-biases exist, but that they are expressed differently in left morph males vs. right morph males.

In this study, we address two questions. First, does *X. umbratilis* show behavioral handedness in response to different types of stimuli? To address this question, we tested if individuals have an eye preference as they approach potential mates, predators, and a novel object. We compared eye preferences across these different stimuli between left morph males and right morph males. Second, we asked if gonopodial morph had an effect on behavioral handedness? That is, does gonopodium morphology predict the direction of detour behavior? To address our second question, we compared preferences of right and left morph males when approaching each of the different stimuli. We found that behavioral handedness does indeed occur in this species, and more importantly, that it is associated with the orientation of the gonopodium.

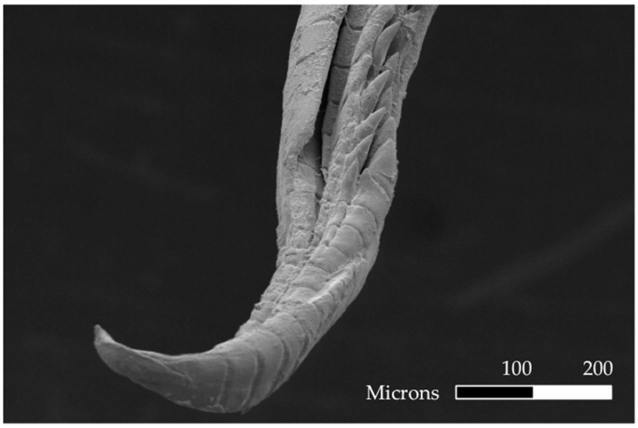

FIGURE 1 | Electron micrograph of a male gonopodium with a sinistral orientation. Scale bar is 100 microns.

MATERIALS AND METHODS

Study System

We addressed our questions using the livebearing fish species, *X. umbratilis* (hereafter referred to as *Xenophallus*). *Xenophallus* is the lone species in a monotypic genus (Parenti and Rauchenberger, 1989; Jones and Johnson, 2009). Its range includes several drainages in the Arenal and Guanacaste provinces of northern Costa Rica (Bussing, 1987). This species—like all members of the family Poeciliidae—give birth to live, free-swimming young that develop from eggs fertilized inside the female. Males transfer sperm directly to females using a modified anal fin called the gonopodium, which they bring in contact with the urogenital pore of the female. This structure varies among species, ranging from relatively simple rod-like morphs to rather complex forms with barbs and hooks (Langerhans, 2011). Yet, the gonopodium of *Xenophallus* is unlike other livebearers. It shows a unique form of asymmetry. At the tip of the shaft, the gonopodium turns in either a dextral or sinistral corkscrew (**Figure 1**). In the wild, both morphs are common within populations; in fact, in all populations that have been adequately sampled (n >5 males) to date, we found both male morphs (with one exception; see **Table 1**). However, the frequency of each morph appears to vary among populations at any given time; of 14 populations with both morphs that we surveyed between 1998 and 2019, we found dextral morph frequency ranged from 0.12 to 0.75 (see Results for details). Hence, gonopodium asymmetry in this species appears to be common and widespread.

To test our hypotheses, we used live fish held in captivity that we collected from two locations in Costa Rica (**Figure 2**). In June 2018, we sampled *Xenophallus* from Quebrada La Palma, a small stream in the Lake Arenal drainage of northern Costa Rica. In April 2019, we sampled *Xenophallus* from Quebrada Chorros, a similar stream, also in the Lake Arenal drainage, near the city of

TABLE 1 | Field collections of *Xenophallus umbratilis* evaluated in this study.

Locality	Population name	Date	Sample	Museum #	GPS	R	L	R:L
1	Tributary to Rio Bijagua	1/05	1-A	009364b,c	N 10° 43.885′ W 85° 03.321′	31	23	0.58
		5/06	1-B	009358b		3	0	1.00
		5/06	1-C	009374		4	0	1.00
		5/07	1-D	009387		4	0	1.00
2	Rio Esquivetto	5/06	2-A	009301b	N 10° 41.231′ W 85° 04.002′	12	23	0.52
3	Quebrada Azul	2/06	3-A	009354	N 10° 29.955′ W 84° 59.138′	6	7	0.46
4	Rio Sabalito	5/06	4-A	009381c	N 10° 32.920′ W 84° 58.837′	4	9	0.31
		5/07	4-B	009395		2	2	0.50
5	Quebrada Jilguero	4/98	5-A	009308b,c	N 10° 33.647′ W 84° 58.151′	22	8	0.73
6	Quebrada La Palma	5/06	6-A	009338b,c	N 10° 33.614′ W 84° 56.442′	6	45	0.12
		5/07	6-B	009392a,b		9	29	0.24
		5/18	6-C	Monitored		2	22	0.08
		6/18	6-D	Monitored		0	24	0.00
		5/19	6-E	Monitored		0	29	0.00
7	Trib. to lake Arenal	4/98	7-A	009345c	N 10° 30.076′ W 84° 50.436′	3	3	0.50
		2/06	7-B	009324		0	1	0.00
8	Quebrada Perez	4/98	8-A	009315b	N 10° 28.411′ W 84° 49.335′	4	3	0.57
		5/06	8-B	009329b		9	3	0.75
9	Vuelta de la Borracho	5/07	9-A	Unassigned	N 10° 25.646′ W 84° 45.135′	11	38	0.22
10	Rio Agua Caliente	5/07	10-A	Unassigned	N 10° 26.103′ W 84° 43.402′	10	23	0.30
11	Quebrada Chorros	5/19	11-A	Monitored	N 10° 28.367′ W 84° 39.450′	46	0	1.00
12	Rio Isla Grande	5/06	12-A	009300c	N 10° 23.581′ W 83° 58.090′	7	10	0.41
13	Rio Corinto	5/06	13-A	009294c	N 10° 12.674′ W 83° 53.114′	23	17	0.58
		5/07	13-B	009385		5	7	0.42
14	Upper Rio Tortuguero	5/07	14-A	009394	N 10° 15.565′ W 83° 48.734′	2	4	0.33

*Locality numbers correspond to those found on the map in **Figure 2**. Date depicts the month/year that the collection was made; where multiple collections were made from the same locality, they are designated by a letter. The museum # indicates the population accession number in the Brigham Young University Monte L. Bean Life Science Museum (MLBM); "Monitored" indicates populations where fish were collected, scored, and returned unharmed to the stream; "Unassigned" indicates samples that have yet to be assigned a number in the MLBM. R and L show the number of individual males with a right morph and left morph gonopodium, respectively, from each sample. R:L indicates the right to left morph ratio.*

Fortuna. We chose these two locations for several reasons: first, they are geographically close to each other, and therefore likely to experience similar micro-climate effects in the wild (**Figure 2**); second, the locations are ecologically similar for a variety of measures (**Supplementary Table 1**); and finally, at the time of collection, the two localities differed in the relative frequency of left- and right-morph males, allowing us to bring both morph types into the lab (**Table 1**). For both sampling events, we collected and transported 150 live fish to the fish breeding facility at Brigham Young University, where they were treated for parasites and held under common laboratory conditions (as described below). By chance, all adult males available for this study from Quebrada La Palma were left morph males and all adult males available from Quebrada Chorros were right morph males (see **Table 1** for collection details). We recognize the potential confounding influence of locality with male morph type; however, our sampling was designed to minimize ecological differences between sites (see above). Hence, if locality effects exist, they are less likely to be ecological than genetic, a topic we will address in future work focused on the inheritance of male gonopodial morphs. Fish held in the lab, both males and females, were fed *ad libitum* twice daily and kept in 10-gallon tanks at 23°C on a 12L:12D light cycle. For both collections, individuals were kept in this common laboratory environment for 4 months prior to testing; in this way, we attempted to minimize any affect that condition might have on behavior during the trials.

Experimental Design

Our study was designed to determine if *Xenophallus* males show a bias with respect to the side that they use to approach different stimuli, and if this bias differs between left morph males and right morph individuals. We isolated 15 males of each morph (30 total fish) by placing them in individual tanks so each fish could be exposed to each stimulus (details explained below). We used a detour test approach similar to the one used by Bisazza et al. (1997a,b) to test for behavior handedness, a technique widely adopted in behavioral research (Kabadayi et al., 2018). This involved placing a single male in an arena built within a tank (**Figure 3**) and allowing him to swim back and forth from one end to the other. At each end of the raceway in the arena the male came to a fork where he was given a choice to proceed to the right or left. In a screened area behind the detour, we placed a visual stimulus. This enabled the individual in the trial to see the stimulus as it approached the detour fork, but in order to clearly

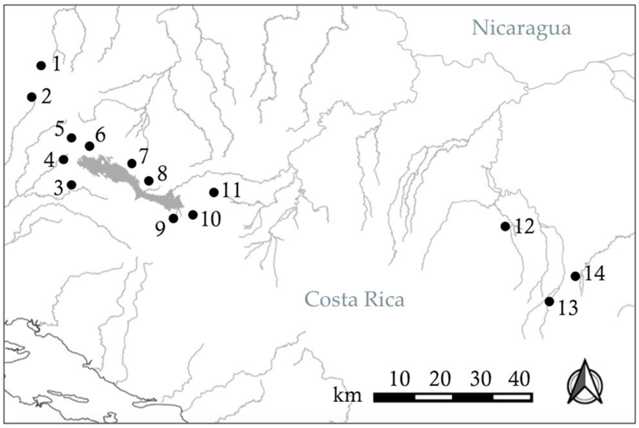

FIGURE 2 | Distribution of 14 populations of *Xenophallus* across their range in northern Costa Rica, sampled or monitored from 1998 to 2019. See **Table 1** for details.

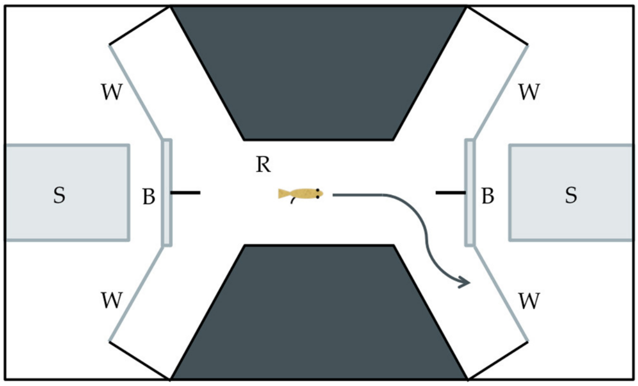

FIGURE 3 | Drawing of the experimental tank used in the detour behavioral assay. (S) The stimulus insert is made of clear glass and used to house the live, physical stimulus. (B) The semi-opaque barrier partially obscures the stimulus from an individual in the raceway area. (W) The viewing window allows an individual to clearly see a stimulus through clear glass. (R) The raceway is where an individual moves toward a stimulus and has the opportunity to make a left or right detour.

view the stimulus, the focal male had to detour either to the right or the left. Individuals were allowed to move in the tank in both directions, making a choice each time they move from one end to the other. They were allowed to move freely without prodding so we could attribute their detour behavior directly to the stimulus. Each fish was given 20 min in the arena and we recorded the number of right and left detours. The male was then removed from the arena and the water was filtered for 10 min prior to the next trial. This was repeated for up to 30 individual males (15 dextral and 15 sinistral) for each visual stimulus, although for some treatments we tested slightly fewer males ($n = 13$–15 per male morph per treatment). Individuals were randomly assigned to each stimulus treatment (see below). We deemed this sample size sufficiently large based on similar studies in other species (Bisazza et al., 1997a; Torres-Dowdall et al., 2019), but not so large as to unnecessarily use live animals as per our approved IACUC protocol (see Acknowledgments). The entire arena was housed in a walk-in, sound-proof room, allowing all observations to be made remotely via a camera mounted over the tank.

We predicted that *Xenophallus* males might respond differently to different stimuli. Hence, we ran our detour assay with three different live stimuli as well as a control. The stimuli we tested were: (1) *Xenophallus* adult females (potential mates); (2), the fish *Parachromis dovii* (a known predator of *Xenophallus*); (3) a novel object (Lego, see explanation below) that *Xenophallus* would have never previously encountered; and (4) a negative control which consisted of an empty stimulus tank. These stimuli were chosen with the idea that males might specialize in the eye that they use to secure this visual information, perhaps as a function of their gonopodial anatomy. The stimuli were physically placed at either end of the tank. When viewed from straight on, the stimulus was partially obscured (see **Figure 3**), requiring the fish to make a lateral choice to view it clearly. Because teleost fish are unable to use both eyes to focus on the same object (Land, 2015), this test reveals which eye a fish actually uses to view the stimulus. We isolated six size-matched females to be used as potential mate stimuli, randomly selecting two of them to use in each trial. Predators were similarly selected for use from four different *P. dovii* individuals held in the lab. Finally, we chose a Lego stack, consisting of orange, yellow, blue, white, and red blocks in the shape of a "Y" as a novel object. This was an item that we thought would attract the interest of *Xenophallus* males, but that was completely foreign to this species. Overall, this design allowed us to determine if there is a behavioral bias in the way that male *Xenophallus* approach different stimuli, and also if this bias is at all affected by male gonopodial morph type.

Statistical Analyses

To determine if these fish show a bias in behavioral handedness, we followed the statistical approach of Torres-Dowdall et al. (2019). This required us to first calculate a laterality index (LI), which we used as a measure of the degree to which an individual shows a bias in right- or left-handed detours. We calculated this metric as follows:

$$\mathrm{LI} = \frac{\text{Detour Right} - \text{Detour Left}}{\text{Detour Right} + \text{Detour Left}} * (100)$$

Positive LI values indicate a right-handed detour bias, consistent with a preferred use of the left eye to view the stimulus. Negative LI values indicate a left-handed detour bias, consistent with a preferred use of the right eye to see the stimulus. A laterality score statistically indistinguishable from "0" indicates no bias in lateralized behavior. We calculated a mean laterality score for each treatment for both left morph and right morph males, resulting in eight tests. We analyzed these results using a two-tailed, one-*sample t*-test to determine if the observed LI in each treatment showed a significant departure from zero. All statistical tests were run in program R (R Core Team, 2020; version 3.6.3).

RESULTS

Detour Tests

Xenophallus shows clear behavioral handedness in detour decisions when approaching various stimuli (**Figure 4**). Of the eight tests that we ran, six showed average LI scores that differed significantly from 0. Only the novel object treatment failed to elicit a significant response, which was true for both left morph ($\text{LI} = -4.96$, $t_{13} = 0.72$, $p = 0.49$) and right morph ($\text{LI} = 3.92$, $t_{12} = 0.62$, $p = 0.54$) males. Left morph males responded to the potential mate stimulus and the predator stimulus completely opposite of the right morph males. Left morph males detoured left in response to potential mates ($\text{LI} = -34.22$, $t_{13} = 4.41$, $p < 0.001$) and in response to predators ($\text{LI} = -33.11$, $t_{13} = 7.39$, $p < 0.001$). In contrast, right morph males detoured right in response to potential mates ($\text{LI} = 20.16$, $t_{13} = 2.07$, $p = 0.05$) and predators ($\text{LI} = 21.65$, $t_{12} = 2.37$, $p = 0.03$). Finally, in response to a no-stimulus control, we found the interesting pattern that both left morph males ($\text{LI} = -23.71$, $t_{14} = 2.49$, $p = 0.03$) and right morph males (-24.73, $t_{14} = 2.35$, $p = 0.03$) preferentially detoured to the left, rather than approaching the control at random.

Male Morph Frequencies in the Wild

Of 14 populations of *Xenophallus* sampled between 1998 and 2019, almost all contained both left morph and right morph males (**Figure 5**; **Table 1**). In fact, we found no evidence that either male morph was fixed in a population where at least 5 males were sampled (see **Table 1**). This was true, regardless of collection locality, drainage, season of collection, or year of collection. The exception during our entire sampling were two samples taken at the La Palma site (sample 6-D, 6-E) that appeared to be fixed for the sinistral morph the last two times we sampled and one sample from Rio Chorros (sample 11-A) that appeared to be fixed for the dextral morph (**Table 1**). Moreover, we found considerable variation in the frequency of dextral and sinistral morphs among populations at the time they were surveyed, with some samples composed primarily of left morph males and others composed primarily of right morph males (**Table 1**). Interestinglym, one location, Rio Corinto, was sampled at two different times with the frequency of male morphs shifting from a right bias when we first sampled in 2006 to a left bias when we later sampled in 2007.

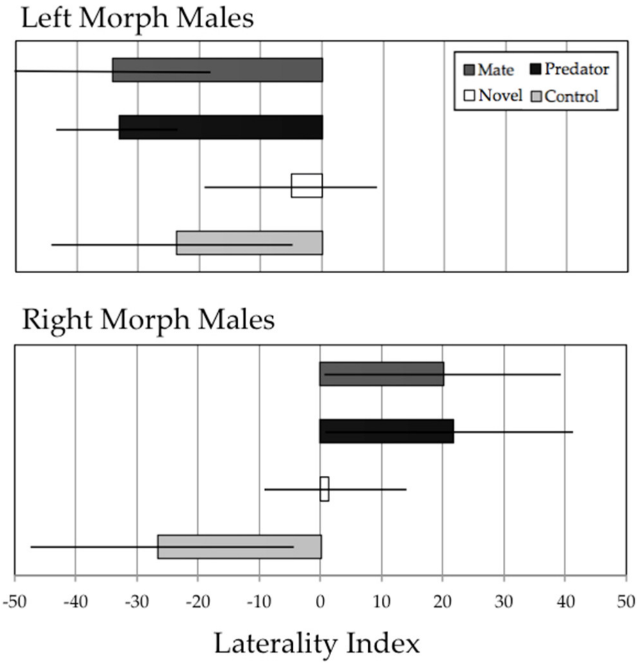

FIGURE 4 | Results of the detour assay for left morph males (upper panel) and right morph males (lower panel). Each group of males was exposed to four experimental stimuli: potential mate, predator, novel object, and empty tank control. The Laterality Index (LI) is defined in the methods; briefly, negative LI scores indicate detour to the left, and positive LI scores indicate detour to the right. Figure shows a bar chart with error bars depicting ± 2 SE.

DISCUSSION

Our study provides insight into the relationship between the anatomic asymmetry of the gonopodium and male behavior. It is perhaps not surprising to find that males approach potential mates in a non-random way—the gonopodium is used in copulation, and there is considerable evidence that the structural anatomy of the gonopodium in other poeciliids is important for successful sperm transfer (Langerhans, 2011). Specifically, in *Xenophallus* we found that males with gonopodia that terminate to the left approach females by detouring left, and the opposite was true for males with gonopodia that terminate to the right. Although we know little about how the gonopodium actually interacts with the female gonopore in *Xenophallus*, our results suggest that there may be some directionality to mating such that males with a particular morph might be more successful in mating on one side of the female than the other side. These results also set up the intriguing possibility that the success of males might depend to some extent on the frequency of each male morph in the population, with rare males having an advantage if females actively try to avoid forced copulations by more common males. Hence, this system may be well-suited for evaluating negative frequency dependent selection in the wild (Palmer, 1996), similar to the classic work on lateralized feeding on scales in cichlid fishes, where individuals with rare morphs had an advantage over their counterparts with the more common morph (Hori, 1993).

We also found that males detoured in a non-random way when evaluating predators. However, unlike previously published work which showed that some poeciliids evaluate predators and potential mates with opposite eyes (Torres-Dowdall et al., 2019), we found that *Xenophallus* males actually detour in the same direction in response to predators as they do to potential mates. Left morph males detour left in response to predators, and right morph males detour right in response to predators. These results are intriguing in two important ways. First, there appears to be an association between gonopodium morphology and predator inspection behavior. This is unexpected—there is

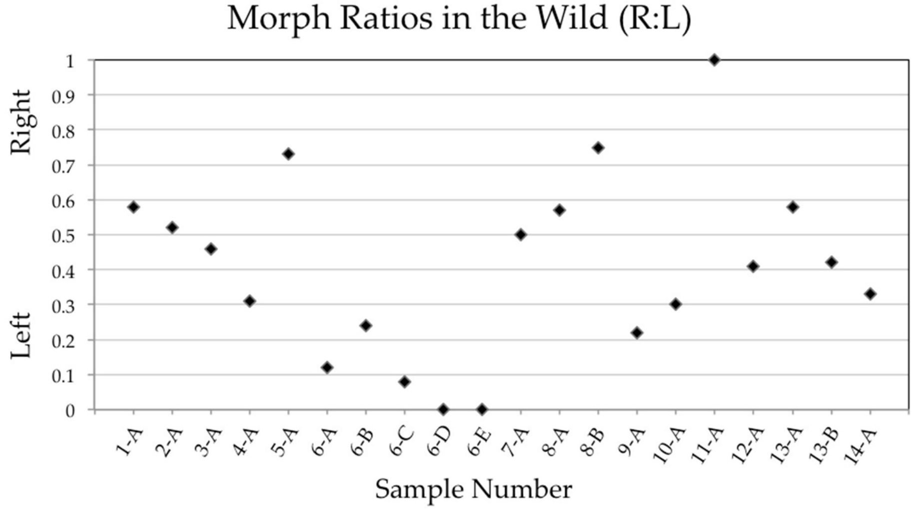

FIGURE 5 | Plot showing gonopodial morph ratios (right to left) of samples taken from wild populations between 1998 and 2019. Sample numbers correspond to those found in **Table 1**. We only include samples here where the number of individuals scored was >5.

no *a priori* reason that gonopodium morphology should favor viewing predators from one direction over another. Rather, previous work has suggested that side-bias in inspection behavior in fishes may have evolved to allow prey species to preferentially use one eye, and one side of the brain, to gather and process information about predation risk. If that is true in *Xenophallus*, then our data indicate that predator inspection behavior is not fixed to one eye or the other, and it appears to be associated with gonopodium morphology. Second, our results are inconsistent with previous work that argued that fish use one eye to specialize in gathering information about potential mates and the opposite eye to gather information about predation risk (Langerhans, 2011). We found that *Xenophallus* males use the same eye for both. Hence, there appears to be no division of labor among eyes and brain processing that some authors have suggested in other species.

Two other interesting patterns emerged from our detour trails. First, we found that neither left morph nor right morph males showed a side bias when approaching a novel object. There was simply no preference. This is important because it shows that *Xenophallus* are not fixed in their detour behaviors irrespective of the stimulus present. In other words, it tells us that males are deliberate in their response to potential mates and predators. Second, we found that in our control tests, where there was no stimulus present, that both left morph and right morph males consistently detoured to the left. That is, when faced with a barrier, and nothing present to inspect, males more often went around that barrier on the left side. At face value, this is a somewhat puzzling result. Why should individuals all show a detour preference in the absence of a stimulus? Our result, however, is consistent with an intriguing hypothesis that in the absence of a target stimulus, individuals in social species might move preferentially to one side to avoid collisions with other individuals (Vallortigara and Rogers, 2005). This so called "lateral locomotor bias" could result in social coordination resulting in the majority of individuals moving to the same side, even when that side is arbitrary. This type of lateralized social coordination has been found in a variety of other systems, including ants and bees (Frasnelli et al., 2012; Rogers et al., 2013), amphibians (Dadda et al., 2003), and fishes (Bisazza et al., 2000). In fact, a similar explanation is found for some lateralized human behaviors such as embracing, kissing, and cradling (Ocklenburg et al., 2018; Packheiser et al., 2019). Hence, our system with *Xenophallus* may also prove useful to test this more general hypothesis that social coordination can play a role in population-level lateralization.

Finally, our work might shed some light on the very fundamental question of why brain lateralization has evolved in so many vertebrate species. It is unclear why the gonopodium in *Xenophallus* is asymmetrical. It could simply have evolved as a randomly dimorphic trait. However, it appears that once this trait is present, it has potentially had an effect on lateralized behavior. We might imagine a scenario in which the evolution of anatomical asymmetries could lead to behavioral handedness, which in turn could lead to a specialization of the brain in processing information acquired from different sides of the body. Fishes have a general body form where the eyes are found on the sides of the head—not forward as in organisms with well-defined binocular vision—encouraging them to orient their body to one side or the other in order to obtain clear visual information. Hence, fishes, including systems like *Xenophallus*, are promising candidates for exploring different aspects of the origins of brain lateralization. Although future work will be necessary to evaluate these ideas, what is clear from our research is that there is a strong relationship between gonopodium morphology and lateralized behavior. Understanding the cause of this relationship may help reveal why so many forms of handedness exist in nature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Brigham Young University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EJ and JJ conceived the idea for the study, designed the statistical analysis, and revised the manuscript in response to reviewer comments. EJ designed and built the test arena, collected the experimental data, and wrote the first draft of the manuscript. All authors reviewed and edited the manuscript, approved its final version, contributed to the preparation, research, writing of the manuscript, and were involved in the field collections.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.606856/full#supplementary-material

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Consistent Behavioral Syndrome Across Seasons in an Invasive Freshwater Fish

Juliane Lukas[1,2*], Gregor Kalinkat[1], Friedrich Wilhelm Miesen[3], Tim Landgraf[4,5], Jens Krause[1,2,4] and David Bierbach[1,2,4]

[1] Department of Biology and Ecology of Fishes, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany, [2] Faculty of Life Sciences, Albrecht Daniel Thaer-Institute, Humboldt University of Berlin, Berlin, Germany, [3] Ichthyology Section, Zoological Research Museum Alexander Koenig, Bonn, Germany, [4] Cluster of Excellence "Science of Intelligence," Technical University of Berlin, Berlin, Germany, [5] Dahlem Center of Machine Learning and Robotics, Institute of Computer Science, Free University of Berlin, Berlin, Germany

Edited by:
Jerald B. Johnson,
Brigham Young University,
United States

Reviewed by:
Spencer J. Ingley,
Brigham Young University–Hawaii,
United States
Jared B. Lee,
Southern Virginia University,
United States

***Correspondence:**
Juliane Lukas
contact@julianelukas.com

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Understanding the linkage between behavioral types and dispersal tendency has become a pressing issue in light of global change and biological invasions. Here, we explore whether dispersing individuals exhibit behavioral types that differ from those remaining in the source population. We investigated a feral population of guppies (*Poecilia reticulata*) that undergoes a yearly range shift cycle. Guppies are among the most widespread invasive species in the world, but in temperate regions these tropical fish can only survive in winter-warm freshwaters. Established in a thermally-altered stream in Germany, guppies are confined to a warm-water influx in winter, but can spread to peripheral parts as these become thermally accessible. We sampled fish from the source population and a winter-abandoned site in March, June and August. Fish were tested for boldness, sociability and activity involving open-field tests including interactions with a robotic social partner. Guppies differed consistently among each other in all three traits within each sample. Average trait expression in the source population differed across seasons, however, we could not detect differences between source and downstream population. Instead, all populations exhibited a remarkably stable behavioral syndrome between boldness and activity despite strong seasonal changes in water temperature and associated environmental factors. We conclude that random drift (opposed to personality-biased dispersal) is a more likely dispersal mode for guppies, at least in the investigated stream. In the face of fluctuating environments, guppies seem to be extremely effective in keeping behavioral expressions constant, which could help explain their successful invasion and adaptation to new and disturbed habitats.

Keywords: animal personality, dispersal, range expansion, invasive species, thermally altered freshwaters, guppy

INTRODUCTION

Consistent behavioral differences among individuals (e.g., behavioral types or animal personality; Réale et al., 2007) seem to be a ubiquitous biological feature (Bell et al., 2009; Bierbach et al., 2017) that was proposed to have substantial ecological and evolutionary importance (Sih et al., 2012; Wolf and Weissing, 2012). For one, behavioral types are thought to be relevant in determining success

(Chapple et al., 2012; Carere and Gherardi, 2013; Canestrelli et al., 2015) and impact of biological invasions (Cote et al., 2010a; Fogarty et al., 2011; Juette et al., 2014), which is becoming an ever more pressing issue in the light of global change and ongoing translocations of species among habitats (Wong and Candolin, 2015; Piria et al., 2017; Seebens et al., 2017, 2018).

It is assumed that individuals that invade novel habitats are a non-random subset of their source population (Shine et al., 2011; Sih et al., 2012; Juette et al., 2014; Canestrelli et al., 2015; Spiegel et al., 2017). These invaders may share certain phenotypic and life-history characteristics that are associated with their behavioral types (Juette et al., 2014). Behavioral types of invading individuals may determine whether they are transported (by humans) or move (by themselves) into new environments (first invasion stage; Lockwood et al., 2011), whether they can survive and establish a non-native population (second stage), and whether they may spread to new habitats thereafter (third stage). Behavioral types shown to be relevant in this regard are boldness (Cote et al., 2011; Myles-Gonzalez et al., 2015), aggression (Duckworth and Badyaev, 2007; Hudina et al., 2015), activity (Brown and Irving, 2014; Thorlacius et al., 2015) as well as sociability (Cote et al., 2010b, 2011; Rasmussen and Belk, 2012; Camacho-Cervantes et al., 2015). Further, it was proposed that individuals carrying correlated suites (i.e., behavioral syndromes, Sih et al., 2012) of bold, aggressive, active and asocial types may predominantly be found at the invasion front (Duckworth and Badyaev, 2007; Cote et al., 2010b, 2011; Juette et al., 2014). However, such spatially and/or temporally assorted compositions of behavioral types among populations have only rarely been investigated along naturally occurring invasion gradients.

The difficulty to study invasion phenomena in the wild is their random and unpredictable occurrence (Lockwood et al., 2011), which makes it nearly impossible to follow active invasion fronts. In the rare cases where range expansions could be tracked in real time (e.g., western bluebirds: Duckworth and Badyaev, 2007; African jewelfish: Lopez et al., 2012; signal crayfish: Hudina et al., 2015; round goby: Thorlacius et al., 2015; cane toad: Gruber et al., 2017), the invasion origin is often unknown or unclear due to multiple introduction events. Thus, we need to identify study systems that allow us to investigate dispersal across sensible temporal and spatial scales. Thermally altered freshwater systems in the temperate zones provide fruitful conditions to study this phenomenon. Here, natural and/or anthropogenic warm water influxes create unique temperature refuges that allow non-native species (especially those of tropical origin) to establish populations (Langford, 1990; Jourdan et al., 2014). While ambient water temperatures drop in winter (e.g., sometimes below 1°C in the river Rhine; Jourdan et al., 2014), areas close to constant warm water influx will remain suitable for tropical non-native species to survive. Once peripheral areas become thermally suitable again in warmer seasons, certain individuals from the sources may disperse into these areas. Such a scenario enables us to investigate the role of behavioral types during *in situ* dispersal of non-native species from a single, well-determined source into new (i.e., winter-abandoned) habitats.

Under a personality-biased dispersal hypothesis, where individuals with a certain set of personality traits are assumed to leave the source population to colonize new habitats, we predicted that *(i)* the source population's average personality traits should change over the course of the year due to the drain of the emigrating individuals, *(ii)* newly founded populations should differ in their average personality traits compared to their source populations, and *(iii)* behavioral variation should decrease both in the source as well as in the newly founded population due to the non-random migration patterns. In addition, *(iv)* population-wide correlations among personality traits (behavioral syndromes) may emerge or disappear because of assumed non-random migration patterns.

In Germany, tropical fishes have established populations in several artificially heated stream systems (Lukas et al., 2017b), the most investigated being the Gillbach near Cologne (see **Figure 1A**, Jourdan et al., 2014; Lukas et al., 2017a; Kempkes et al., 2018). To estimate seasonal fluctuations in temperature-associated abiotic and biotic conditions within the sampled range of the stream, we continuously recorded water temperatures with submerged data-loggers. We sampled feral guppies (*Poecilia reticulata*) from the Gillbach during different seasons (temporal scale: spring, early summer, late summer) and, if possible, at different sites (spatial scale: core of warm water influx vs. more peripheral downstream area; see **Figure 1A**) and repeatedly assessed behavioral types in terms of boldness, sociability and activity in a laboratory setting.

MATERIALS AND METHODS

Study Site

The Gillbach (Rhine catchment, Germany) has been under the influence of thermal pollution and anthropogenic modifications for over six decades. Its headwaters are exclusively fed by the warm water discharge of the brown coal power plant Niederaußem (**Figure 1A**). This creates a temperature regime that is highly dependent on the volume and temperature of the plant's water discharge. Generally exhibiting above ambient water temperatures (**Figure 1B**), the Gillbach harbors several established populations of non-native fish, invertebrate and plant species, many of which are of tropical or subtropical origin (see **Supplementary Table 1** for a list of native and non-native fish species found in the Gillbach).

Here, feral guppies (*Poecilia reticulata*) are highly abundant and as successful global invaders (Deacon et al., 2011) they are well-suited to investigate the expression of behavioral traits across time and space. First records of guppies at the Gillbach date back to 1978 and the population has existed continuously for a conservative minimum of 10 years (Lukas et al., 2017b; Kempkes et al., 2018). The Gillbach population likely originated from a small number of released domesticated guppies (see **Supplementary Figure 4**, Jourdan et al., 2014; Lukas et al., 2017b), and thus would have undergone a demographic bottleneck (i.e., founders' effect) with potential consequences on genetic diversity. The Gillbach has since been subject to repeated introduction of guppies through intentional release (personal observation of JL, GK, FWM and DB). However, release points downstream of the source are unlikely due to limited access to the waterbody, thus all individuals captured below the source site

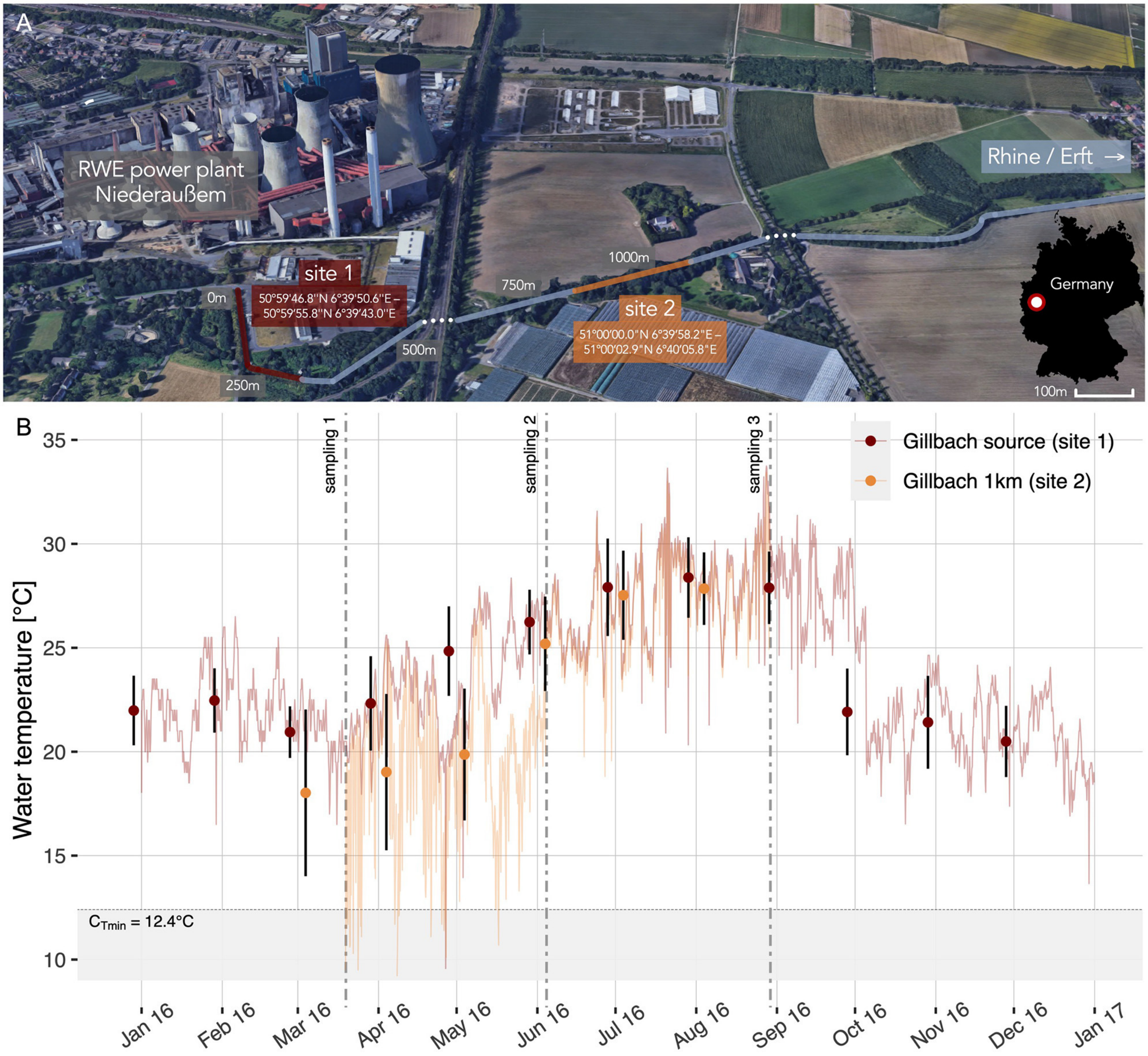

FIGURE 1 | The Gillbach stream and its altered temperature regime. **(A)** Warm water discharge from a power plant feeds into the Gillbach (map created using ©Google Earth; Imagery ©2019 Google, Map data ©2009 GeoBasis-DE/BKG) and generates a thermal gradient above ambient water temperatures that is preserved for several kilometers (site 1–site 2). **(B)** 4-hourly water temperature with error bars indicating monthly means ± SD for each site (site 1 Gillbach source: red; site 2 one kilometer downstream: orange). Dotted lines correspond to the three sampling occasions. The shaded region indicates temperatures below the critical minimum temperature (C_{Tmin}) of Gillbach guppies. Note that due to a loss of loggers, continuous monitoring at site 2 was interrupted (no data from 01.01.2016 to 19.03.2016 or from 29.08.2016 to 31.12.2016).

(site 1, **Figure 1A**) will have dispersed from the source at some point. The population's maximum spatial range is estimated to be < 2 km downstream, as no guppies have been found beyond this point during multiple samplings over multiple years (see **Supplementary Tables 1**, **2**, Höfer and Staas, 1998; Jourdan et al., 2014; Lukas et al., 2017a).

Sampling

Sampling was conducted at two sites (∼300 m transects, see **Figure 1A**) in March, June and August 2016. The Gillbach's source population (site 1) grew from a small core population surviving the winter to more than 4000 individuals by June, but decreased again in late August to a few hundred (**Supplementary Table 2**). This extreme population decline was likely predation-induced as densities of convict cichlids (*Amatitlania nigrofasciata*) and European chub (*Squalius cephalus*) also increased during this period (**Supplementary Table 1**). Sampling commenced until sufficient numbers for behavioral testing were reached (i.e., ∼40 adult individuals). Captured fish were transferred into well-aerated coolers and kept separately by sampling site. To reduce a possible sampling bias of certain behavioral types, we collected specimens by seine and dip netting and actively drove fish into the net as recommended by Biro and Dingemanse (2009). While we acknowledge some size selectivity against juvenile guppies introduced by the 2 mm mesh size, our population sample was representative of adult body size variation (see **Figure 3B** and **Supplementary Figure 3**), thus avoiding indirect bias of behavioral traits that are correlated with size (Polverino et al., 2016).

Water temperatures at the source (site 1) and one kilometer downstream (site 2) were monitored using HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA) for a period of 12 months (**Figure 1B**). For the period of sampling and behavioral testing, the Gillbach's thermal gradient resulted in a between-site temperature difference of 4°C in spring (March – May 2016, mean ± SD, site 1: 23.1 ± 2.6°C; site 2: 19.2 ± 3.6°C; **Figure 1B**) and ∼1°C in summer (June – August, site 1: 27.5 ± 2.2°C; site 2: 26.8 ± 2.4°C; **Figure 1B**). However, we expect the temperature difference between site 1 and 2 to be even more pronounced in colder months [e.g., difference between site 1 and 1 km downstream of site 2: 7°C in November (Klotz et al., 2013); 8.4°C in February (Jourdan et al., 2014)]. With temperatures below the population's critical minimum temperature (∼12.4°C after Jourdan et al., 2014, see **Figure 1B**), survival of guppies outside the core area during winter is highly unlikely.

Husbandry

Guppies collected for behavioral testing were transferred to the lab at Humboldt Universität zu Berlin, Germany. We determined sex and body size distribution of each population sample before introduction to the housing tank (pre-acclimation; see **Supplementary Figure 3**). Females and males obtained from the same site were housed communally in separate compartments of a 250-l fiberglass holding tank (LxWxH: 120 × 58 × 40 cm). Each compartment contained artificial java moss to serve as a shelter. The water was kept under continuous aeration and filtration, while the temperature was maintained at 26°C. Fish were held under diurnal lighting (12:12 h light:dark cycle). Offspring was removed twice a week. Adults were fed commercially available flake food (TetraMin® Tetra GmbH) twice a day. Feeding was halted 24 h before experiments and commenced afterwards. To acclimate the fish to the new laboratory conditions, no behavioral testing was conducted for at least 30 days (**Figure 2A**). Previous studies on poeciliids indicated that 4 weeks are sufficient for thermal up- or downward acclimation of the magnitude of 5–6°C (as experienced by spring-caught fish, see Chung, 2001; Seebacher et al., 2014). One week prior to testing, fish were marked using VIE tagging (see Jourdan et al., 2014 for procedure) to allow for individual recognition throughout the behavioral assessment.

Behavioral Assessment

We tested a total of 159 guppies (95 females, 64 males; for population-specific sample sizes see **Figure 2A**). Fish were included if their body size exceeded 15 mm standard length (SL) and if they showed external signs of maturity (females: gravid spot, males: fully developed gonopodium, see **Supplementary Figure 4**). Test fish were introduced to an open field test arena (88 × 88 cm, 75 mm water depth) filled with aged tap water kept at 26°C. To reduce disturbing stimuli, the arena was enclosed in black, opaque plastic and illuminated with artificial light reproducing the daylight spectrum. Behavioral observations were recorded via a camera (Ximea 4K USB 3.1 camera) mounted above the arena. Position information was extracted using EthoVision 10 XT software (Noldus Information Technology, Wageningen, Netherlands).

(1) Boldness: Latency to emerge from a shelter was used as a measure of boldness with a low latency time indicating high levels of boldness. Fish were introduced into a gray opaque shelter box (diameter: 100 mm; top left of each image in **Figure 2B**) and allowed to acclimate. After 1 min, a researcher removed the sponge from the entrance (40 × 25 mm), allowing the fish to emerge and explore the test arena. A fish was scored as having emerged when its full body was visible outside of the shelter. If fish did not emerge after 5 min (15 cases in 477 trials), the lid of the shelter box was removed. Fish that failed to emerge within 8 min (11 cases in 477 trials) were given the maximum score of 480 s and the box was removed entirely. As these interventions could have disturbed fish and influenced subsequent measures, we confirmed that the results were qualitatively similar if we fitted models excluding these data points (results not shown).

(2) Sociability: A three-dimensional fish replica was stationed 1 cm away from the shelter entrance. The replica, which resembled a large-sized guppy female (SL = 30 mm; left fish in **Figure 2B**), could be moved by a robot via a magnetic base (see Landgraf et al., 2016; Bierbach et al., 2018 for details). Upon the fish's emergence (or the removal of the shelter box), the biomimetic robot started to move through the arena in a standardized sequence (**Figure 2B**) imitating a conspecific. We calculated the mean distance the fish kept to the biomimetic robot as a proxy of sociability with lower values indicating higher levels of sociability. Consequently, fish that followed the robot closer were assumed to be more sociable (see Bierbach et al., 2018).

(3) Activity: After completion of the robotic fish's predefined path, the replica was removed from the arena by hand. Once the fish resumed swimming behavior (e.g., after a period of freezing), it was allowed to explore the arena. As a measure of activity, we calculated the mean velocity with which the fish explored the open arena during 3 min.

To minimize handling and exposure to stress (Animal Behaviour, 2020), all three traits were assessed consecutively (same test sequence) and within the same arena. We are aware that testing multiple traits within one behavioral assay and in the same order can introduce carryover effects and give rise to correlation artifacts (Carter et al., 2013; Seebacher et al., 2014), both of which are common caveats in personality research, but neither affected our interpretation of between-population differences. After the completion of the first behavioral assay, we sexed each individual and recorded its body length by photographing the fish in a water-filled petri dish with a reference grid. Fish were transferred back to their respective compartment and allowed 72 h resting before being

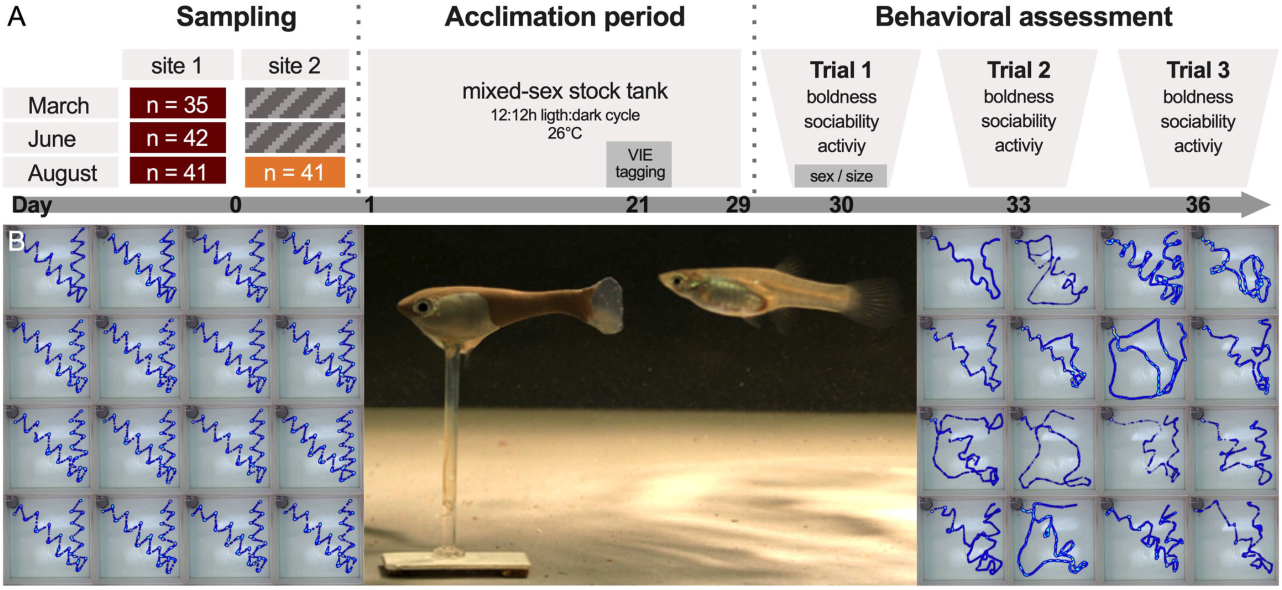

FIGURE 2 | Behavioral testing with robotic fish. **(A)** Test fish were collected three times and, when possible, at two sites (site 1 and 2, see **Figure 1A**). After an initial 30-day acclimation period, fish were tested a total of three times (trial 1–3, sample sizes indicate tested individuals, for total catch numbers see **Supplementary Table 2**). **(B)** During trials, the fish replica was moved remotely through the test tank (center). Once the focal individual had emerged from its shelter, the biomimetic robot initiated its predefined path (left) and the fish's swimming trajectory was recorded (right).

retested following the same protocol. To reduce acclimatization effects, we only assessed short-term repeatability by testing fish a total of three times within the span of a week. The order in which fish were tested was randomized for each trial.

Statistical Analysis

To test for differences in average trait expression over a temporal and/or spatial scale, we used linear mixed models (LMM, *GENLINMIXED* procedure) with each behavioral trait (boldness, sociability, activity) as a separate response variable. Boldness and sociability were $\log_{10}$-transformed to fulfill normality assumptions. All models included sample "population" as the fixed effect of interest (four levels: March site 1, June site 1, August site 1, August site 2). The low sample size collected at site 2 during March and June ($n = 8$ and $n = 2$, respectively; see **Supplementary Table 2**) made these estimates uncertain. After verifying that there were no substantial differences found when running the analyses with and without the above population samples, these estimates were excluded from final analyses. Behavior in poeciliids is often size- and/or sex-dependent (Brown and Braithwaite, 2004; Harris et al., 2010), thus we included "standard length" (SL) as a covariate and "sex" as an additional fixed factor. To account for the repeated testing of individuals, we further included "trial" as a fixed factor and "fish ID" as a random factor. We allowed for trial effects to vary across populations (interaction "population x trial"), but removed the interaction term when it did not significantly improve the model (results not shown). *Post-hoc* LSD tests were employed in case fixed factors were found to have a significant effect. For the covariate "body size," we also performed Pearson's correlations to better evaluate the relationship.

(Co)Variance structure was nested within "population," which enabled us to calculate the total behavioral variance as well as within- and among-individual variance components in each population. Based on this, we calculated behavioral repeatability for each of the four populations as a measure of consistency. We assumed that repeatability and/or variance estimates differed among populations when 95% confidence intervals (CIs) were not overlapping (see Nakagawa and Schielzeth, 2010).

To evaluate the existence of behavioral syndromes (i.e., correlation among behavioral traits) at certain times and/or sites, we first estimated the raw phenotypic correlation (Pearson's correlation). To partition phenotypic correlation coefficients into a between-individual and within-individual correlation component (after Dingemanse and Dochtermann, 2013), we used multivariate mixed-effect models (MMM, *MIXED* procedure) separated by sample population. In order to minimize the effects of mean differences in behavioral traits on trait correlation, we mean-centered each behavioral trait within "trial" and "population" (z-transformation). Each mixed model included "sex" and "body length" as covariates and "fish ID" as a random factor. We assumed that trait correlations differed among populations when 95% confidence intervals (CIs) were not overlapping (see Nakagawa and Schielzeth, 2010).

All statistical analyses were performed with SPSS 25.0 software (IBM Corp., NY, USA) and visualization of the results was done with GraphPad Prism 8.01 (GraphPad Software Inc., CA, USA) or R (v. 4.0.2; R Core Team, 2020).

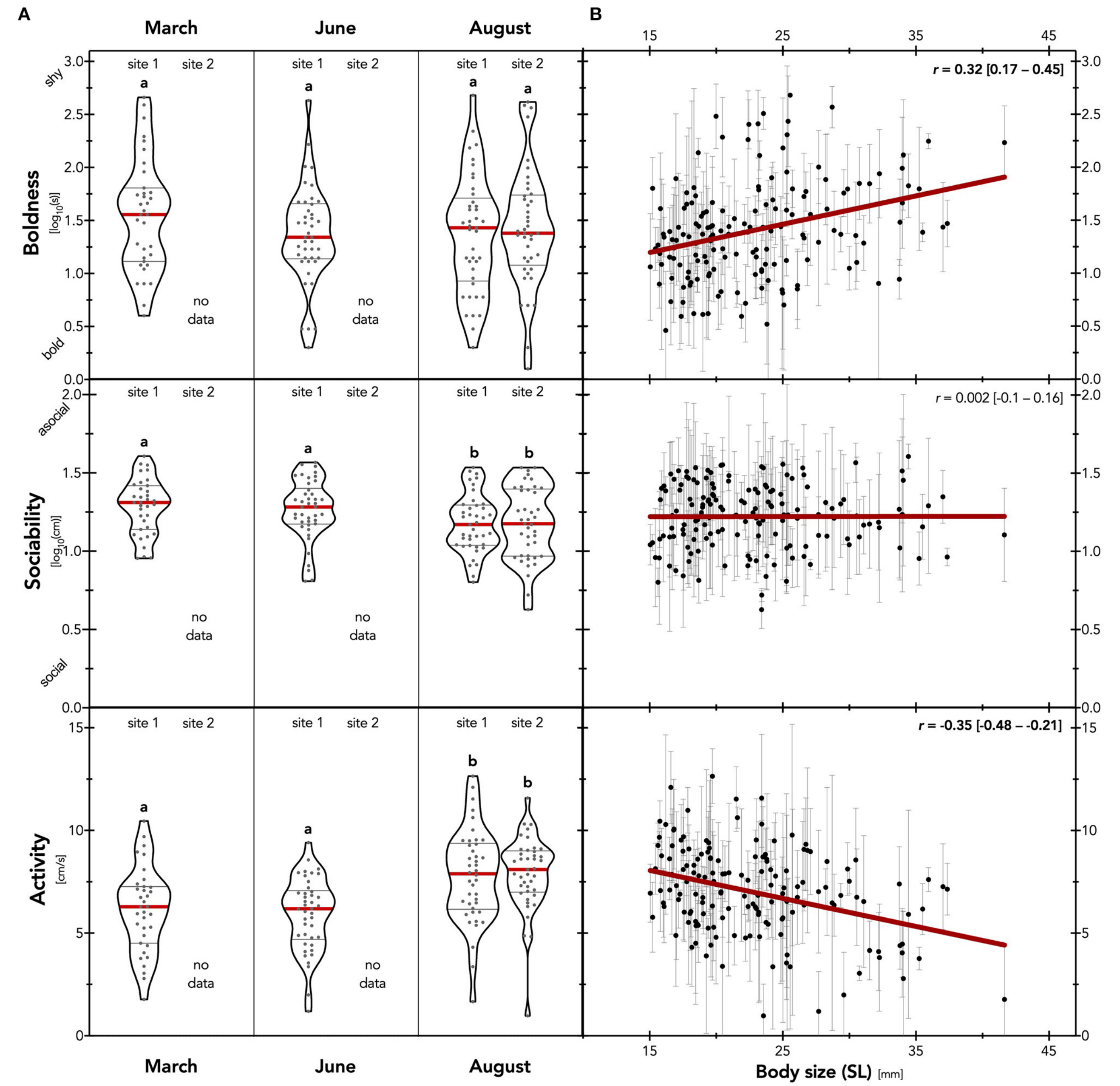

FIGURE 3 | Average personality traits across temporal and spatial scale. **(A)** Mean trait scores (median ± IQR) for boldness (i.e., time to emerge from shelter), activity (i.e., velocity during open field exploration) and sociability (i.e., distance to biomimetic robot). Different letters depict significant differences based on *post-hoc* LSD tests after global linear mixed models ($p < 0.05$). (**B**) Mean trait scores (±SD) in relation to body size (standard length; $n = 159$). To visualize the relationships, we show linear regressions (red line) calculated from individual means (parameters significantly different from zero at the 95% confidence level are indicated in bold; see main text for detailed analysis). Note that low scores of boldness and sociability indicate high trait expression.

RESULTS

Differences in Average Trait Expression Among Populations

Expression of average sociability and activity differed between populations, while boldness did not ("population" effect in **Table 1**; **Figure 3A**). On a temporal scale, fish sampled at site 1 in August showed higher levels of sociability (i.e., reduced distance to robotic fish) compared to March and June populations (*post-hoc*, August site 1 *vs*. March site 1: $p = 0.003$, *vs*. June site 1: $p = 0.025$). Similarly, fish sampled at site 1 in August were more active than populations sampled in March and June (*post-hoc*, August site 1 *vs*. March site 1: $p = 0.002$, *vs*. June site 1: $p < 0.001$). On a spatial scale, we found no evidence that fish sampled in August

TABLE 1 | Results from linear mixed models.

	Boldness ($\log_{10}$)		Sociability ($\log_{10}$)		Activity	
Effect	$F_{df1,df2}$	p	$F_{df1,df2}$	p	$F_{df1,df2}$	p
Population	$F_{3,469} = 0.57$	0.634	$F_{3,469} = 3.8$	**0.01**	$F_{3,469} = 8.44$	**< 0.001**
Trial	$F_{2,469} = 1.72$	0.18	$F_{2,469} = 135.9$	**< 0.001**	$F_{2,469} = 42.32$	**< 0.001**
Sex	$F_{1,469} = 0.06$	0.81	$F_{1,469} = 2.28$	0.132	$F_{1,469} = 0.24$	0.623
Body size (SL)	$F_{1,469} = 8.43$	**0.004**	$F_{1,469} = 0.02$	0.884	$F_{1,469} = 3.58$	0.059

For each behavioral trait, the LMM included "population," "trial," and "sex" as fixed effects and "body size" (SL in mm) as covariate with the random factor Fish ID. Significant effects (p < 0.05) are indicated in bold.

TABLE 2 | Repeatability estimates and total behavioral variance estimates.

	Boldness ($\log_{10}$)			Sociability ($\log_{10}$)			Activity		
Population	**Repeatability [95% CI]**	***p***	**Total variation [95% CI]**	**Repeatability [95% CI]**	***p***	**Total variation [95% CI]**	**Repeatability [95% CI]**	***p***	**Total variation [95% CI]**
March site 1	0.49 [0.40–0.57]	**0.004**	0.24 [0.15–0.4]	0.26 [0.15–0.4]	0.05	0.06 [0.04–0.1]	0.27 [0.17–0.41]	**0.042**	6.86 [4.3–11.87]
June site 1	0.40 [0.31–0.5]	**0.005**	0.31 [0.2–0.5]	0.32 [0.23–0.43]	**0.011**	0.07 [0.05–0.11]	0.30 [0.21–0.42]	**0.015**	5.75 [3.74-9.33]
August site 1	0.54 [0.47–0.6]	**0.001**	0.34 [0.22–0.54]	0.34 [0.25–0.45]	**0.009**	0.06 [0.04–0.09]	0.38 [0.29–0.47]	**0.005**	7.15 [4.61-11.48]
August site 2	0.53 [0.46–0.6]	**0.001**	0.3 [0.19–0.48]	0.49 [0.42–0.57]	**0.001**	0.08 [0.05–0.13]	0.36 [0.27–0.46]	**0.007**	5.92 [3.81-9.57]

p-values are derived from Wald's z statistics (see ***Supplementary Table 5*** *for within- and among-individual variation estimates). Significant differences among populations (bold) can be assumed when 95%CI do not overlap.*

at site 1 and site 2 differed in boldness, sociability, nor activity (*post-hoc*, August site 1 *vs.* site 2 boldness: $p = 0.76$, sociability: $p = 0.92$, activity: $p = 0.89$; **Figure 3A**).

Boldness and activity showed a size-dependency (**Figure 3B**) with smaller individuals being bolder (i.e., reduced emergence time; Pearson's $r = 0.32$; $p < 0.001$) and more active (Pearson's $r = 0.35$; $p < 0.001$) than larger ones. We found no such relation between body size and sociability (Pearson's $r = 0.002$; $p = 0.98$). While sex is correlated with size in the sexually dimorphic guppy, sex did not affect any of the measured behavioral traits (non-sig. "sex" effect; **Table 1**). We further observed a habituation to the repeated testing during sociability and activity assays (sig. "trial" effect; **Table 1**), which was uniform across population samples (non-sig. interaction "population x trial" for all three traits; p never below 0.25). Fish were considerably less social and less active with each consecutive measurement. We note that the habituation effect observed for activity but not boldness serves as a validation that emergence into and activity within an open field measured two different traits.

Among- and Within-Individual Variation in Behavioral Traits

Fish within each sampled population differed consistently in their expression of boldness, sociability and activity across trials (sig. repeatability estimates; **Table 2**). Only fish sampled in August at site 2 had a significantly higher repeatability estimate for sociability than fish from the March sampling at site 1 (non-overlapping CIs; **Table 2**). There was no significant difference among populations in estimated overall behavioral variances in any of the examined traits (see "total variation" in **Table 2**; for within- and among-individual variance estimates see **Supplementary Table 5**).

Behavioral Syndrome Structure

Boldness and activity were correlated in all four population samples (significant phenotypic correlation, r_{raw} in **Figure 4**). This was driven by high significant among-individual correlations (r_{among} in **Figure 4**) that did not differ among populations (overlapping CIs). This indicated that fish with high average activity levels also left the shelter quicker, confirming that activity and boldness are part of a larger behavioral syndrome (**Figure 4**).

There was no evidence for raw phenotypic or among-individual correlations between sociability and boldness, nor between sociability and activity among populations (non-significant r_{raw} and r_{among}). Thus, sociability measured as distance kept to the moving robotic fish does not seem to be part of a behavioral syndrome with either boldness or activity. Nevertheless, we found several traits to show significant within-individual correlations (r_{within}) which indicates that fish showing high variation in one trait also exhibited high variation in the other trait (for within- and among-individual variance estimates see **Supplementary Table 6**).

DISCUSSION

Our study follows the annual range shifts of non-native fish in an artificially heated stream in temperate Germany. As guppies dispersed into winter-abandoned areas in warmer months, we expected this process to be driven by dispersal-enhancing behavioral traits. We found similar consistent among-individual

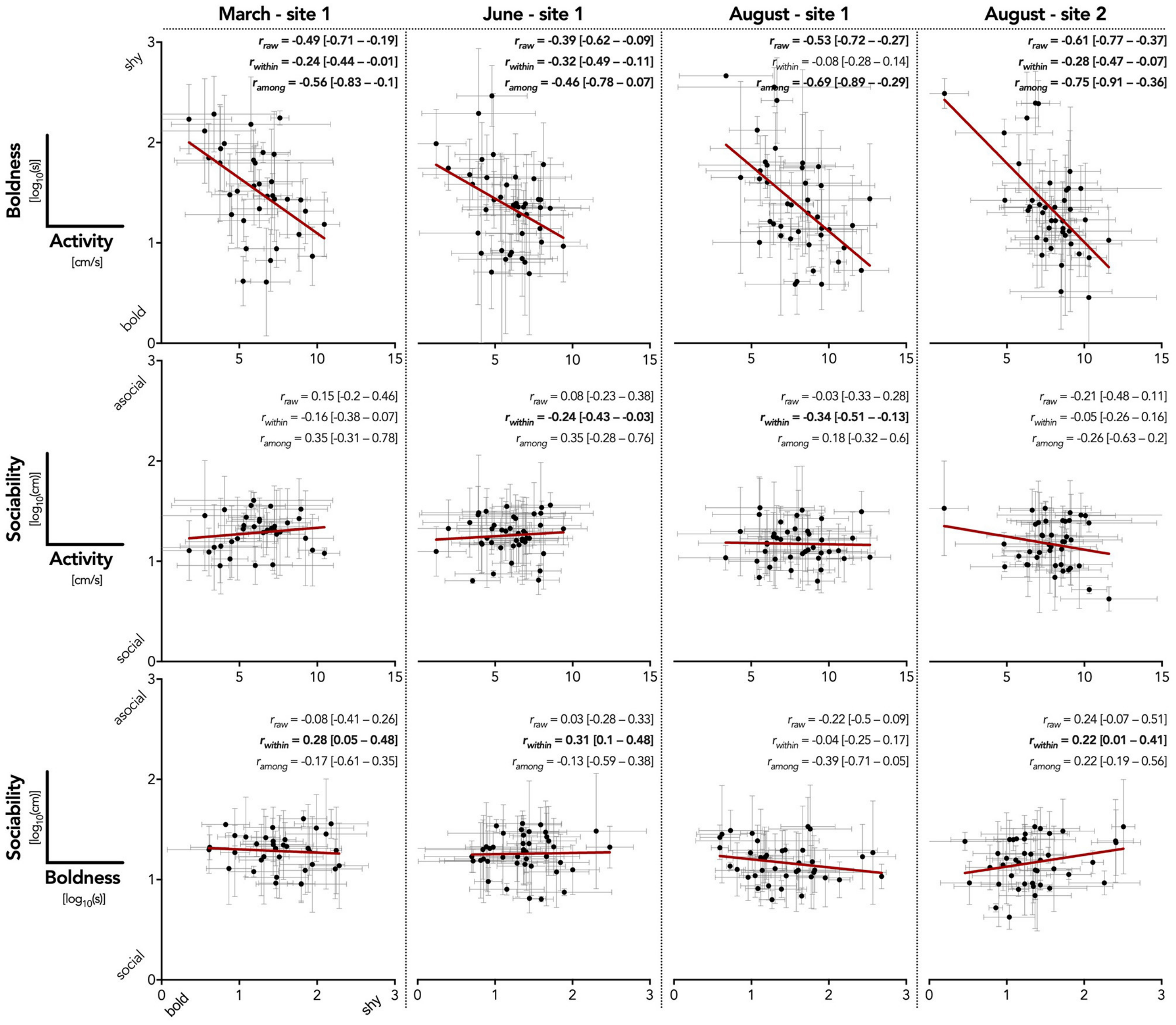

FIGURE 4 | Correlations between behavioral traits across temporal and spatial scale. Mean trait scores (±SD) of individual test fish are shown for each population. Linear regressions (red line) and mixed model estimated correlation coefficients visualize the relationships between traits (parameters significantly different from zero at the 95% confidence level are indicated in bold; see main text for detailed analysis). Note that for boldness and sociability lower values indicate a high trait score.

variation (e.g., significant behavioral repeatability) in the behaviors "boldness," "sociability," and "activity" within all temporal and spatial samples (except a difference between sociability in March at site 1 and August at site 2). Contrary to our predictions, we did not detect a decrease in total behavioral variation of the sampled populations indicative of a "thinning out" of disperser's behavioral types. Further, we found no evidence that the source and the newly-founded population differed in terms of average behavioral trait expression, though a seasonal change in activity and sociability (but not boldness) was detectable in the source population: August-sampled individuals were more active and more sociable than those sampled earlier in the year. Instead, we found a behavioral syndrome among activity and boldness that was consistently detectable throughout the year and across sampled populations. To sum up, we found no strong evidence for a personality-biased dispersal in this feral guppy population and the detected seasonal differences in average trait expression (sociability, activity) are likely due to changed environmental factors such as temperature, predator abundance and/or resource availability.

Differences in behavioral traits across seasons but not between sites as found in our study could be explained by seasonal variation in environmental conditions rather than by a drain of individuals carrying certain behavioral types from source to downstream populations. The Gillbach's water temperatures show strongest differences between sites in the colder months (∼4°C between site 1 and 2), but relatively small differences during the warmer months (<1°C between site 1 and 2; **Figure 1B**). Thus, when winter-abandoned peripheral sites are colonized again in summer, these habitats do not differ much in temperature from the source at this point in time. Nevertheless, the Gillbach still shows a strong seasonality in temperature which, in combination with a variation in day length, food quality/quantity and predation pressure, may likely cause population-wide changes in behaviors across seasons (e.g., Biro et al., 2010; Eccard and Herde, 2013; Spiegel et al., 2015; Uchida et al., 2016; Barbosa et al., 2018; Dhellemmes et al., 2020) as seen in the source population. For example, prior temperature acclimation during sensitive developmental phases can alter the phenotypes of subsequent adult phases (Beitinger et al., 2000; Seebacher et al., 2014). This might explain the increased activity exhibited by fish in August, as higher developmental temperatures lead to more active phenotypes (Seebacher et al., 2014). However, teasing apart the effects of abiotic (e.g., temperature) and biotic (e.g., predation pressure) fluctuations in environmental conditions will require additional experimentation.

Besides seasonal differences in average behavioral traits, we found stable behavioral syndromes among sampled populations that involved a strong positive correlation between boldness and activity. Such a boldness-activity syndrome has been documented in various taxa (van Oers et al., 2004; Pintor et al., 2008; Wilson and Godin, 2009; Cote et al., 2010b; Eccard and Herde, 2013; Muraco et al., 2014; Bierbach et al., 2015), including guppies (Brown and Irving, 2014). Natural selection through predation favors the evolution of behavioral correlations (Dingemanse et al., 2007; Harris et al., 2010; Dhellemmes et al., 2020). The Gillbach's guppy population underwent strong seasonal changes in size (**Supplementary Appendix 2**) and the observed population decline at the end of the summer, while primary production is highest in temperate regions, suggests strong predation pressure. A positive boldness-activity correlation might thus represent an optimal trait combination in the face of predation experienced by both populations (Smith and Blumstein, 2010). However, the lack of differences in the level of (individual) behavioral variation among populations might be a result of a random downstream drift and a low overall behavioral plasticity in this population. This can be a consequence of the assumed bottleneck origin of this feral population that most likely stems from an unknown number of released domesticated guppies (see Gertzen et al., 2008 for estimates of propagule pressure for popular aquarium species). Investigations involving other populations of the guppy and more distantly related species are highly recommended, especially as several environmental factors are predicted to impact behavioral variation, especially in fish (e.g., Harris et al., 2010; Laskowski et al., 2016; Barbosa et al., 2018). Alternatively, guppies and especially those from the Gillbach that experience highly fluctuating temperature regimes may have the ability to adjust their behavioral expressions within very short times, e.g., within the 30-days acclimatization period in the laboratory used here. It is known that guppies have a remarkable capacity for behavioral plasticity (Deacon and Magurran, 2016) and thermal adaptation (Chung, 2001; Jourdan et al., 2014), and thus have successfully established populations in environments with very different (thermal) regimes compared to their native habitats (Deacon et al., 2011). Testing of individuals after different acclimation periods could be a possibility to investigate this issue further.

Many large-scale field studies, especially on taxa of small-sized organisms, have the inherent problem that it is not (yet) feasible to track individuals continuously in real time. Without it, studies in the wild always carry an element of uncertainty concerning the dispersal process and our study is no exception. We assume that peripheral areas (site 2) are depopulated in winter and (re)colonized by dispersing individuals from the source (site 1) in warmer months based on the following: First, temperature data of site 2 (**Figure 1B**) suggests that several temperature drops occurred during spring (and likely many more in winter), thus inhibiting winter survival of guppies more than 1 kilometer downstream of the source. Second, the unique conditions of the Gillbach system render colonization from other areas than the source unlikely. Although our mark-recapture experiment (**Supplementary Appendix 2**) was not suited to find individuals dispersing between site 1 and 2, we argue that the Gillbach's source provides only one direction of dispersal simply because upstream dispersal to site 2 cannot take place as there are no guppy reports further downstream. Third, introductions by humans are similarly unlikely as this part of the stream is fenced and not well-accessible and the introduction of ornamental guppies to site 2 would have led to noticeable changes in fish's color patterns which was not the case. We are therefore confident that our assumptions on the dispersal route from source to downstream areas is justified and thus the reported results present an unbiased snapshot of the dispersing guppies' behavioral types during this journey.

Theoretical models (Cote et al., 2010a; Spiegel et al., 2017) as well as several studies across taxa (Duckworth and Badyaev, 2007; Pintor et al., 2009; Cote et al., 2010b; Myles-Gonzalez et al., 2015; Coates et al., 2019) provide evidence for the prominent role of individual differences in behavior during dispersal and invasion processes (Bowler and Benton, 2005; Chapple et al., 2012; Canestrelli et al., 2015). Nevertheless, the picture might be more complicated and context-dependent: spatial sorting of behavioral types did not emerge under predation pressure (Cote et al., 2013) and was lost as the population aged (Thorlacius et al., 2015). Moreover, even if there are significant (but small) effects of behavioral trait variation affecting relevant ecological phenomena, they might be non-detectable in benign laboratory environments (Schröder et al., 2016; Moiron et al., 2020). Our current study exemplifies that population-wide behavioral analysis can fail to provide evidence for behaviorally-mediated migration patterns and future analysis may focus on tracking individuals along their travel routes across seasons and locations. On top of this, several other feral guppy populations are known in Germany (Lukas et al., 2017b) and across Europe (Kempkes et al., 2018) for which similar investigations are highly advisable in order to test the generality of our herein presented results. Such analyses should strive to incorporate the invasion history, environmental factors as well as the population-specific genetic backgrounds which all can affect behavioral variation in wild populations (Sih and Bell, 2008; Bergmüller and Taborsky, 2010; Stamps and Groothuis, 2010).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article is available at: https://doi.org/10.6084/m9.figshare.13292750.

ETHICS STATEMENT

The animal study was reviewed and approved by LaGeSo Berlin (Reg. 0117/16 to DB).

AUTHOR CONTRIBUTIONS

JL and DB designed the study. JL, FM, GK, and DB jointly collected the fish. DB, TL, and JK developed the robotic fish system "Robofish." JL and DB conducted the behavioral experiments, analyzed the data, and wrote the manuscript. All authors approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.583670/full#supplementary-material

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What Drives Life-History Variation in the Livebearing Fish *Poeciliopsis prolifica*? An Assessment of Multiple Putative Selective Agents

Andrea J. Roth-Monzón[1]*†, Mark C. Belk[1], J. Jaime Zúñiga-Vega[2] and Jerald B. Johnson[1,3]

[1] Evolutionary Ecology Laboratories, Department of Biology, Brigham Young University, Provo, UT, United States, [2] Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México, Mexico, [3] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT, United States

Edited by:
Wayne Iwan Lee Davies,
Umeå University, Sweden

Reviewed by:
Ingo Schlupp,
University of Oklahoma, United States
Eugenia Zandona,
Rio de Janeiro State University, Brazil

***Correspondence:**
Andrea J. Roth-Monzón
rothmonzon@gmail.com

†Present address:
Andrea J. Roth-Monzón,
Department of Ecology and
Evolutionary Biology, University of
Connecticut, Mansfield, CT,
United States

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Life-history traits are directly linked to fitness, and therefore, can be highly adaptive. Livebearers have been used as models for understanding the evolution of life histories due to their wide diversity in these traits. Several different selective pressures, including population density, predation, and resource levels, can shape life-history traits. However, these selective pressures are usually considered independently in livebearers and we lack a clear understanding of how they interact in shaping life-history evolution. Furthermore, selective pressures such as interspecific competition are rarely considered as drivers of life-history evolution in poeciliids. Here we test the simultaneous effects of several potential selective pressures on life-history traits in the livebearing fish *Poeciliopsis prolifica.* We employ a multi-model inference approach. We focus on four known agents of selection: resource availability, stream velocity, population density, and interspecific competition, and their effect on four life-history traits: reproductive allocation, superfetation, number of embryos, and individual embryo size. We found that models with population density and interspecific competition alone were strongly supported in our data and, hence, indicated that these two factors are the most important selective agents for most life-history traits, except for embryo size. When population density and interspecific competition increase there is an increase in each of the three life-history traits (reproductive allocation, superfetation, and number of embryos). For individual embryo size, we found that all single-agent models were equivalent and it was unclear which selective agent best explained variation. We also found that models that included population density and interspecific competition as direct effects were better supported than those that included them as indirect effects through their influence on resource availability. Our study underscores the importance of interspecific competitive interactions on shaping life-history traits and suggests that these interactions should be considered in future life-history studies.

Keywords: resource availability, interspecific competition, density, life history, poeciliids, stream velocity

INTRODUCTION

Life-history traits can be highly adaptive (Roff, 2002; Chapuis et al., 2017), and can evolve in response to a variety of selective pressures, both biotic and abiotic (Johnson and Bagley, 2011). Some demonstrated selective agents known to affect the evolution of life histories are population density (Reznick et al., 2002, 2012), predation (Martin, 1995; Johnson and Belk, 2001; Reznick et al., 2001; Roff, 2002; Chapuis et al., 2017), resource availability (Reznick and Yang, 1993; Grether et al., 2001; Roff, 2002; Pérez-Mendoza et al., 2014; Zani and Stein, 2018), and other environmental effects, such as water flow, elevation, toxicity, etc. (Badyaev and Ghalambor, 2001; Ghalambor et al., 2004; Zúñiga-Vega et al., 2007; Riesch et al., 2010, 2014; Johnson and Bagley, 2011; Banet et al., 2016; Heins and Baker, 2017; Santi et al., 2019). The family Poeciliidae has a staggering diversity in reproductive adaptations and heterogeneity in life-history traits, emerging as a model system for life-history research (Pollux et al., 2009). A wealth of knowledge has been accumulated in several species of this family (Pollux et al., 2009; Johnson and Bagley, 2011), however, most studies tend to focus on a single selective factor, although it is clear that life-history traits can be affected and shaped simultaneously by several factors (Moore et al., 2016).

Studies that have examined how several factors affect life-history traits in poeciliids have shown that life histories respond in a predictable and repeatable way to certain selective agents, but not to all of them (Moore et al., 2016). For example, in several livebearing fish species predation drives life-history evolution in a predictable and repeatable manner, but resource availability has less consistent effects (Johnson and Belk, 2001; Reznick et al., 2001; Moore et al., 2016). However, the response to other selective factors can depend on the population studied (Moore et al., 2016). For some populations, it is clear that several pressures may be acting in concert (Johnson, 2002; Moore et al., 2016), whereas in others one factor may be dominant. Nevertheless, we still have a lack of studies that allow us to draw general predictions about responses to several selective pressures, and which selective pressures have primacy in wild populations.

Here, we evaluate several hypotheses about the relative importance of biotic and abiotic factors in shaping four life-history traits using a multi-model inference approach in a species of livebearing fishes (Johnson and Bagley, 2011). We focus on four well known life-history traits: superfetation (number of simultaneous broods carried by a female), individual embryo size, number of embryos (across all broods; Frías-Alvarez and Zúñiga-Vega, 2016), and reproductive allotment. We build specific hypotheses of how each factor could affect the evolution of the life-history traits using the wealth of knowledge available for poeciliids (Johnson and Bagley, 2011). However, some selective agents have only received modest consideration in this type of study (interspecific competition and stream velocity; Johnson and Bagley, 2011). Given this modest inclusion, it is unknown how important interspecific competition and stream velocity are in comparison to other more commonly studied selective agents in livebearers. Thus, we model four hypothetical drivers of life-history variation: resource availability, population density, stream velocity, and interspecific competition (see **Table 1** for hypotheses), each of which is a potential agent of life-history evolution in livebearing fishes (*Poeciliidae;* Johnson and Bagley, 2011). We compare these putative selective agents to evaluate the relative importance of each on the evolution of life histories of *Poeciliopsis prolifica* and test if the effect of each selective agent is in accordance with previous hypotheses (**Table 1**).

MATERIALS AND METHODS

Study System and Collection Sites

Poeciliopsis prolifica is distributed through northwestern Mexico on the Pacific slope from the Rio Yaqui, Sonora south to near Las Varas, Nayarit (Miller et al., 2005). Populations exist under a variety of environmental conditions that include differences in fish density, stream velocity, fish community structure, and habitat characteristics. This provides a range of selective conditions under which populations might evolve local adaptations. We collected 298 *P. prolifica* females using hand-held seine nets (1.3 m × 5 m; 8 mm mesh size) from 12 populations (**Figure 1**) during the dry season (**Table 2**; Permits FAUT-0117, DGOPA/1864/210205/-0765, and DGOPA-005/16). All individuals were euthanized and preserved in alcohol following guidelines of the ethics committee of Brigham Young University (although Ethics Committee approval is not required according to national law in Mexico).

For each locality, we quantified four environmental parameters that represent four putative selective agents: interspecific competition, resource availability, stream velocity, and population density (**Table 2**). All selective agents are predicted to affect life-history traits, either directly or indirectly (Johnson and Bagley, 2011). In this study, we assume that these four selective agents are unmeasured factors that are correlated with measurable environmental characteristics. To characterize the selective agent we attempt to identify environmental parameters that are correlated with the unmeasured true selective agent. We then estimate the correlation between observed characters and unobserved factors through path analysis.

We estimated relative population density of *P. prolifica* by calculating the average number of *P. prolifica* fish observed per seining attempt, a standardized catch per unit effort (CPUE) found to be positively correlated with actual population density (Johnson, 2002). CPUE can allow comparison of localities with different seining efforts and has been used in several studies as a proxy to population density (Kobza et al., 2004; Cruz et al., 2020). We estimated interspecific competition as the number of co-occurring species of the same genus. *P. prolifica* can co-occur with up to three species of the same genus: *P. viriosa*, *P. latidens*, and *P. presidionis* (Mateos et al., 2002; Miller et al., 2005). Thus, competition will have four factors from no competitors to three co-occurring competitors (0,1,2,3). To our knowledge there are no published accounts of the degree of similarity or the niche overlap among these four species. However, general accounts of these four species indicated that they are ecologically similar—they all inhabit the mid-water column in streams and small rivers, they are similar in body form,

TABLE 1 | Potential effects of the four putative selective agents on the life history of *P. prolifica*.

Selective Agent	Path ID[a]	Effect on life history	Prediction	Rationale	References
Resources (R)	1	Direct	An increase in resources will result in an increase in number of embryos and reproductive allocation, but a decrease in superfetation and embryo size	Higher resources should result in greater body condition and thus greater investment in reproduction. An increase in number of embryos should result in a reduction in the size of each individual embryo (trade-off between number and size of offspring). Partitioning a large reproductive bout into smaller broods (superfetation) is less necessary if resources are abundant	Reznick and Yang, 1993; Zúñiga-Vega et al., 2010; Johnson and Bagley, 2011
Population density (D)	2	Direct	An increase in population density will result in an increase in superfetation and embryo size, but a decrease in number of embryos and reproductive allocation	Higher population density may increase competition for other environmental factors, such as habitat and not through resource availability. Competition should still negatively affect body condition and result in lower investment in reproduction. Both superfetation and large offspring size are advantageous in competitive environments	Johnson and Bagley, 2011
	5	Indirect trough resources	An increase in population density will result in an increase in superfetation and embryo size, but a decrease in number of embryos and reproductive allocation	Higher population density increases competition for resources resulting in lower resources per individual, which causes lower body condition and lower investment in reproduction. If resources are scarce, superfetation may reduce peak cost of reproduction by partitioning a large reproductive bout into smaller broods	Reznick et al., 2002, 2012; Zúñiga-Vega et al., 2010; Johnson and Bagley, 2011
Interspecific competition (C)	3	Direct	An increase in competition will result in an increase in superfetation, number of embryos and reproductive allocation, but a decrease in embryo size	Higher interspecific competition causes a pressure for higher reproduction to compete and coexist. Life history acts as a tradeoff for competition that allows coexistence. Simply said there is a trade-off between competitive abilities and reproduction	Hutchinson, 1957; Levine and Rees, 2002; Kneitel and Chase, 2004; Leibold et al., 2004; Calcagno et al., 2006; Chapuis et al., 2017
	6	Indirect trough resources	An increase in competition will result in an increase in superfetation and embryo size, but a decrease in number of embryos and reproductive allocation	Higher interspecific competition increases overall density that in turn decreases resources. As resources are lower per individual this causes low body condition and lower investment in reproduction. Superfetation is beneficial when resources are scarce	Scott and Johnson, 2010; Wilson, 2013
Stream velocity (S)	7	Direct	An increase in stream velocity will result in an increase in superfetation, number of embryos and reproductive allocation, but a decrease in embryo size	Fast flow environments result in more streamlined body shapes that should increase superfetation, allowing reproductive effort to be higher or at least unchanged. More embryos should result in a reduction in the size of each individual embryo	Zúñiga-Vega et al., 2007, 2010; Johnson and Bagley, 2011

[a] *Path ID numbers correspond to those shown in* ***Table 4*** *and* ***Figure 2****.*

and they are omnivorous, consuming plant, and animal matter (Miller et al., 2005). Furthermore, we collected these species in the same microhabitat and it has been shown that *P. prolifica* body shape converges with that of its congeners when co-occurring due to interspecific competition (Roth-Monzón et al., 2020). Hence, we conclude that due to previous evidence, their close phylogenetic relationship, and ecological similarity there is high potential for competitive interactions. We used number of co-occurring species as an indicator of level of interspecific competition in our analysis. We chose number of co-occurring species to focus on answering the effect of the addition of species in life-history traits. Furthermore, it is a known measure that has affected *P. prolifica* (Roth-Monzón et al., 2020). Additionally, number of competitors is a general measure that can characterize interspecific competition throughout several years. We found no piscivorous predators in the localities sampled. However, in all locations, we found another species of livebearer (*Poecilia butleri*). We also found one location with a very low density (only 16 individuals collected) of an introduced livebearer (*Gambusia affinis*).

We use stream slope to characterize stream velocity. We calculated stream slope in ArcMap 10.6 (Enviromental Systems Research Institute [ESRI]., 2014), as the difference between upper elevation and lower elevation of a 2-km segment of stream for each locality sampled. It is known that stream velocity increases as stream slope increases, thus this indirect measure should be a good proxy for stream velocity (Gore and Banning, 2017). We recognize that there is variation among sites in the number of pools and riffles, regardless of the overall slope across the focal 2-km segment of the stream. Frequently, poeciliids use pools of stagnant water for foraging activities and social interactions (Mazzoni et al., 2011). However, these pools are highly dynamic, some of them dry out, and new ones are constantly formed. Thus, our proxy for stream velocity does not represent these

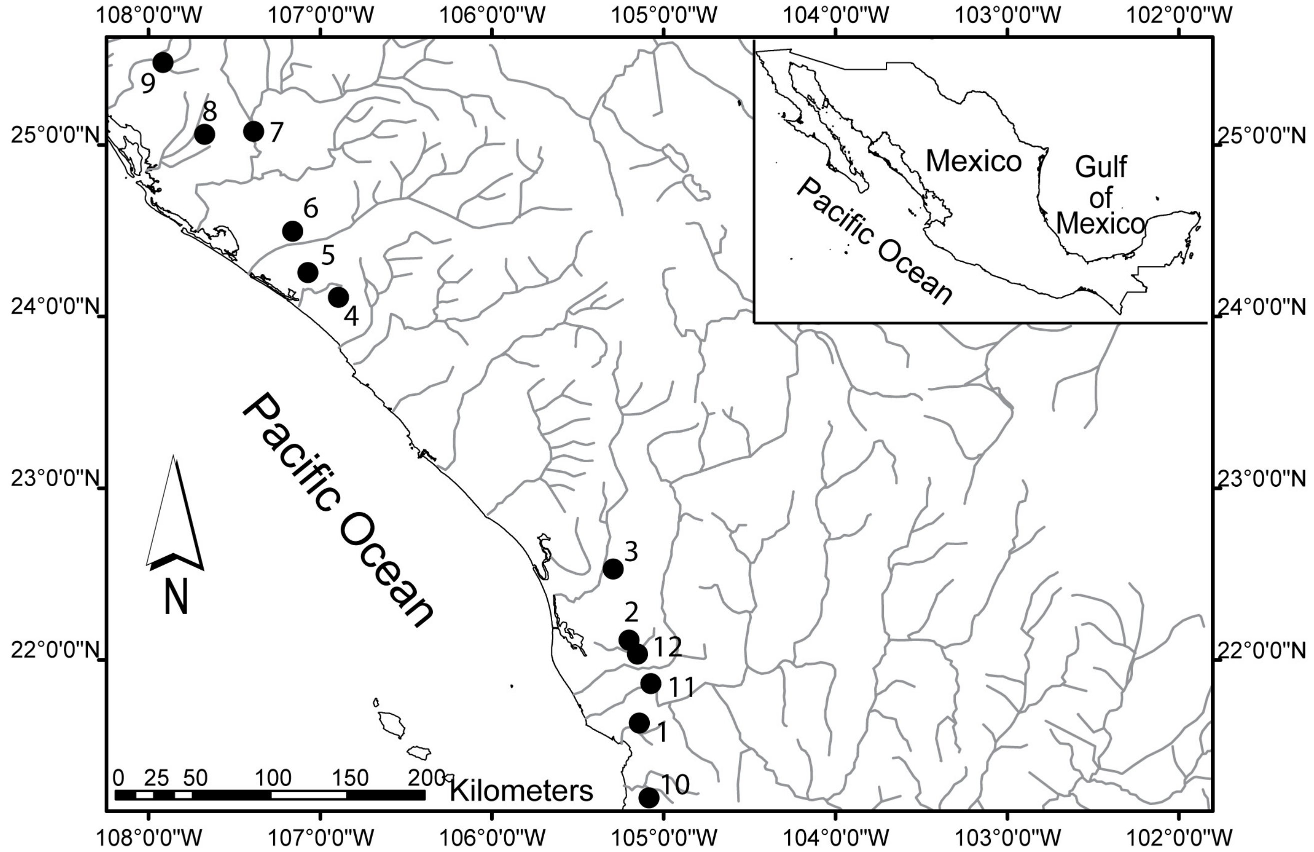

FIGURE 1 | Map showing the localities sampled for *Poeciliopsis prolifica.* Inset map shows Mexico and the two states (Nayarit and Sinaloa) that were sampled. Numbers correspond to location ID (also found in **Table 2**).

changing microhabitats, which may be temporarily available to fish depending on the amount of daily precipitation or water runoff, but instead represents an estimate of the general water velocity that fish experience all throughout the year. Individuals that inhabit a steep river may indeed search for pools, but they certainly deal with fast currents when moving among pools. In contrast, individuals that inhabit a stream that runs through a plain landscape will never experience fast water currents, not even when moving between pools. Therefore, selection for more streamlined phenotypes with enhanced swimming abilities, which in turn impose constraints on reproductive allotment (Zúñiga-Vega et al., 2010), must be overall stronger in streams located in steep terrains than in streams located in relatively plain areas.

We estimated resource availability measuring canopy cover with a hand-held densiometer. Canopy cover is an indicator of primary productivity (Grether et al., 2001; Zimmermann and Death, 2002; Kiffney et al., 2004; Schiesari, 2006). High canopy cover indicates lower primary productivity that has been correlated with lower secondary productivity such as invertebrates, thus being a reliable measure of resources in a stream (Grether et al., 2001; Zimmermann and Death, 2002; Kiffney et al., 2004; Schiesari, 2006). Canopy cover has been a good proxy for resource availability in other studies with streams similar to ours (Grether et al., 2001; Collins et al., 2015). Two populations had missing data on canopy cover (localities four and seven); to avoid the exclusion of these in the analysis we used aerial images from Google Earth to calculate percent cover in ImageJ. This approach has been used before and found to strongly correlate with field measurements of canopy cover (Inskeep et al., 2011). In our localities, we also found a strong correlation between our field measurements of canopy cover and our calculations of canopy cover from aerial images ($R^2 = 0.76$, $P = 0.010$). To understand if competition, resources, stream slope, and population density covaried, we conducted a pairwise correlation tests. We found no significant correlations among these putative selective agents (**Table 3**).

Life-History Traits

We quantified four different life-history traits: superfetation (number of simultaneous broods carried by a female), individual embryo size, number of embryos (across all broods; Frías-Alvarez and Zúñiga-Vega, 2016), and reproductive allotment. We choose these traits because the four selective pressures of interest are thought to affect them, and clear predictions can be made of their effects (see **Table 1**). We classified embryo development stage using the 11-stage scale developed by Haynes

TABLE 2 | Sample size, selective agents, and collection year for all twelve locations in the study.

Location ID	Collection year	Sample size	Number of competitors	Canopy cover (%)	Stream slope (km)	Population density (CPUE)
1	2007	44	2	100	7.5	7.38
2	2007	21	1	41	2.5	17.75
3	2007	39	3	37	9	21.71
4	2007	16	1	98	3.5	3.5
5	2007	17	1	100	5.5	4.14
6	2007	36	1	82	4.5	42.2
7	2007	29	0	82	22.5	41.29
8	2007	35	1	100	2.5	16.36
9	2007	19	1	100	3	9.86
10	2015	21	1	69	7	6.31
11	2015	11	2	36	1	27
12	2015	10	0	69	0	24.14

*Population density is measured as catch per unit effort. Location ID corresponds to map in **Figure 1**.*

(1995). We defined a brood as all the embryos that share the same developmental stage (Haynes, 1995). We measured individual embryo size by drying an entire brood of offspring in a desiccating oven for 48 h at 55°C and dividing the brood dry mass by the number of embryos in the brood. To avoid non-independence in individual embryo size due to the fact that females can have more than one brood, we only considered the brood at the most advanced developmental stage of each female for the calculation of individual embryo size. We obtained female somatic dry mass by drying the female soma (minus the intestinal tract and offspring) for 48 h at 55°C. For reproductive allocation, we used the total dry mass of all broods of each female relative to somatic dry mass of the female following Tomkins and Simmons, 2002.

To account for the effect of maternal body size differences on each life-history trait, we adjusted each trait by an analysis of covariance (ANCOVA). We adjusted individual embryo size by including maternal somatic dry mass and developmental stage as covariates, to obtain comparable "size-free" and "stage-free" least squares means for analysis. We only adjusted superfetation, number of embryos, and reproductive allocation by using maternal somatic dry mass as a covariate. To meet assumptions of normality for the ANCOVAs, we used the following transformations on the life-history traits: superfetation and total number of embryos were square root transformed, whereas embryo size and reproductive allocation were log transformed. Hence, the comparison among populations were done using the adjusted least squares means generated by the ANCOVA models allowing us to compare "size-free" and "stage-free" life-history traits.

TABLE 3 | Pairwise correlation coefficients with *P*-values of the four putative selective agents.

Environmental variable	Competition	Resources	Stream slope	Population density
Competition		0.20	0.67	0.53
Resources	−0.39		0.79	0.27
Stream Slope	−0.14	0.09		0.23
Population density	−0.20	−0.35	0.37	

Values below the diagonal are correlation coefficients, and values above the diagonal are the corresponding P-values.

Before using the adjusted life histories in a model selection approach, we tested for population differences in all four life-history traits by employing a multivariate analysis of covariance (MANCOVA). Likewise, we included maternal somatic dry mass and stage of development as covariates in the MANCOVA. We found significant differences among the 12 populations of *P. prolifica* in all life-history traits ($F_{11,1136} = 14.75, P \leq 0.001$), so we proceeded with the model selection approach using the adjusted least squares means. All of these analyses were implemented in R software (R Core Team., 2020).

Model Selection

We generated a set of 14 candidate models that represent competing biological hypotheses of the way the four putative selection agents could act to shape life-history traits (**Figure 2**, **Table 4**, and **Supplementary Figure 1**). We did not include all possible interactions between factors because we wanted to include only those that represent plausible hypotheses taken from theory (**Table 1**). We used a structural equation modeling approach (i.e., path analysis) because it allowed us to assess both direct and indirect effects of the selective agents. Assessing indirect effects is important in our study because some selective agents (e.g., competition and population density) are usually only considered in previous studies as acting through indirect effects (Scott and Johnson, 2010; Reznick et al., 2012).

We know that life-history traits can co-vary and its theory suggests that it may evolve as an integrated suite of traits (Fisher, 1930; Reznick, 1985). This assumption is important as it affects whether life histories can be evaluated as a collective strategy or if each life-history trait should be considered separately. To assess this assumption we used a confirmatory factor analysis that allowed us to test if a single factor (i.e., a single life-history variable) could adequately summarize all four life-history traits measured (Burnham and Anderson, 2002). During this confirmatory analysis, we found a negative error for reproductive

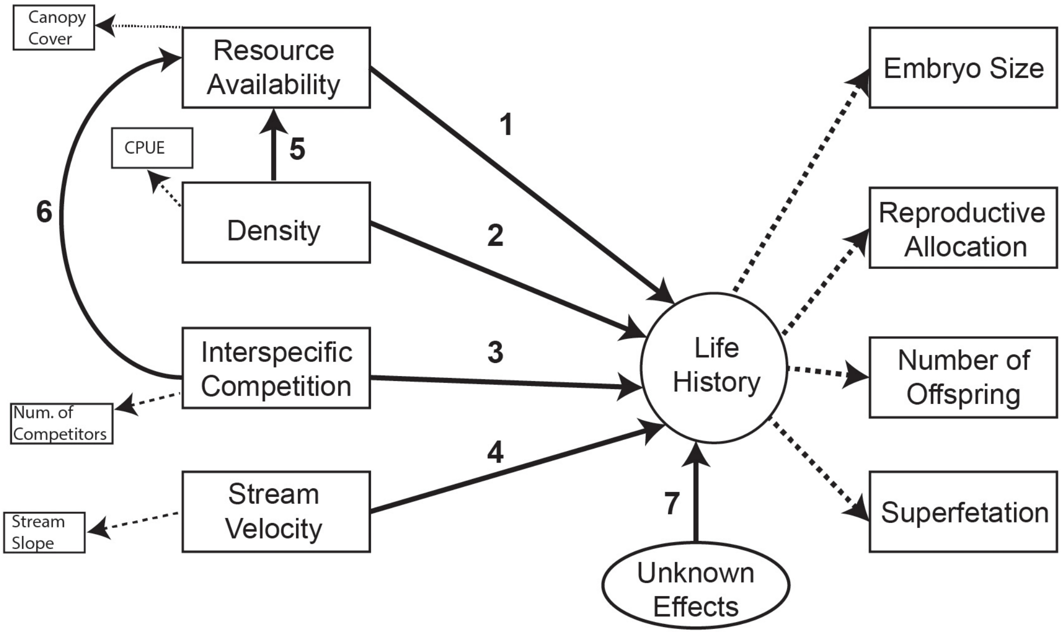

FIGURE 2 | Path diagram of the global model with both direct and indirect effects of four putative selective agents on life histories. Numbers above each path line correspond to path ID (also found in **Table 1** and **Table 4**).

allocation. We determined that the negative error was due to sample variance (Van Driel, 1978; Chen et al., 2001), thus we restricted the error to a small positive number (0.01) following Van Driel (1978). Fixing the error term as a small positive number allowed us to continue running a factor analysis and retain the potential for error in the measurement of reproductive allocation. We found that three of the four life-history traits appear to behave as an integrated suite of traits in *P. prolifica*, as they all had a positive association and loadings greater than 0.50 in the calculated life-history suite (**Figure 3**), corroborating the idea that certain life-history traits evolve in an integrated fashion (Fisher, 1930; Reznick, 1985). These three correlated life-history traits were number of embryos, reproductive allocation, and superfetation and, hence, our life-history suite is a combination of these three variables in such a way that large positive values of this variable correspond to females that produce many embryos, high reproductive allocation, and several simultaneous broods. In turn, large negative values of our life-history suite indicate females with the opposite set of traits (fewer embryos, low reproductive allocation, and fewer simultaneous broods). In contrast, embryo size had a small loading and very little variation explained by this life-history suite. Thus, our life-history suit was not a good reflection of embryo size, so for all the models we treated embryo size separately. We therefore proceeded to test the different hypotheses through a multi-model inference approach with two life histories measures as response: the life-history suite (which is a combined measure of number of embryos, reproductive allocation, and superfetation) and embryo size alone.

TABLE 4 | List of the *a priori* 14 candidate models of the effect of the four putative selective agents.

Selective agents[a]	Model	Paths
R	1	1,7
D	2	2,7
C	3	3,7
S	4	4,7
RD	5	1,2,7
RD	6	1,5,7
RD	7	1,2,5,7
RC	8	1,3,7
RC	9	1,6,7
RC	10	1,3,6,7
RCD	11	1,2,3,7
RCD	12	1,5,6,7
RCD	13	1,2,3,5,6,7
RCDS	14	1,2,3,4,5,6,7

[a] *Selective agents are abbreviated as follows: R, resources; D, population density; C, competition; S, stream velocity.*

We ran all 14 candidate models in path analysis using the software Amos (Arbuckle, 2013). All models were run using a maximum likelihood estimator. For each model, we generated an Akaike Information Criteria score (corrected for small sample sizes; AICc). We used AICc scores to identify models that best fit the data. Models in which AICc scores differ by less than two are generally considered indistinguishable (Burnham and Anderson, 2002; Burnham et al., 2011). We also calculated the model-averaged standard total effect for each selective agent using all 14 models and their associated AIC weights. The model-averaged standard total effect represents the amount of change

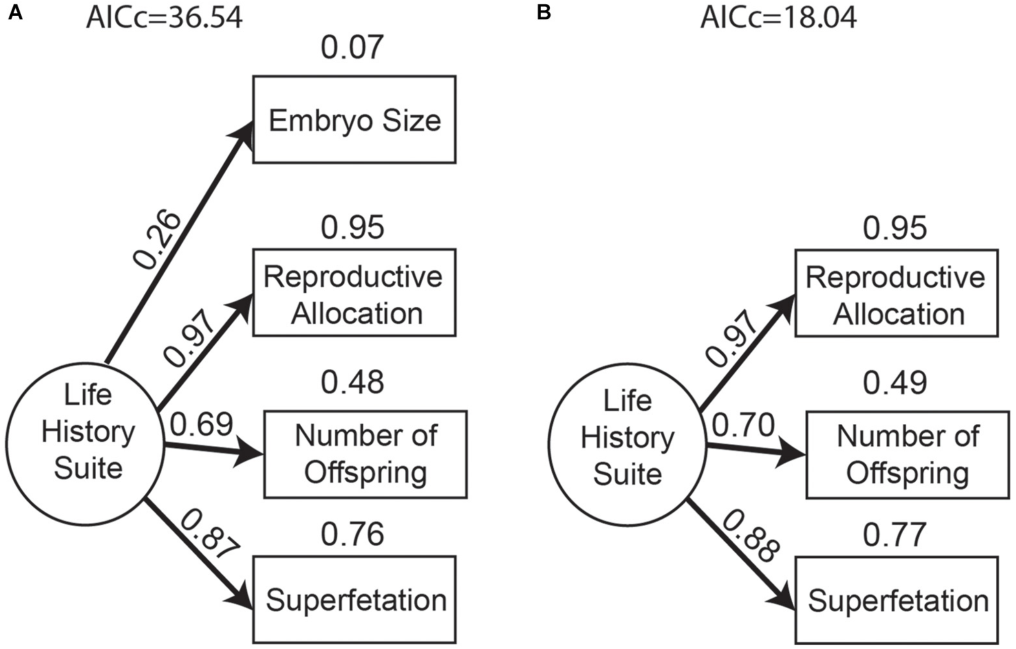

FIGURE 3 | Path diagrams of confirmatory factor analysis for **(A)** all life-history traits as a single measure and **(B)** excluding embryo size. Path diagram shows loadings above each path and proportion of variance accounted for (R^2) above each life-history trait.

in either the life-history suite or embryo size given a standard deviation unit change in each of the selective agent included in the models.

RESULTS

Population density and interspecific competition alone were the best predictors of number of embryos, reproductive allocation, and superfetation in *P. prolifica.* For this life-history suite, the two models with population density and interspecific competition alone as direct effects had lower AICc scores than all other models. However, it was not possible to distinguish between these two models as they differed by less than two AICc score units (**Table 5**). For embryo size, all models that included a single selective agent had the lowest AICc values and were equivalent in terms of AICc (**Table 6**).

Although the two models with population density and interspecific competition alone were undistinguishable based on AICc scores for the life-history suite, they differed in the strength of their effects on life histories. Population density had a stronger effect than interspecific competition (**Figure 4**), but both models revealed a positive influence of these two environmental factors on the life-history suite. In other words, increasing population density or interspecific competition results in an increase in all three life-history traits (number of embryos, RA, and superfetation).

For embryo size, all selective pressures, except interspecific competition, had the predicted effect from theory (**Table 1** and **Figure 5**). This means that embryo size increased as population density increased (**Figure 5A**), and decreased as resources and stream gradient increased (**Figures 5B,D**). In contrast, interspecific competition had a positive effect on embryo size (**Figure 5C**). This said, the model-averaged standard total effect was generally low for all four selective agents suggesting the strength of these selective pressures was overall weak on embryo size (**Figure 5**).

TABLE 5 | List of the *a priori* 14 candidate models of the effect of the four putative selective agents on the life-history suite with corresponding AICc values, ΔAICc, and AICc weight (W).

Selective agents[a]	Model[b]	Paths	AICc	ΔAICc	W
D	**2**	**2,7**	**31.08**	**0**	**0.416**
C	**3**	**3,7**	**31.37**	**0.29**	**0.360**
S	4	4,7	33.29	2.21	0.138
R	1	1,7	34.22	3.14	0.086
RC	9	1,6,7	45.92	14.84	<0.001
RD	5	1,2,7	46.91	15.83	<0.001
RC	8	1,3,7	47.33	16.25	<0.001
RC	10	1,3,6,7	48.95	17.87	<0.001
RD	6	1,5,7	49.02	17.94	<0.001
RD	7	1,2,5,7	49.06	17.98	<0.001
RCD	11	1,2,3,7	60.97	29.89	<0.001
RCD	12	1,5,6,7	63.18	32.1	<0.001
RCD	13	1,2,3,5,6,7	63.38	32.3	<0.001
RCDS	14	1,2,3,4,5,6,7	86.03	54.95	<0.001

Bolded model are the best models according to the AICc for the life-history suite.
[a] *Selective agents are abbreviated as follows: R, resources; D, population density; C, competition; S, stream velocity.*
[b] *Model numbers correspond to those shown in* ***Table 3****.*

TABLE 6 | List of the *a priori* 14 candidate models of the effect of the four putative selective agents on embryo size with corresponding AICc values, ΔAICc, and AICc weight (W).

Selective agents[a]	Model[b]	Paths	AICc	ΔAICc	W
R	**1**	**1,7**	**10.46**	**0**	**0.245**
D	**2**	**2,7**	**10.46**	**0**	**0.245**
C	**3**	**3,7**	**10.46**	**0**	**0.245**
S	**4**	**4,7**	**10.46**	**0**	**0.245**
RD	6	1,5,7	17.43	6.97	0.008
RD	5	1,2,7	18.76	8.3	0.004
RC	8	1,3,7	19.29	8.83	0.003
RC	9	1,6,7	20.35	9.89	0.002
RD	7	1,2,5,7	20.91	10.45	0.001
RC	10	1,3,6,7	20.91	10.45	0.001
RCD	12	1,5,6,7	28.42	17.96	<0.001
RCD	11	1,2,3,7	29.43	18.97	<0.001
RCD	13	1,2,3,5,6,7	31.84	21.38	<0.001
RCDS	14	1,2,3,4,5,6,7	40.77	30.31	<0.001

Bolded model are the best models according to the AICc for embryo size.

[a] *Selective agents are abbreviated as follows: R, resources; D, population density; C, competition; S, stream velocity.*

[b] *Model numbers correspond to those shown in **Table 3**.*

DISCUSSION

Overall we found mixed results, for embryo size selective agents were indistinguishable in their ability to predict life-history variation as in a previous study with the livebearing fish *Brachyrhaphis rhabdophora* (Johnson, 2002). However, for the other three life-history traits studied two selective agents independently, competition and population density, were equally likely to explain the variation in *P. prolifica*.

As interspecific competition increased, we found an increase in number of embryos, reproductive allocation, and superfetation. These results are consistent with theoretical predictions suggesting that poor competitive abilities can be compensated by a large reproductive effort (Hutchinson, 1957; Levine and Rees, 2002; Kneitel and Chase, 2004; Leibold et al., 2004; Calcagno et al., 2006; Chapuis et al., 2017). Hence, our findings indicate that *P. prolifica* may have low competitive abilities, but is likely better at reproduction. However, we did not measure competitive abilities which will be needed to confirm the hypothesis of a trade-off between reproduction and competitive abilities (Kneitel and Chase, 2004; Calcagno et al., 2006). Nevertheless, a trade-off is the most likely explanation for the observed positive association between the life-history suite and interspecific competition, otherwise a decrease in number of embryos and reproductive allocation would be expected (Scott and Johnson, 2010). Furthermore, interspecific competition was also positively associated with embryo size, meaning that for *P. prolifica* an increase in interspecific competition causes an increase in all four life-history traits, which also supports a trade-off between competitive ability and reproduction. Noticeably, the fact that intense competition can increase both number and size of offspring indicates that the common trade-off between these two life-history traits, which has been previously documented in other poeciliids (e.g., *Poeciliopsis gracilis* and *P. infans*; Frías-Alvarez et al., 2014), does not occur in *P. prolifica*.

Population density was also positively associated with each of the life-history traits evaluated here (reproductive allocation, number of embryos, superfetation, and embryo size). However, for embryo size the effect of population density was small (**Figure 5**). Nevertheless, results are still somewhat puzzling for the three other life-history traits in that increasing population density is expected to result in a decrease in number of embryos and in reproductive allocation (Johnson and Bagley, 2011; Moore et al., 2016). Higher population density should result in a reduction in per capita resource availability, resulting in lower reproductive allocation and number of embryos, but higher superfetation (**Table 1**). Two possibilities could account for our observations. First, competition in our system may not be through competition for available food resources as appears to be the case in other studies (Reznick, 1989; Reznick et al., 2002). In other words, life-history traits in *P. prolifica* may be shifting to improve competitive ability in a context different from competition for resources, such as microhabitat (**Table 1**). Second, most studies examining the effects of population density on life history have been conducted in species that lack superfetation (e.g., Smith, 2007; Reznick et al., 2012). The ability to carry multiple broods simultaneously in *P. prolifica* might confer advantages that alter the effect of population density on life history (Zúñiga-Vega et al., 2017). For example, higher superfetation is related to higher reproductive allocation in other livebearing fishes (Zúñiga-Vega et al., 2017), and it has been suggested that superfetation allows these species to overcome morphological constraints in terms of reproductive investment (Frías-Alvarez and Zúñiga-Vega, 2016; Zúñiga-Vega et al., 2017). Thus, it is possible that species that superfetate can have more offspring without the typical trade-off relative to non-superfetating species (Olivera-Tlahuel et al., 2015). This could allow for the positive relationship between population density and the life-history traits observed in our study.

Resource availability was not a good predictor of the life history suite, despite the fact this is a known selective agent for other poeciliids (Zúñiga-Vega et al., 2010; Johnson and Bagley, 2011). One possibility is that food resources are simply not limiting in this system and therefore play a lesser role in shaping life histories. Moreover, food resources availability can change through time (Reznick and Yang, 1993) and can be affected by several abiotic factors (i.e., substrate disturbance, stream velocity, etc., Zimmermann and Death, 2002; Hall et al., 2015) and it is possible that when we sampled did not capture the effects of resource availability. Another explanation could be that canopy cover, although a useful surrogate for primary productivity in streams, does not adequately reflect the resource dynamics in this system. If so, then future studies could focus on the inclusion of different measures to construct a more direct estimate of resource availability.

We were similarly surprised to find that stream velocity did not predict life history in our system, given that *P. prolifica* is a superfetating species and stream velocity is known to affect this life-history-trait in other superfetating poeciliid fish species

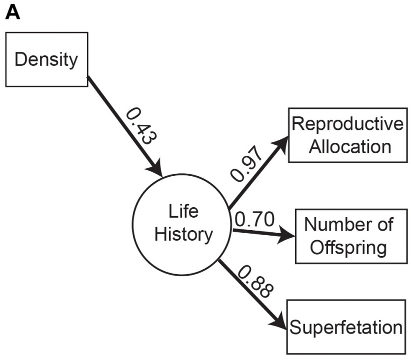

FIGURE 4 | Path diagrams of best models for the life-history suite showing **(A)** population density and **(B)** competition as direct effect on the life histories. Path diagram shows model-averaged standard total effect above the path line.

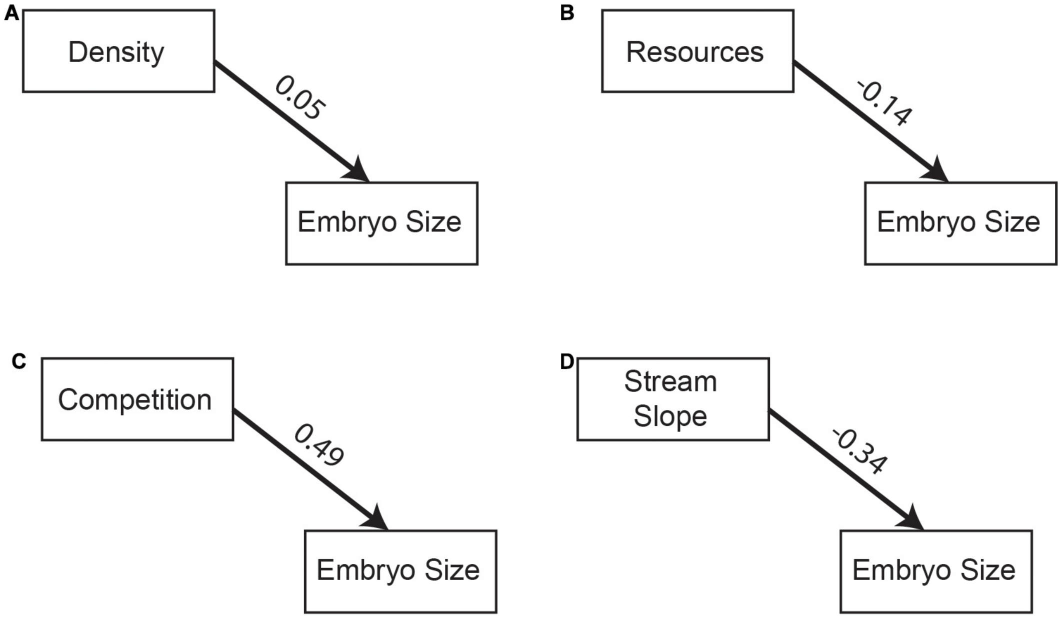

FIGURE 5 | Path diagrams of best models for embryo size showing **(A)** population density, **(B)** resource availability, **(C)** competition, and **(D)** stream slope as direct effect on embryo size. Path diagram shows model-averaged standard total effect above the path line.

(Zúñiga-Vega et al., 2007, 2010). One possible reason for this is that our use of stream slope may not capture the actual water velocity that these populations encounter. It is possible that *P. prolifica* females spend their majority of time in pools, rather than in the current where stream slope would be a better predictor of flow rate (Croft et al., 2003). Thus, measuring water velocity in the particular pool where individuals are observed could better account for the stream velocity that the species is experiencing. Additionally, stream velocity is a complex measure that can vary in time and space, especially depending on space complexity and heterogeneity (Lake, 2000; Palmer et al., 2010). Several measures may be needed to truly account for stream velocity variability, and basic information about species-specific microhabitat preferences could aid in considering what is the best measure for a particular species. It is also important to note that the effect of stream velocity on superfetation has not been conclusive, as some studies have found positive effects (Zúñiga-Vega et al., 2007) while others have found no effect (Frías-Alvarez and Zúñiga-Vega, 2016; Zúñiga-Vega et al., 2017). More research will be needed to understand how generally stream velocity can affect life histories. Finally, stream velocity is potentially a gradient in each population due to spatial heterogeneity. This gradient may cause individual fish to experience high variability in the direction and magnitude of selection. Adaptation to stream velocity could only occur in environments that are consistent in streamflow allowing selection time to act. Furthermore, the effect of stream velocity may not be detectable on those populations that experience gradients (either spatially or temporally) with a single point in time and several collections will be needed to reflect this variability.

The effect of all selective pressures on embryo size were small in *P. prolifica*. It is unclear why this is the case. It is possible that the selective agents that we examined here are simply weak in their ability to shape embryo size. Alternatively, embryo size may be a trait with little variation among populations such as has been previously observed in other poeciliids (e.g., *Poecilia butleri*; Zúñiga-Vega et al., 2011), perhaps as a result of genetic constraints. Despite the modest responses of offspring size to the putative selective agents, the pattern of change observed here was consistent in the direction predicted by theory for all selective agents (**Table 1**).

We found that the effect of interspecific competition was generally weaker than the effect of population density in predicting life history. This supports a body of theory that argues that intraspecific competition in general should be stronger than interspecific competition (Chesson, 2000, 2013; Adler et al., 2018). Although this was true for the majority of life-history traits examined here, this was not the case for embryo size where interspecific competition was stronger than the effects of population density. We note that studies which consider interspecific competition as a selective pressure on life histories of livebearers are uncommon (Scott and Johnson, 2010; Chapuis et al., 2017). Hence, our findings point to a promising area for future research, especially in understanding the interplay of ecological interactions both within and among species.

Finally, it is interesting to note that both population density and interspecific competition best explained our data when included as direct effects on life histories. Usually, when competition (both intraspecific and interspecific) is considered in life-history research, it is usually explained in terms of its indirect effect on life history mediated through food resource availability (Reznick and Yang, 1993; Scott and Johnson, 2010; Johnson and Bagley, 2011; Wilson, 2013). However, this was not the case in our study. Several possibilities could account for this result. First, food resource availability may not be as important in our system as in others; and it is possible that competition could be acting through some other environmental variable, such as habitat use. Second, this could simply be an artifact of the model selection framework used in our analysis. Models with indirect effects required more parameters, and consequently these models are penalized for the inclusion of additional parameters. Hence, the models may not be favored not because indirect effects are unimportant, but because they are not sufficiently important to offset the cost of including them in the model. When comparing models with direct and indirect effects at comparable nesting (models 5 vs. 6, 8 vs. 9, and 11 vs. 12), most were indistinguishable (**Table 4** and **Table 5**), so penalization for number of parameters may play an important aspect on preference for direct and indirect effects. Finally, it is possible that competition indeed has a strong direct effect on life history in *P. prolifica*. If this is the case, we need to consider how competition could act as a direct selective agent on life histories, an area where we still lack a strong theoretical foundation to make predictions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Brigham Young University Ethics Committee.

AUTHOR CONTRIBUTIONS

AJR-M, JJ, and MB conceived the study and interpreted the data analysis. AJR-M and JJZ-V collected all samples and processed samples. AJR-M collected and analyzed the data, and wrote the first draft of the manuscript. All authors contributed to manuscript corrections, editing, and improved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.608046/full#supplementary-material

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REVIEW
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The Biology of Polymorphic Melanic Side-Spotting Patterns in Poeciliid Fishes

Tanja C. Zerulla and Philip K. Stoddard*

Institute of Environment, Department of Biological Sciences, Florida International University, Miami, FL, United States

Melanin-based color patterns are an emerging model for studying molecular and evolutionary mechanisms driving phenotypic correlations. Extensive literature exists on color patterns and their correlated traits in the family Poeciliidae, indicating that these fishes are tractable models. We review the biology of polymorphic melanic side-spotting patterns characterized by macromelanophores forming irregular spotted patterns across fishes' flanks. These patterns are present in the genera *Gambusia*, *Limia*, *Phalloceros*, *Poecilia*, and *Xiphophorus*. Their presence is controlled by dominant genes on autosomes or sex chromosomes. Variation in expression is under polygenic control; however, these genes' identities are still largely unknown. In some *Gambusia holbrooki* and *Poecilia latipinna*, expression is dependent on low temperature exposure, but underlying molecular mechanisms are unknown. Spotted fish develop melanoma in rare cases and are a well-developed model for melanoma research. Little is known about other physiological correlates except that spotted *G. holbrooki* males exhibit higher basal cortisol levels than unspotted males and that metabolic rate does not differ between morphs in some *Xiphophorus* species. Behavioral differences between morphs are widespread, but specific to population, species, and social context. Spotted *G. holbrooki* males appear to be more social and more dominant. Juvenile spotted *G. holbrooki* have lower behavioral flexibility, and spotted *X. variatus* exhibit greater stress resistance. Findings conflict on whether morphs differ in sexual behavior and in sexual selection by females. Melanic side-spotting patterns are uncommon (<30%) in populations, although extreme high-frequency populations exist. This low frequency is surprising for dominant genes, indicating that a variety of selective pressures influence both these patterns and their correlated traits. Little is known about reproductive life history traits. Spotted *G. holbrooki* are larger and have higher survival when uncommon, but underlying mechanisms remain unknown. Spotted morphs appear to have a strong selective advantage during predation. Predators prefer to attack and consume unspotted morphs; however, this preference disappears when spotted *G. holbrooki* males are common, indicating negative frequency-dependent selection. Spotted morphs are preferred socially under turbid conditions, but other environmental factors that shape phenotypic correlations and morph fitness have not been studied. Finally, we present questions for future studies on melanic side-spotting patterns.

Keywords: color polymorphism, coloration, melanism, phenotypic integration, pigmentation, pleiotropy, trait associations

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Jerald B. Johnson,
Brigham Young University,
United States

Reviewed by:
Helen Rodd,
University of Toronto, Canada
Zachary Culumber,
University of Alabama in Huntsville,
United States

****Correspondence:***
Tanja C. Zerulla
tzerulla@fiu.edu;
Tanja.Zerulla@gmail.com

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INTRODUCTION

Poeciliidae are live-bearing fish of the sub-tropical and tropical waters of the Americas and Caribbean, a family that includes mollies, guppies, swordtails, platyfish, mosquitofish, limia, and others (Meffe and Snelson, 1989; Evans et al., 2011). Melanin polychromism has been observed in poeciliids since the late 1800's (Myers, 1925; Gordon and Gordon, 1957). These polymorphic patterns correlate with other traits, indicating that poeciliids have strong potential as a model taxonomic group for understanding the mechanisms, trends, and evolution of pigmentation-phenotype correlations common across the animal kingdom. A wealth of literature exists on poeciliid genetics, developmental biology, physiology, behavior, ecology, and evolution (for overviews, we recommend: Meffe and Snelson, 1989; Evans et al., 2011). Furthermore, poeciliids are an amenable group for laboratory and field experiments, being relatively easy to capture. Their small size and quick acclimatization to laboratory conditions, as well as their well-documented animal husbandry, makes them easy to maintain and handle for experiments. Poeciliids are also relatively easy to breed and have short generation times, which permits large-scale genetic and evolutionary studies. These features make them attractive over other taxa that may be more difficult to study, such as birds or mammals.

Previous reviews have focused on multiple color patterns and their related traits within a single species or genus of Poeciliidae (revs.: Basolo, 2006; Culumber, 2014). Here we focus on a polychromism that has arisen repeatedly across poeciliid species: the melanic side-spotting pattern, in which fish develop irregularly distributed black spots across their flanks. We propose that this polychromism is a potential model for investigating trait correlation patterns across populations and species, as well as for understanding the molecular and evolutionary mechanisms that lead to these similar correlations. Specifically, we describe the development, inheritance, and genetics of the melanic side-spotting pattern. We review trends in correlations with physiological and behavioral traits, as well as ecological selective pressures and evolutionary origins that possibly shape the polychromism. Although the melanic side-spotting pattern exists in multiple poeciliid species, most research has been focused on the swordtails, genus *Xiphophorus*, due to their development as a laboratory model of melanoma, and on Eastern Mosquitofish (*Gambusia holbrooki*) because it is a common and easily accessible species. Consequently, a species bias is present in this review, so we recommend that any noted trends be thoroughly investigated in other poeciliids. Furthermore, most studies have not been repeated within a species and are limited to only a few populations or breeding lines within a species, so we encourage further studies to validate the limited trait correlations identified and presented in this review within and across species. We highlight what is still unknown about the melanic side-spotting pattern and suggest areas of further research throughout the review.

MECHANISMS OF PIGMENTATION-PHENOTYPE CORRELATION

Correlations between color pattern and other phenotypic traits can arise through a variety of molecular and evolutionary mechanisms. The first potential evolutionary mechanism is **correlational selection**, in which similar selective pressures acting on separate genes results in a non-functional correlation between the melanic side-spotting pattern and another trait (**Figure 1A**; revs.: Sinervo and Svensson, 2002; Gray and McKinnon, 2007; Forsman et al., 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). Correlational selection is rare (rev.: McKinnon and Pierotti, 2010), as it usually leads to the second potential mechanism: **co-adaptive selection**. If these traits experience similar selective pressures, fish expressing both the pattern and the other trait would have increased fitness over those expressing one phenotype alone, so the separate genes for each trait are favored to be inherited together (**Figure 1B**; revs.: Sinervo and Svensson, 2002; McKinnon and Pierotti, 2010). Alternatively, these separate genes can be inherited together if the fitness of the color pattern depends on the expression of the other trait or vice versa (**co-dependence**; **Figure 1C**; revs.: Gray and McKinnon, 2007; Forsman et al., 2008; Peiman and Robinson, 2017; San-Jose and Roulin, 2018), or if the color pattern and other trait must both be expressed and working together for a shared function (**co-specialization**; **Figure 1D**, rev.: Peiman and Robinson, 2017).

Correlations can also arise due to shared molecular mechanisms (revs.: Ducrest et al., 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). Traits controlled by separate genes may be correlated through **sequential causation**, in which one trait is the precursor for another trait (**Figure 1E**, rev.: Peiman and Robinson, 2017). For example, a physiological change in hormones may be required first to initiate expression of the color pattern. Alternatively, these separate genes may have similar transcriptional regulation, such as through a shared promoter, that results in **co-expression** of the two different traits (**Figure 1F**; rev.: McKinnon and Pierotti, 2010). Genes that act on separate traits can also be inherited together if they are located close together on the same chromosome, as in **linkage disequilibrium** (**Figure 1G**; revs.: Sinervo and Svensson, 2002; Ducrest et al., 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). For example, the *P* (*pituitary*) locus is associated with differences in sexual maturity and mating strategy in *Xiphophorus* (Kallman and Borkoski, 1978; Lampert et al., 2010) and is close to the pigmentation locus on the *Y* chromosome (Kallman, 1983). Finally, genes and molecules rarely have a single biological function. Genes or molecular systems with multiple functions can thus affect multiple, separate traits through **pleiotropy** (**Figure 1H**; revs.: Sinervo and Svensson, 2002; Ducrest et al., 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). The melanocortin system is a key candidate that has been proposed to pleiotropically regulate pigmentation and other traits (revs.: Ducrest et al., 2008;

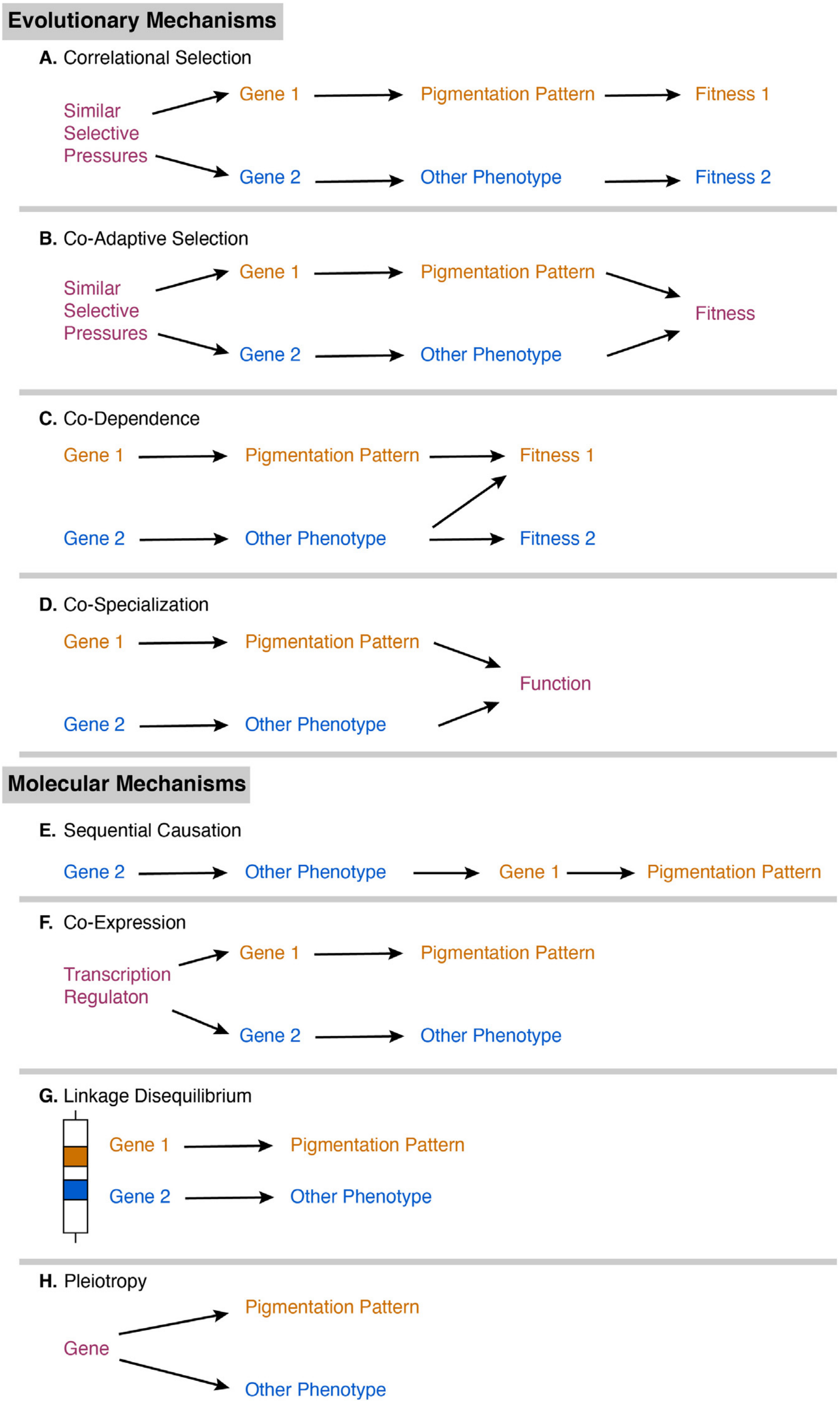


FIGURE 1 | Diagram of proposed evolutionary mechanisms **(A–D)** and molecular mechanisms **(E–H)** of phenotypic correlation. Pathway component colors indicate whether they are specific to the pigmentation pattern (orange), specific to a correlated phenotype (blue), or function in both pathways (purple).

San-Jose and Roulin, 2018). Melanocortins are pituitary peptide hormones that regulate melanin synthesis, rapid distribution of pigment in a cell, glucocorticoid release, energy balance, food intake, aggression, and sexual activity. However, melanocortins are not the only possible mechanism for pleiotropy; many endocrine and neuroendocrine substances are involved in the regulation of both pigmentation and other traits, including other peptide hormones, amino acid hormones (e.g., thyroid

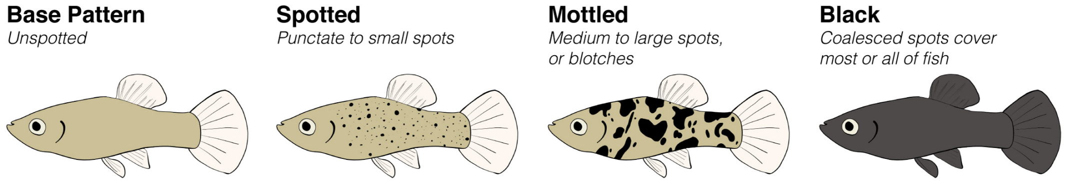


FIGURE 2 | Representation of the poeciliid pigmentation patterns included in this review. The base pattern lacks macromelanophores, so we interchangeably refer to it as "unspotted." Melanic side-spotting patterns include any pattern in poeciliid fishes that consists of macromelanophores forming spots that are irregularly distributed on the fish's flank. Included melanic side-spotting patterns may also extend into fins or be restricted to only a portion of the flank, not pictured here. The black morph is an extreme version of the spotting pattern.

hormone, GABA), monoamines (e.g., serotonin, dopamine), and sex steroids (e.g., testosterone). Neural crest cells have recently been proposed as a developmental mechanism for pleiotropy, as they differentiate into a variety of cells including pigment cells, neural cells, and endocrine cells (revs: Wilkins et al., 2014; San-Jose and Roulin, 2020). Determining which mechanism (or combination of mechanisms) underlies the specific correlations between melanic side-spotting patterns and other traits is not possible in a single study (San-Jose and Roulin, 2017), and will require multiple research groups working in parallel on different aspects of the biology of melanic side-spotting patterns and their correlated traits across different populations and species.

MELANIC SPOTTING PATTERNS

Pattern Description

The base pigmentation pattern of poeciliids is a gray-olivaceous color, created by small pigment cells called micromelanophores outlining the fish's scales in a reticulate pattern (Gordon, 1927, 1928; Anders et al., 1984). Because this base pattern does not have any defined spots, we use the term "unspotted" interchangeably throughout this review (**Figure 2**). Other color patterns overlay this base pattern, including those created by large pigment cells called macromelanophores (Gordon, 1927, 1928). This review focuses on what we term "melanic side-spotting patterns" that encompass a polychromism with a similar phenotype across poeciliid species and genera in which macromelanophores form irregularly distributed black spots on the fish's flanks (**Figure 2**). These melanic side-spotting patterns can vary in amount and size of spots and in whether they are composed of punctate spots or spots that have coalesced into blotches (**Figure 2**; Bellamy, 1936; Gordon and Gordon, 1957; Regan, 1961; Atz, 1962; Borowsky, 1973; Trendall and Johnson, 1981; Angus, 1983). To meet our criteria, the spotting pattern has to occur at minimum somewhere on the flank, but location of spotting on the flank can vary among species. While some species express spots over their whole body including head and fins (e.g., *G. holbrooki*), spots in other species are restricted to specific sections of the flank, such as dorsally of the mid-lateral line (e.g., *Xiphophorus birchmanni*). We do not include micromelanophore or macromelanophore spotting patterns expressed only in fins, that form single or twin spots on the flank or caudal peduncle, or that form stripes or bars. These patterns either overlap with species that express the pattern as a fixed trait (e.g., stripe in *Heterandria formosa*) or have only been studied in depth for a single genus (e.g., tail spots on the caudal peduncle of *Xiphophorus*).

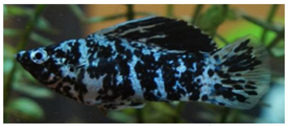
Although phenotypically similar, melanic side-spotting patterns can differ from each other in terms of genetics, inheritance, sex, development, and organization, which is why some patterns have unique names to differentiate them in the literature (**Table 1**). We thus recommend that researchers always include images and specific descriptions and features of the pattern that include key terms like "side-spotted" to ensure that published literature is searchable and accessible for those not familiar with species-specific color patterns. Spotted morphs also have been referred to as melanic, melanistic, or black in the past; we advise that these terms be reserved for all-black morphs to avoid confusion, and recommend using descriptors like spotted, mottled, or blotched instead.

Pigment Cells

Melanophore differentiation has been reviewed extensively (revs.: Anders and Anders, 1978; Vielkind and Vielkind, 1982; Anders et al., 1984; Parichy and Spiewak, 2015). Briefly, all melanophores originate from neural crest cells (Humm and Young, 1956; Vielkind et al., 1976, 1982). Pigment progenitor cells ("chromatoblasts") from the neural crest migrate across the body. These pigment progenitor cells then differentiate into melanophore progenitor cells ("melanoblasts"), which can either divide clonally or differentiate further. Melanophore progenitor cells differentiate irreversibly into either micro- or macromelanophore progenitor cells, which eventually differentiate terminally into micro- and macromelanophores (**Figure 3**).

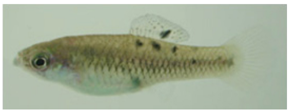
While micromelanophores differentiate in waves during embryonic stages to create the base pigmentation pattern, macromelanophores begin differentiation simultaneously and do so later in the fish's life to create spotting patterns (Humm and Young, 1956; Vielkind et al., 1976, 1982). Macromelanophores are 150–300 μm in diameter, approximately three times the size of micromelanophores measured in *G. holbrooki* (Regan, 1961; Kottler et al., 2020). Unlike micromelanophores, macromelanophores do not appear to have distance-dependent regulation, so they can overlap each other to form larger spots or blotches (Vielkind et al., 1982; Anders et al., 1984). Both melanophore types have dendritic or stellate shapes, but macromelanophores have

TABLE 1 | Summary of the specific poeciliid pigmentation patterns included under the “melanic side-spotting pattern” definition of this review.

Species	Pattern name	Representative photo	Pattern description	Wild or lab?	Sex	Inheritance	References
Gambusia holbrooki	Spotted, mottled, melanistic, melanic	 Photo by the authors	Small to large blotches on flanks, head, and fins	Wild	Male	Y chromosome	Myers, 1925; Regan, 1961; Angus, 1989; Horth, 2006; Kottler et al., 2020
Limia vittata	Spotted	 Photo T.M. Rodriguez-Cabrella	Small to large blotches on flanks	Lab	Both	Unknown	Haskins et al., 1960
Phalloceros caudimacultus	Spotted, melanistic, melanic, mottled, blotched	 Photo licensed under a Creative Commons agreement[1].	Small to large blotches on the flanks, head, and fins	Wild	Both	Autosomal	Myers, 1925; Trendall and Johnson, 1981; McDowall, 1999; Gutiérrez and García, 2007, 2011; McNeil and Wilson, 2008
Poecilia formosa	Spotted	Refer to photo for spotted *P. latipinna*	Small to large blotches on the flanks, head, and fins	Lab	Clonal female that develops as male	Paternal micro-chromosomes	Haskins et al., 1960; Schultz and Kallman, 1968; Turner et al., 1980; Schartl et al., 1997; Lampert et al., 2007; Nanda et al., 2007; Lamatsch et al., 2010, 2011
	Black	Refer to photo for black *P. latipinna*	Extreme form of spotting in which spots coalesce to cover almost or all of the body				
Poecilia latipinna	Spotted, mottled, melanistic, melanic	 Photos licensed under a Creative Commons agreement.	Small to large blotches on the flanks, head, and fins	Wild	Both	Autosomal (single gene)	Schröder, 1964; Angus, 1983; Angus et al., 1999
	Black	 Photos licensed under a Creative Commons agreement.	Extreme form of spotting in which spots coalesce to cover almost or all of the body	Lab	Both	Autosomal (single gene)	Schröder, 1964
Poecilia mexicana	Spotted	Refer to photo for spotted *P. latipinna*	Small to large blotches on the flanks, head, and fins	Wild	Both	Autosomal	Schröder, 1964; Menzel and Darnell, 1973; Culumber et al., 2014

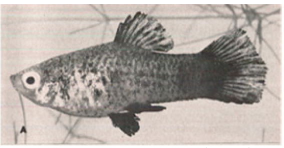
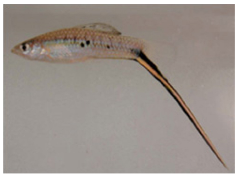
(Continued)

TABLE 1 | Continued

Species	Pattern name	Representative photo	Pattern description	Wild or lab?	Sex	Inheritance	References
	Black	Refer to photo for black *P. latipinna*	Extreme form of spotting in which spots coalesce to cover almost or all of the body	Lab			
Poecilia sphenops	Spotted	Refer to photo for spotted *P. latipinna*	Small to large blotches on the flanks, head, and fins	Wild	Both	Autosomal (at least two independently segregating genes)	Myers, 1925; Schröder, 1964
	Black	Refer to photo for black *P. latipinna*	Extreme form of spotting in which spots coalesce to cover almost or all of the body	Lab			
Poecilia velifera	Spotted	Refer to photo for spotted *P. latipinna*	Small to large blotches on the flanks, head, and fins	Lab	Both	Unknown	Schröder, 1964
	Black	Refer to photo for black *P. latipinna*	Extreme form of spotting in which spots coalesce to cover almost or all of the body				
Xiphophorus birchmanni	Carbomaculatus	 *X. birchmanni* (cam)[2]	Very few, large blotches on dorsoposterior of flank	Wild	Both	Autosomal?	Rauchenberger et al., 1990; Walter et al., 2006
X. cortezi	Atromaculatus, spotted	Photo not available	Many spots that later fuse into blotches similar to carbomaculatus; located on dorsoposterior of flank and dorsal fin	Wild	Both	Autosomal	Atz, 1962; Zander, 1965, 1967; Kallman, 1971; Rauchenberger et al., 1990
	Carbomaculatus, spotted	 *X. cortezi* (cam)[2]	Very few, large blotches on dorsoposterior of flank	Wild	Both	Autosomal (single gene)	Atz, 1962; Zander, 1965; Kallman, 1971; Rauchenberger et al., 1990; Walter et al., 2006
X. couchianus	Weakly spotted	Photo not available [3]	Weakly spotted	Wild (extinct)	Both	Unknown	Myers, 1925; Gordon, 1943
X. evelynae	Spotted, speckled-1, speckled-2	 *X. evelynae* (eve) with speckled-2[2]	Punctate to small spots on dorsoposterior of flank	Wild	Both	?	Weis and Schartl, 1998; Walter et al., 2006
X. helleri	Spotted, dabbed-1	 *X. helleri* (BxII)[2]	Few punctate to small spots on flank	Wild	Both	Autosomal (single gene)	Atz, 1962; Kallman and Atz, 1966; Kallman, 1975; Franck et al., 1998; Weis and Schartl, 1998; Walter et al., 2006
X. maculatus	Pulchra, spotted	 *X. maculatus* (Up-2) with spotted pattern-4[2]	Punctate spots to large blotches on flank	Wild	Both	*X* chromosome	Bellamy, 1924, 1936; Gordon, 1927, 1931a, 1937, 1943, 1946, 1951; Fraser and Gordon, 1929; Atz, 1962; Anders et al., 1973; Walter et al., 2006

(Continued)

TABLE 1 | Continued

Species	Pattern name	Representative photo	Pattern description	Wild or lab?	Sex	Inheritance	References
	Fuliginosus, fury	 Figure A from Mac Intyre (1961)	Undefined spots that appear diffusely on flank and fins to give a "sooty" look	Lab	Both	*Y* chromosome	Koßwig, 1938; Mac Intyre, 1961
	Nigra	 *X. maculatus* (nigra)[2]	A few large blotches coalesce into a black band or "smear" on the flank	Wild	Both	*X* chromosome	Bellamy, 1924; Gordon, 1927, 1931a,b; Atz, 1962; Kallman and Borkoski, 1978; Walter et al., 2006
	Black	 Photos licensed under a Creative Commons agreement.	Extreme form of spotting in which spots coalesce to cover almost or all of the body	Lab	Both	?	Gordon, 1927
X. meyeri	Spotted[4]	 *X. meyeri* (meyeri)[2]	Punctate to medium spots on flank	Wild	Both	Autosomal	Schartl and Schröder, 1987; Weis and Schartl, 1998; Walter et al., 2006
X. montezumae	Spotted	 *X. montezumae* (Rascon)[2]	Small punctate spots on flank by the mid-lateral line, but occasionally also on dorsal fin	Wild	Both	Autosomal	Gordon, 1943; Kallman and Atz, 1966; Zander, 1967; Kallman, 1983; Rauchenberger et al., 1990; Weis and Schartl, 1998; Walter et al., 2006
	Marmoratus	Photo not available	Large blotches on flank located ventrally of mid-lateral line	Wild	Both	?	Weis and Schartl, 1998
X. nezahualcoyotl	Spotted	 *X. nezhualcoyotl* (El Salto)[2]	Small to medium spots on flanks	Wild	Both	*X* and *Y* chromosome	Kallman, 1983; Rauchenberger et al., 1990; Weis and Schartl, 1998; Walter et al., 2006; Fernandez and Bowser, 2008
X. variatus	Punctatus (1–3), spotted, blotched, speckled	 *X. variatus* (Zarco) with punctatus-2[2]	Punctate to large blotches depending on the punctatus pattern (2 is heavier than 1) that are restricted to on or above mid-lateral line on flank	Wild	Both	*X* and *Y* chromosome	Gordon, 1943; Atz, 1962; Kallman and Atz, 1966; Borowsky, 1973; Kazianis and Borowsky, 1995; Schartl et al., 1995; Walter et al., 2006
X. xiphidium	Spots, spotting, flecked	 *X. xiphidium* (RP)[2]	Punctate to small blotches restricted to on or above mid-lateral line on flank	Wild	Both	*X* chromosome	Gordon, 1943; Atz, 1962; Kallman and Atz, 1966; Walter et al., 2006

Patterns are listed by species with name of the pattern in published literature, a brief description and representative photo, whether the pattern exists in the wild, the sex that the it primarily occurs in, and the mode of genetic inheritance. 1. Creative commons photos licensed under the following agreement: https://creativecommons.org/licenses/by-sa/3.0/legalcode. 2. Images provided by the Xiphophorus Stock Center at Texas State University (Walter et al., 2006). 3. The spotted pattern has not been observed in X. couchianus since 1943. This species is extinct in the wild (Walter et al., 2006). 4. X. meyeri also exhibits a spotted pattern. The cell types have been considered intermediate between micro- and macromelanophores (Schartl and Schröder, 1987; Weis and Schartl, 1998) and as macromelanophores (Walter et al., 2006), so it is tentatively included in this review.

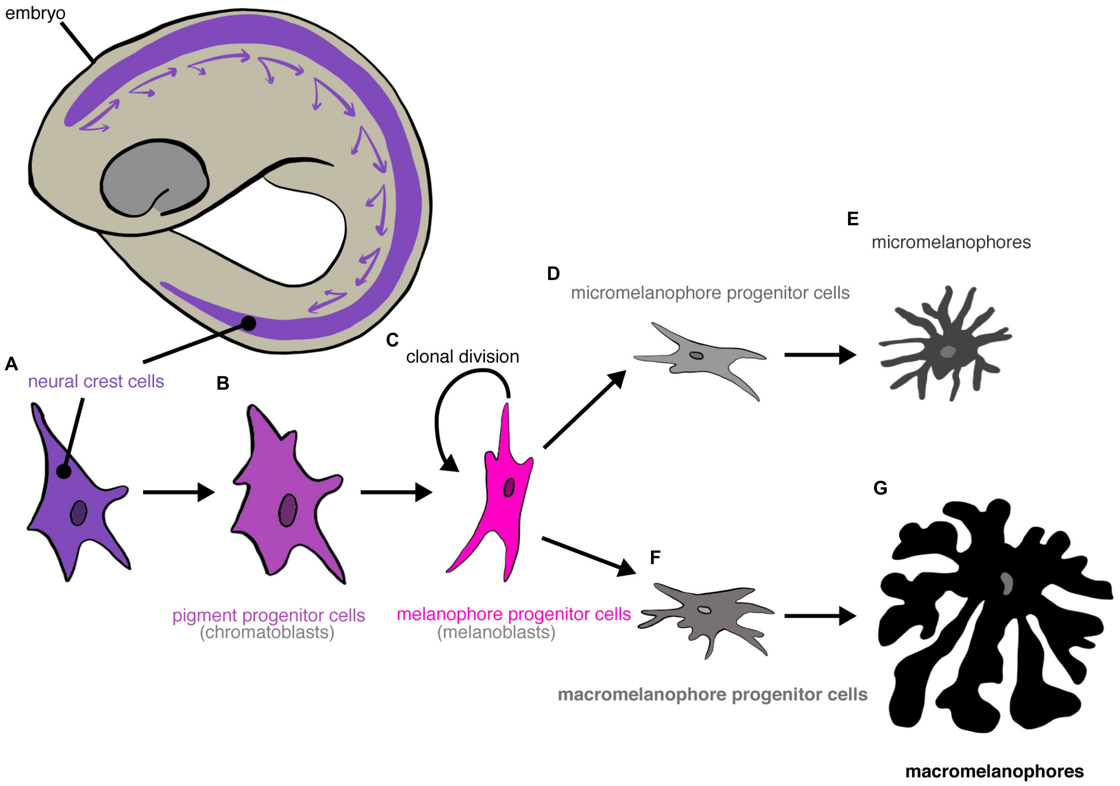


FIGURE 3 | Differentiation of poeciliid melanophore cells. Neural crest cells **(A)** migrate in a ventral-posterior direction. They differentiate into pigment progenitor cells **(B)** that then further differentiate into melanophore progenitor cells **(C)**. Melanophore progenitor cells either divide clonally or terminally differentiate into micromelanophore **(D)** or macromelanophore progenitor cells **(F)**, which differentiate terminally into micromelanophores **(E)** or macromelanophores **(G)**, respectively.

thicker and more branched dendritic arms (Gordon, 1931b; Regan, 1961; Vielkind, 1976; Anders et al., 1984). Because they also contain higher numbers of melanosomes, organelles that synthesize and store melanin, macromelanophore spots appear intensely pigmented (Gordon, 1931b; Regan, 1961; Vielkind, 1976; Anders et al., 1984). When *G. holbrooki* skin is treated with adrenaline, micromelanophores aggregate their melanosomes so skin appears lighter, whereas melanosomes in macromelanophores do not move and remain distributed so spots stay dark (Kottler et al., 2020). This difference in adrenaline response suggests that some function of melanosome regulation is altered in macromelanophores (Kottler et al., 2020). Macromelanophores can inhibit differentiation and initiate destruction of micromelanophores, but not other pigment cell types (Gordon, 1928; Schmidt, 1978). Since destruction does not require direct cell contact, macromelanophores seem to be releasing an unidentified diffusible factor that specifically targets and eliminates micromelanophores (Schmidt, 1978). The function of this destruction is unknown, although it may serve to amplify the pattern as a visual signal by creating a halo effect around the spots (Schmidt, 1978; Vielkind et al., 1982).

Developmental Timeline

Poeciliid growth rate and age of sexual maturation is strongly influenced by environmental factors (Meffe and Snelson, 1989), so we describe development in terms of sexual maturation rather than time. Unspotted morphs are born without spots and remain unspotted for their entire life (Schröder, 1964; Angus, 1983). In the wild, poeciliids are born unspotted; spots often appear as secondary sexual characteristics begin to develop, such as the gonopodium in immature males, but the melanic side-spotting pattern is not fully established until after the fish is sexually mature, after which it can increase with age in some species (Bellamy, 1924, 1928; Myers, 1925; Gordon, 1931b; Regan, 1961; Atz, 1962; Kallman and Atz, 1966; Kallman, 1971; Menzel and Darnell, 1973; Angus, 1983; Martin, 1984; Franck et al., 1998; Angus et al., 1999; Meyer et al., 2006; Gutiérrez and García, 2007). For some spotted or all-black laboratory strains, spotted morphs can be born unspotted or spotted and either develop into spotted or completely black morphs after sexual maturity (Schröder, 1964; Angus, 1983).

ECOLOGY

Frequency

For poeciliid species in which a melanic side-spotting pattern has been identified, the spotted morph is not present in every population (Gordon, 1948; Gordon and Gordon, 1957; Kallman, 1971; Martin, 1984; Horth, 2004; Culumber et al., 2014). When present, the frequency of spotted individuals varies depending on the population and species. In wild populations within their native range, spotted patterns ranged from a frequency of <0.5 to 28% of individuals in *Xiphophorus maculatus*, *X. helleri*, *X. variatus*, *X. cortezi*, *G. holbrooki*, *Phalloceros caudimaculatus*, and *Poecilia mexicana* (Gordon, 1943, 1948; Gordon and Gordon, 1957; Regan, 1961; Kallman, 1971; Snelson et al., 1986; Karplus and Algom, 1996; Horth, 2004; Gutiérrez and García, 2007; Culumber et al., 2014). Rarely, extreme populations occur with higher frequencies of spotted morphs ranging from 40 to over 70%, as seen in *X. cortezi*, *X. variatus*, and *X. xiphidium* (Gordon, 1943; Kallman, 1971). Frequencies were often determined from a small subset of populations, and thus further sampling is required to understand frequency distributions for melanic side-spotting patterns across the entire native range of a species. Some invasive populations of *Poecilia latipinna* and *Phalloceros caudimaculatus* also exhibit frequencies >50%; however, these populations were founded by black or spotted individuals, which may partially explain the pattern's maintenance at a higher frequency (Nelson, 1983; McDowall, 1999; McNeil and Wilson, 2008; Petrescu-Mag et al., 2008; Koutsikos et al., 2017). Future comparisons of low-frequency and high-frequency populations may be interesting to investigate how population-level differences affect the frequency and maintenance of spotted morphs within a species.

Melanic side-spotting patterns likely arose through random, spontaneous mutations in the pigmentation pathway. Spontaneous mutations can maintain novel phenotypes at low frequencies within populations if the mutations occur at a similar or higher rate than they are selected out of a population (Gordon and Gordon, 1957; Angus, 1983; Horth, 2006; Gutiérrez and García, 2007); however, spontaneous mutation rates are very low, and the frequencies seen in most poeciliid populations would require an improbably high mutation rate, suggesting the wide taxonomic distribution and persistence of poeciliid side-spotting is instead maintained by other processes (Huxley, 1955; Horth, 2006; Gray and McKinnon, 2007; Forsman et al., 2008; McKinnon and Pierotti, 2010). Furthermore, melanic side-spotting patterns appear to be evolutionarily stable, as macromelanophore-based patterns have possibly existed within poeciliid species for millions of years (Kazianis and Borowsky, 1995). Frequency of spotted morphs often varies seasonally and annually by population, but ultimately stabilizes around a mean over time, indicating that the pattern is an evolutionarily stable phenotype (Gordon, 1943; Gordon and Gordon, 1957; Horth and Panayotova, 2012). Since melanic side-spotting persists stably across populations and species, various molecular constraints (e.g., pleiotropy) and selective pressures (e.g., predation) likely act upon these patterns and their correlated traits to maintain them at a low frequency in the population (Gordon and Gordon, 1957; Horth, 2004), and those that have been identified in the literature are reviewed in the following sections.

Life History Traits

Life history traits are important components of fitness, so differences between morphs reflect variation in the trade-offs as well as molecular, ecological, and evolutionary constraints that govern morph life history strategies, which in turn affect the frequency and maintenance of melanic side-spotting patterns. Unspotted and spotted female *Poecilia sphenops* have similar gestation times, regardless of the sire's pattern (Schröder, 1964). Although most *G. holbrooki* broods have equal sex ratios, some male-biased broods sired by spotted males have been observed (Horth, 2006). The spotted pattern locus could contain or be linked to a selfish gene element that directly or indirectly drives the production of spotted males, which would maintain the morph in some populations. Further research into brood sex ratios is required across populations and species. Other reproductive traits like fecundity, brood size, neonate size at birth, and energy investment during gestation by females have not been systematically investigated. *Xiphophorus* males with heavier pigmentation often have reduced survival (Koßwig, 1927; Mac Intyre, 1961), which could reflect a fitness disadvantage that drives down the frequency of spotted morphs. In *G. holbrooki*, spotted males are larger than unspotted males, which could give spotted males a dominance or mating advantage that contributes to the maintenance of this polymorphism (Martin, 1977; Horth et al., 2010; Culumber et al., 2018). However, this trend cannot be generalized to every population, as temperature-sensitive siblings of the same genotype but different phenotype were the same size (Horth, 2003), or to every species, since *X. helleri* morphs did not differ in body mass (Meyer et al., 2006). Although feeding behavior does not differ between morph juveniles in *G. holbrooki* (Culumber et al., 2018), other correlated physiological (e.g., growth rate) or behavioral (e.g., food competition) traits could still underlie the increased size of spotted males (Martin, 1977; Culumber et al., 2018), while also explaining why this size difference is not a general trait across populations and species. Further research into morph trade-offs among life history traits is required.

Life history traits do not vary due to genetic and physiological differences alone; variation in social and physical environment also influences these traits, so each morph's life history strategy may only be apparent in certain conditions. For example, different frequencies of spotted and unspotted morphs alter the social environment, as the likelihood of encountering and interacting with each morph will also be different. Juvenile body condition in *G. holbrooki* was better when individuals were raised with a preponderance of the opposite morph, which indicates negative frequency-dependent selection (Culumber et al., 2018). Unspotted juveniles were affected to a greater degree, so morphs do seem to have underlying genetic or physiological differences that shape this trait (Culumber et al., 2018). Survival rates also differed based on the frequency of the spotted morph in *G. holbrooki* (Horth and Travis, 2002). When spotted males were uncommon, they survived better than unspotted males; however, as frequency of spotted males increased, their survival decreased to the same level as unspotted males (Horth and Travis, 2002; Horth, 2004). The spotted morph thus seems to have a survival advantage when uncommon, again indicating negative frequency-dependent selection. Furthermore, higher frequencies of spotted males led to higher mortality of juveniles and females (Horth and Travis, 2002), which can lower a population's reproductive capacity. Altered behavior in the presence of spotted males is hypothesized to drive this lower survival (Horth and Travis, 2002). For example, juvenile feeding was lower in the presence of spotted males compared to unspotted males (Culumber et al., 2018). The effect of other social environmental factors like density or sex ratio and of physical environmental factors on morph life history traits remains to be investigated across species.

Predation

Predation is a major selective pressure on animal color patterns (rev.: Endler, 1988). Studies have found that predators prefer unspotted over spotted poeciliid morphs, which may contribute to a selective survival advantage for melanic side-spotting patterns. Pike topminnow (*Belonesox belizanus*) ate more unspotted than spotted *X. helleri* and aimed more hunting behaviors toward unspotted model fish than spotted (Dürr, 1996 and Becker, 1997 in: Franck et al., 2001). Similarly, sunfish (*Lepomis* sp.) ate more unspotted than spotted *G. holbrooki* (Martin, 1977; Bonner, 1980 in: Martin, 1986), while both dragonfly naiads (*Libellulid* sp.) and crayfish (*Procambarus* sp.) tended to eat the unspotted morph first (Horth, 2004).

Predation is hypothesized to maintain melanic side-spotting patterns at low frequencies in poeciliid populations through negative frequency-dependent selection (Gordon and Gordon, 1957; Martin, 1977; Horth, 2004). At a low frequency of spotted *G. holbrooki*, Largemouth Bass (*Micropterus salmoides*) ate more unspotted males (Horth, 2004). This preference disappeared when the bass had access to a higher frequency of spotted males, indicating that the spotted morph only has a selective survival advantage when it is uncommon (Horth, 2004). A lack of preference by bass when the morphs were equally present (Humphrey, 2019) could be because the spotted morph frequency was above a selection threshold for these predators. Modeling negative frequency-dependent predation of *G. holbrooki* morphs confirmed its role in stabilizing the pattern polymorphism in populations (Horth and Panayotova, 2012).

This advantage at low frequencies could result from predators using a search image (Endler, 1988), in which they seek unspotted fish as their major prey and overlook or ignore spotted fish because they are not a common and reliable food source. When the spotted morph becomes more common, the predators adjust their search images to include them, and the former advantage disappears (Horth, 2004). Alternatively, spotted morphs could have an advantage due to other correlated traits that have not yet been identified, such as anti-predator behaviors (Horth, 2004). In a preliminary experiment, spotted and unspotted *G. holbrooki* males did not differ in their response to predator odorants (Kraft, 2016). Both morphs also exhibited similar decreases in mating behavior and increases in freezing behavior when a restrained Largemouth Bass was present (Humphrey, 2019). Although *G. holbrooki* morphs from a single population do not appear to exhibit differences in anti-predator behavior, this result may differ by population, poeciliid species, and predator species. Crypsis may be more important in altering predation rate. Black mollies prefer shoals that were against a black background over shoals against a white background, suggesting that background matching is important for poeciliid color morphs (Bradner and McRobert, 2001a), but crypsis has not been studied in relation to the melanic side-spotting pattern.

Physical Environment

If environmental factors differentially affect the fitness of spotted and unspotted morphs, then frequency of the melanic side-spotting pattern may vary temporally and spatially with these factors (Gordon and Gordon, 1957; Horth and Travis, 2002). Some *X. helleri* live in seasonally variable streams that are clear in the dry season and turbid in the rainy season, while *P. mexicana* populations are found in both non-sulfidic springs with clear water and in sulfidic springs with cloudy water that creates a white background (Franck et al., 2001; Culumber et al., 2014). Both unspotted *X. helleri* females in clear water and unspotted *P. mexicana* females from non-sulfidic springs preferred unspotted social partners (Franck et al., 2001; Culumber et al., 2014). In turbid water, unspotted female *X. helleri* switched their preference to spotted social partners, while unspotted female *P. mexicana* from cloudy sulfidic springs showed no preference between morphs (Franck et al., 2001; Culumber et al., 2014). In both species, spotted morphs preferred spotted social partners (Franck et al., 2001; Culumber et al., 2014). Spotted fish therefore seem to have a social advantage in low visibility conditions, most likely driven by the increased visibility of spotted individuals compared to unspotted (Franck et al., 2001). Since spotted *P. mexicana* were only obtained for sulfidic springs, these populations may alternatively differ in genetic preferences (Culumber et al., 2014). Selective advantage of the spotted morph thus seems to vary spatially and temporally depending on environmental conditions (Franck et al., 2001), and could explain different frequencies of the pattern across species' populations.

Environmental factors can also affect the expression of melanic side-spotting patterns (Regan, 1961; Schröder, 1964; Anders and Klinke, 1965). For example, a small number of populations of *G. holbrooki* and *P. latipinna* are temperature-sensitive, exhibiting greater expression of the spotted pattern at low water temperatures experienced during the winter (<22°C; Schröder, 1964; Angus, 1983, 1989; Angus et al., 1999; Horth, 2006). In simulated population models of *G. holbrooki*, temperature affected population frequency of the spotted morph both seasonally and long-term under different climate change scenarios (Horth and Panayotova, 2012; Panayotova and Horth, 2018). Variation in temporal expression of the spotted pattern could then influence how and when selective pressures affect this morph's fitness. A fish born in the spring will be unspotted, then mature and reproduce during the summer, all before winter temperatures induce expression of the spotted phenotype (Angus, 1983). The spotted pattern gene(s) would thus be inherited and maintained in the population regardless of fitness costs later in the fish's life because natural selection did not act upon the phenotype before the bulk of reproduction took place (Angus, 1983). Alternatively, the spotted and unspotted morphs may differ in fitness in warm and cool waters, possibly through other correlated traits, which would favor inducible phenotypic expression (Horth, 2003; Meyer et al., 2006). The effect of other physical factors on pattern expression and morph fitness remains unexplored, including oxygen conditions, salinity, precipitation, seasonal flooding and drying out of areas (hydroperiod), habitat fragmentation, vegetative cover, food availability, ultraviolet light exposure, behavioral thermoregulation, and parasitism (Gordon and Gordon, 1957; Schröder, 1964; Borowsky, 1973; Nelson and Planes, 1993; Meyer et al., 2006; Ducrest et al., 2008; Petrescu-Mag et al., 2008; Horth et al., 2013; San-Jose and Roulin, 2018).

SOCIAL BEHAVIOR

Social Conflicts: Aggression and Dominance

Behavioral studies on the melanic side-spotting pattern exhibit a strong species bias to Eastern Mosquitofish (*G. holbrooki*), likely due to its widespread distribution and accessibility. Behavioral differences between *G. holbrooki* morphs are easily observable, and had already been noted in wild populations a century ago (Myers, 1925), which likely further sparked the multitude of studies aimed at understanding spotted morph behavior. Below we present trends in social behavior, but we advise caution in generalizing these trends and encourage further research into behaviors correlated with the melanic side-spotting pattern in other poeciliid species.

Melanin-based color patterns are associated with higher aggression, bold behavior, and social dominance across taxa (revs.: Ducrest et al., 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). In *G. holbrooki*, one morph is not inherently more aggressive than the other; rather, male *G. holbrooki* morphs alter their behavior depending on their social partner and the composition of their social group (Martin, 1977; Horth, 2003; Kraft et al., 2018). In same-morph groups, unspotted males exhibited higher aggression toward each other than spotted males did (Martin, 1977). In mixed-morph groups, unspotted and spotted males directed similar levels of aggression toward unspotted males, but unspotted males directed less aggression back to spotted males (Martin, 1977; Horth, 2003; Kraft et al., 2018). Spotted male dominance over unspotted males thus seems to be driven by behavioral changes of unspotted males (Martin, 1977; Horth, 2003; Kraft, 2016). A dominance advantage for spotted males could maintain the pattern in populations if it also confers a fitness advantage. However, this correlation may not be present in all poeciliids. Within male *X. helleri* dyads, neither unspotted nor spotted males achieved dominance over the other (Franck et al., 2001). As well, spotted *X. variatus* were more shy in new environments (Culumber, 2016), rather than bold as would be expected of dominant fish.

Unspotted *G. holbrooki* juveniles exhibited more submissive behaviors toward spotted adults than unspotted adults, whereas spotted juveniles did not change their behavior based on color composition of the social group (Kraft et al., 2018). Unyielding dominance behaviors by spotted males could be a tactic to win more social conflicts against unspotted males, as the unspotted males would be more likely to switch to submissive behaviors and thus lose. This behavioral inflexibility may also confer stress resistance, which is typically a fitness advantage (rev.: Wingfield and Sapolsky, 2003), as the spotted morph would maintain their normal behavior in a variety of contexts. For example, spotted *X. variatus* exhibited greater behavioral resistance to stress than unspotted morphs (Culumber, 2016). Alternatively, this inflexibility may be a fitness disadvantage if spotted morphs do not avoid stressful or costly conflicts.

Precopulatory Sexual Selection: Mating and Sexual Activity

Findings conflict on whether morphs differ in sexual behavior and in precopulatory sexual selection by females. Where differences do exist, they seem to be influenced by population, species, morph frequency, experience, and environmental factors. (The reader interested in larger patterns may safely skip to the next section, "Social Groups"). In *G. holbrooki*, spotted males sometimes exhibited lower sexual activity than unspotted males (Nelson and Planes, 1993; Kraft et al., 2018), no difference (Martin, 1977; Karplus and Algom, 1996), or higher sexual activity (Martin, 1977; Karplus and Algom, 1996; Horth, 2003; Humphrey, 2019; Culumber et al., 2020). Results may depend on which specific sexual behaviors are measured. For example, Karplus and Algom (1996) found that morphs did not differ in time spent following females but spotted males were more likely to associate with females and interfere with other individuals' sexual interactions. These conflicting results may also be a function of different densities and morph frequencies (Martin, 1977), or selective pressures particular to the origin population that strongly influence the presence and direction of the pigmentation-behavior correlation.

Female behavior toward males can influence mating success for both courtship and coercive mating strategies in poeciliids (Bisazza et al., 2001). Female preferences for these morphs vary widely, most likely because responses are shaped by female morph identity, population, experience, and social context. For example, female *G. holbrooki* either directed more aggression toward spotted males and avoided them (Martin, 1977; Taylor et al., 1996), or were equally aggressive to both morphs (Horth, 2003). Female *G. holbrooki* preferred free-swimming unspotted males (Nelson and Planes, 1993; Taylor et al., 1996; Horth, 2003), but showed no preference when a barrier separated the sexes (Martin, 1986; Nelson and Planes, 1993) and have even preferred spotted and black stationary models (Gould et al., 1999). Even if *G. holbrooki* females have an innate preference for spotted males, other traits that are correlated with the melanic side-spotting pattern, such as sexual activity, may be more important in setting these preferences in the wild. Local environmental selective pressures may also strongly influence morph preferences because female preferences from different origin populations or environmental conditions differ in several species: *G. holbrooki* (Bisazza and Pilastro, 2000; Bisazza et al., 2001), *X. helleri* (Franck et al., 2001), and *P. mexicana* (Culumber et al., 2014). Finally, it is unknown whether females prefer traits that only sometimes correlate with the melanic side-spotting pattern instead of the pattern itself, which may also explain these conflicting results.

Higher sexual activity or female preference for a color morph could lead to higher mating success and thus higher reproductive fitness, which would contribute to that morph's maintenance in a population (Martin, 1977). In contrast, higher sexual activity could be disadvantageous if the males over-harass and stress females, leading to increased female mortality or decreased reproductive output (Horth and Travis, 2002). *G. holbrooki* females paired with size-matched spotted and unspotted males produced broods with frequencies of each phenotype that matched the expectation that neither morph had a mating advantage that translated into a fitness advantage (Horth et al., 2010); however, reproductive fitness may depend on morph frequencies in a population and should still be investigated. To our knowledge, postcopulatory sexual selection has not been studied in species with side-spotted morphs.

Social Groups

Spotted and unspotted morphs do not encounter and interact with color morphs and sexes at similar rates. Each morph may thus have a different social group, which in turn affects social dynamics. Both *G. holbrooki* morphs interacted with the same number of unspotted males, but spotted males had more social partners and more interactions with females than unspotted males in the lab and in the wild (Kraft et al., 2016). Both white and black mollies preferred phenotypically similar shoal mates (McRobert and Bradner, 1998; Bradner and McRobert, 2001b). Although white and black mollies are the result of domestication and hybridization among *Poecilia* species (McRobert and Bradner, 1998; Bradner and McRobert, 2001b), black mollies represent an extreme form of spotting and may still provide some insights, but these preferences should still be investigated in *Poecilia* spotted morphs. In clear water, unspotted and spotted female *X. helleri* also preferred phenotypically similar schools (Franck et al., 2001). Asymmetric social interactions between morphs could lead to assortative schooling, influencing dominance and mating dynamics, which would in turn affect morph fitness. However, social groups are also influenced by experience, as both black and white mollies preferred to shoal with the morph with which they were raised over phenotypically similar fish (Ledesma and McRobert, 2008). Given that spotted morphs are usually uncommon, both morphs may prefer to shoal with the familiar and common unspotted morph, and thus assortative schooling and mating may not occur in the wild. To our knowledge, social interactions with heterospecific individuals have not yet been investigated.

GENETICS

Modes of Inheritance

The melanic side-spotting pattern is always a genetically dominant trait (Gordon, 1927, 1937; Fraser and Gordon, 1929; Gordon and Smith, 1938; Gordon and Gordon, 1957; Atz, 1962; Schröder, 1964; Zander, 1965; Kallman and Atz, 1966; Kallman, 1971; Angus, 1983, 1989; Horth, 2006; Gutiérrez and García, 2007). Long-term persistence of a dominant trait indicates that multiple positive and negative selective pressures likely act upon the pattern and its correlated traits (Gordon and Gordon, 1957). Mode of inheritance differs across species and for each specific melanic side-spotting pattern (**Table 1**). In some species, the pattern is controlled by one or more autosomal genes (Atz, 1962; Schröder, 1964; Zander, 1965; Kallman and Atz, 1966; Kallman, 1971; Angus, 1983). In others, the patterns are linked to a sex chromosome (Gordon, 1927, 1946, 1951; Fraser and Gordon, 1929; Bellamy, 1936; Regan, 1961; Anders et al., 1973; Kallman, 1983; Angus, 1989; Horth, 2006; Fernandez and Bowser, 2008). Sex-linkage may evolve in certain species through sexually antagonistic selection (Kottler and Schartl, 2018); the melanic side-spotting pattern or its correlated traits may be advantageous to male fitness but disadvantageous to female fitness, resulting in strong selective pressure for pattern expression to be greater in males than females (Kottler and Schartl, 2018). For melanic side-spotting patterns that are primarily expressed in males, spotted females are rare (Fraser and Gordon, 1929; Gordon, 1947; Snelson et al., 1986). These spotted females could arise through sex chromosome crossover, which is also a rare event (Kallman, 1965). Alternatively, these exceptional females have atypical sex determination and are genetically male, as was likely the case for a rare spotted female *G. holbrooki* (Snelson et al., 1986; Angus, 1989).

Polygenic Control

The melanic side-spotting pattern is heritable, controlled primarily by genetics (Koßwig, 1929). At least one gene exists that controls the presence of macromelanophore pigmentation, and this locus has been dubbed the *Macromelanophore-determining locus* or *Mdl* (Vielkind, 1976; Vielkind et al., 1976, 1982; Anders and Anders, 1978; Weis and Schartl, 1998; Gutbrod and Schartl, 1999). In some species, degree of pigmentation expression may be the result of gene dosage, the number of pigmentation alleles inherited (Schröder, 1964; Kallman, 1971; Gutiérrez and García, 2007, 2011). In others, specific melanic side-spotting patterns can vary in amount and size of spots, in developmental timing of expression, and in location of spots on the fish's body (**Table 1**), indicating that spotting patterns have different genetic origins and/or that additional regulatory genes play a role in determining pattern expression (Gordon, 1931b; Kallman and Atz, 1966). Hybridizations across populations and species of *Xiphophorus* result in altered pattern expression, usually leading to heavier pigmentation and sometimes to melanomas (Koßwig, 1929; Gordon and Smith, 1938; Gordon, 1951; Atz, 1962; Anders et al., 1973). This altered pigmentation is the result of mismatches between the pattern genes of one population or species and the regulatory genes of another, indicating that these patterns are under polygenic control (Gordon and Smith, 1938; Gordon, 1951; Atz, 1962; Anders et al., 1973). The number of genes and the location of the loci involved may differ across poeciliid populations and species (Anders et al., 1973). One to a few genes control the pattern in some species (Schröder, 1964; Kallman, 1971; Horth, 2006), while others involve multiple genes across chromosomes interacting together (Kallman and Atz, 1966; Anders et al., 1973; Borowsky, 1973).

Candidate Genes for Melanic Spotting Patterns

Most genetic work on macromelanophore pigmentation patterns has been conducted in the *Xiphophorus* melanoma model.

Knowledge about the genetics underlying these patterns outside of *Xiphophorus* is practically non-existent with the notable exception of *G. holbrooki* (Kottler and Schartl, 2018; Kottler et al., 2020). At this time, no gene has been explicitly identified as the macromelanophore-determining gene in poeciliids (Kottler et al., 2020). Candidate genes for the development and regulation of melanomas are reviewed elsewhere (rev.: Schartl, 2008; Schartl and Walter, 2016), so we will focus on the genetic mechanisms that appear to be related to the presence and expression of melanic side-spotting patterns.

The presence of macromelanophores is controlled by the *Mdl* in *X. maculatus* and in *G. holbrooki* (Vielkind, 1976; Vielkind et al., 1976, 1982; Anders and Anders, 1978; Weis and Schartl, 1998; Gutbrod and Schartl, 1999; Kottler et al., 2020). This locus is hypothesized to consist of co-dominant alleles, including macromelanophore and other regulatory genes (Kallman and Atz, 1966; Anders et al., 1973; Vielkind and Vielkind, 1982; Weis and Schartl, 1998). The "macromelanophore gene(s)" that control the presence or absence of the melanic side-spotting pattern likely act on the melanophore progenitor cells, affecting whether they are assigned to the micro- or macromelanophore fate (Vielkind and Vielkind, 1982). In *G. holbrooki*, the presence of the spotted pattern was linked to an allele on the *Y* chromosome for *GIPC1* (*GIPC PDZ domain containing family member 1*), which functions in vesicle trafficking and sorting proteins to melanosomes for pigment synthesis (Liu et al., 2001; Kottler et al., 2020); however, its specific function in the melanic side-spotting pattern has not yet been determined. Because of its role in macromelanophore proliferation during melanoma formation, the *Xiphophorus* "macromelanophore gene" was thought to be X*mrk* (*Xiphophorus* receptor tyrosine kinase), a homolog of *epidermal growth factor receptor b* (Wittbrodt et al., 1989; Schartl, 2008). However, X*mrk* is found in species both with and without the spotting pattern, so it does not define the pattern's presence (Weis and Schartl, 1998; Schartl, 2008). At this time, X*mrk*'s known relation to spotting is as a marker of *Mdl*, although it may still have an undefined function related to pattern expression; X*mrk* has been proposed to control the migration of pigment progenitor cells from the neural crest and to inhibit certain cell lines from differentiating into macromelanophores (Adam et al., 1991; Weis and Schartl, 1998; Schartl, 2008).

Regulatory genes linked to the *Mdl* appear to control the type of melanic side-spotting pattern and the pattern's location on the fish's flank, as these features do not change with hybridization in *Xiphophorus* (Anders and Anders, 1978; Schartl, 1990). Non-linked regulatory genes appear to control the number of melanophore progenitor cells that differentiate into macromelanophores, the developmental timing of macromelanophore appearance, as well as the size and number of spots in *Xiphophorus* (Anders et al., 1973, 1984; Vielkind et al., 1976; Anders and Anders, 1978; Vielkind and Vielkind, 1982; Gutbrod and Schartl, 1999). The identities of these regulatory genes are still unknown, but some studies have identified candidates for the *Xiphophorus* autosomal locus *R(Diff)*, named for "*regulatory* or *differentiation*," that appears to control the terminal differentiation of melanophore progenitor cells into macromelanophores (Vielkind, 1976; Vielkind and Vielkind, 1982; Vielkind et al., 1982), and may have additional functions in pattern expression (Schartl, 2008). Although not yet investigated for the melanic side-spotting pattern, 22 candidates for melanoma regulation were identified as part of the *R(Diff)* region in *Xiphophorus* hybrids with the spotted-dorsal fin pattern (for a detailed description, see: Lu et al., 2017). Many of the identified genes have known functions that could affect pattern expression and other physiological or behavioral traits, including cell cycle regulation, cell differentiation, cell signaling, cell migration, cell structure and adhesion, and transcription and translation factors (Lu et al., 2017).

Few candidate genes have been identified that are linked to melanic side-spotting patterns, and their specific functions in patterning or in phenotypic correlations are still unknown. Since a variety of molecular systems can regulate both pigmentation and other traits (e.g., melanocortins, monoamines; see: section "Mechanisms of Pigmentation-Phenotype Correlation"), we suggest that parallel research studies be conducted to identify each gene system's contribution to the pattern's expression and to its correlation with other traits. Non-protein-coding mechanisms that affect gene expression and protein function should also be considered, including epigenetic regulation, gene promoters, non-coding RNAs, and post-translational modifications (Ducrest et al., 2008; Kottler and Schartl, 2018).

Temperature-Sensitivity Mechanism

Gene x environment interactions can affect presence and expression of the melanic side-spotting pattern (Vielkind and Vielkind, 1982). In a subset of *G. holbrooki* and *P. latipinna* populations, the pattern is temperature-sensitive with reduced penetrance at higher temperatures (Schröder, 1964; Angus, 1983, 1989; Angus et al., 1999; Horth, 2006). Temperature-sensitivity has not been identified in many populations or in other species, although expression of the spotted-dorsal fin pattern in *Xiphophorus* hybrids also exhibited incomplete penetrance at high water temperatures (Perlmutter and Potter, 1988). We propose a few evolutionary hypotheses for why temperature-sensitivity is limited to only a subset of populations within a species. First, the constitutively expressed and temperature-sensitive melanic side-spotting patterns may have evolved independently within a species through different genes (Angus, 1989). Second, these populations share the same melanic side-spotting pattern locus but have different alleles, only some of which confer temperature-sensitivity (Angus, 1989). For example, a hypothetical protein important for macromelanophore determination might function constitutively in one population, but a mutation in that protein could have evolved in another population that makes its splice variation or tertiary structure temperature-sensitive. Finally, temperature-sensitivity could be an additional regulatory gene or locus that has evolved independently in a subset of populations.

Temperature-sensitivity appears to be genetically controlled, as it was inherited paternally in *G. holbrooki* (Horth, 2006).

The molecular mechanism for temperature-sensitivity could be either in the *Mdl* or in regulatory loci. Molecular interactions, gene expression, and protein instability generally increase with temperature. *G. holbrooki* and *P. latipinna* show an unusual trend in the opposite direction, similar to mammals that express a Himalayan pattern in which they are normally white but body areas exposed to low temperatures express melanin pigmentation; increased melanin synthesis via increased tyrosinase activity at low temperatures is a key mechanism for melanism in mammals (Kidson and Fabian, 1981; Kwon et al., 1989). Angus (1989) found tyrosinase activity in temperature-sensitive *P. latipinna* was elevated at higher temperatures when expression of the spotting pattern was weak; therefore, tyrosinase activity does not seem to be the temperature-sensitivity mechanism. In spotted-dorsal fin *Xiphophorus* hybrids, higher temperatures led to multi-vesiculated macromelanophores, which Perlmutter and Potter (1988) suggest is due to inhibition of melanin synthesis within vesicles. Alternatively, temperature-sensitivity could result from failure to transport tyrosinase to the melanosomes in these pigment cells (hence the empty vesicles), as retention of tyrosinase by the endoplasmic reticulum was higher at elevated temperatures in *himalayan* mouse cells (Halaban et al., 2000). Mutations that lead to increased stability of tertiary protein structure at low temperatures are another potential mechanism. Finally, we should consider that temperature-sensitivity may not be due to polymorphisms in protein-coding genes; for example, temperature-sensitive epigenetic regulation of pigmentation gene expression is possible. A shared temperature-sensitive molecular system could lead to the correlated expression of the melanic side-spotting pattern and other traits, as seen in a temperature-sensitive population of *G. holbrooki*. Males of the same genotype were exposed to different temperatures, so they were either unspotted or spotted, and these expressed patterns correlated with behavioral differences (Horth, 2003).

EVOLUTIONARY ORIGINS

Although melanic side-spotting patterns appear across Poeciliidae, the differences in inheritance and pattern features within and across species suggests that many of these patterns have different genetic mechanisms (Gordon, 1931b; Kallman and Atz, 1966). It is not clear at this time whether melanic side-spotting patterns arose through homologous, convergent, and/or parallel evolution within Poeciliidae, within specific genera, or even within species.

Here, we consider the possible evolutionary origins of melanic side-spotting patterns using the genus *Xiphophorus* as an example. First, pigmentation loci may have evolved independently in some species while others share a common ancestor. For example, the sex chromosomes of *X. variatus* and *X. maculatus* are homologous so they may share an ancestral spotted pattern, but the *X. helleri* autosome is not homologous with those two, and its spotted pattern may have evolved independently (Kallman and Atz, 1966; Schartl, 1990). Alternatively, the pattern genes were originally all on the same homologous chromosome and then translocated to another chromosome in a subset of species (Kottler and Schartl, 2018). Second, *Xiphophorus* may have a shared ancestral spotted pattern that was repeatedly lost in some species, which would explain why *Mdl* homologs exist in species without macromelanophore patterns (Weis and Schartl, 1998). Regulatory genes could have then evolved independently in different populations and species through parallel evolution, which would alter the development, expression, and location of the patterns (Anders et al., 1973; Vielkind and Vielkind, 1982; Schartl, 1990). *Mdl* on *Xiphophorus* sex chromosomes is linked to regions that may be hotspots for gene duplication, structural rearrangements, and recombination (Volff and Schartl, 2001), suggesting that a high incidence of parallel evolution is possible for some *Xiphophorus* species. Thus, a similar combination of homologous, convergent, and parallel evolution is hypothesized to explain the origin of multiple melanic side-spotting patterns in Poeciliidae.

PHYSIOLOGY

Androgens

Many endocrine and neuroendocrine substances are involved in the regulation of pigmentation, so physiological correlates are expected within morphs. Because the expression of the melanic side-spotting pattern is male-biased in some species (Angus, 1989; Horth, 2006), androgens have been hypothesized to affect the pattern's presence. Mature unspotted *G. holbrooki* females treated with methyl-testosterone developed male gonopodia but did not develop the spotted pattern (Angus, 1989; Horth, 2006). Although these results suggest that the pattern is sex-linked and not sex-limited in *G. holbrooki*, some caution should be applied. Methyl-testosterone is not a potent androgen in teleosts; if the pattern requires higher androgenic activity to appear in females, then 11-keto-testosterone is more likely to produce observable effects (Hishida and Kawamoto, 1970). Furthermore, secondary sexual characteristics have late life plasticity, but that does not necessarily mean that expression of the pattern also has late life plasticity. Expression of androgen receptors is often primed early in life, so testosterone may only induce pattern expression in females when applied before sexual maturity.

In species where both sexes express the melanic side-spotting pattern, males often have higher expression and heavier pigmentation (Gordon, 1927, 1951; Häussler, 1928; Bellamy, 1936; Mac Intyre, 1961; Borowsky, 1973; Angus et al., 1999; McDowall, 1999), so androgen levels are hypothesized to affect pattern variation among individuals and sexes, but circulating levels have not yet been measured in relation to initiation of spotting patterns. Individuals expressing melanin-based coloration often have higher circulating levels of sex steroids (rev.: San-Jose and Roulin, 2018), but whether differences between spotted and unspotted morphs in poeciliids exist are still unknown. Hormones often have pleiotropic effects (Ketterson and Nolan, 1999), and high levels of sex steroids are associated with higher social dominance (rev.: Oliveira et al., 2002); thus, if higher pigmentation expression in males is due to higher levels of sex steroids, then it may represent a pleiotropic mechanism for the correlation of the melanic side-spotting pattern and behavior in poeciliids. Alternatively, sex steroid levels are correlated to behavior as opposed to the melanic side-spotting pattern, as suggested by the "challenge hypothesis" (Wingfield, 1984; Oliveira et al., 2002). Briefly, spotted and unspotted morphs experience different social environments; if spotted morphs experience more aggressive social interactions, they might respond with higher circulating androgen levels.

Metabolism and Cortisol

Melanocortins were previously identified as a key candidate for pleiotropy between melanin-based color patterns and physiological traits. Melanocortins regulate melanin production, energy balance, and glucocorticoid release, among other traits (revs.: Ducrest et al., 2008; San-Jose and Roulin, 2018). Although spotted *G. holbrooki* males had higher basal cortisol levels than unspotted males, no significant difference in cortisol responses to an introduced stress (presence of a predator) was observed between morphs (Humphrey, 2019). This negative result may be a function of low sample size. To our knowledge, no study has measured other circulating hormones, such as melanocortins like α-melanocyte stimulating hormone. Metabolic rate also does not appear to correlate with the melanic side-spotting pattern in poeciliids, as spotted and unspotted morphs of *X. variatus* and *X. helleri* did not differ in metabolic rate (Meyer et al., 2006; Culumber, 2016). Spotted morphs might have a physiological compensation mechanism that counteracts changes in metabolic rate associated with pigmentation expression, or metabolic rate may not be a major outcome of melanocortin systems.

Melanoma

Macromelanophore-based patterns in Poeciliidae have been heavily studied as a model for skin cancer, particularly in hybrid *Xiphophorus* species; hybridization across populations or species creates a mismatch between the pigmentation pattern genes and their regulatory genes, which results in the unregulated differentiation of macromelanophores and the formation of tumors (revs.: Anders et al., 1973, 1984; Anders and Anders, 1978; Vielkind and Vielkind, 1982; Schartl and Walter, 2016). However, melanomas have been observed in senescent individuals from natural populations of *X. variatus* and *X. nezahualcoyotl*, as well as a non-hybrid lab strain of *X. variatus* (Borowsky, 1973; Schartl et al., 1995; Fernandez and Bowser, 2008), so we will discuss the possible influence of melanomas on the evolution of melanic side-spotting patterns.

Melanoma is a potential mechanism for reducing the frequency of spotted individuals in a population (Gordon and Gordon, 1957). When malignant melanomas occur in hybrid *Xiphophorus*, they lower fish health and reduce survival, decreasing the fitness of spotted individuals; however, only a small proportion of these hybrid fish develop malignant melanomas, and melanomas are extremely rare in natural populations, so this disadvantage is not likely to be a general feature of this pattern (Häussler, 1928; Anders et al., 1973; Anders and Anders, 1978; Schartl, 2008); however, the rarity of melanomas in natural populations may also be a sampling artefact, as these fish are unlikely to survive and thus unlikely to be sampled. In *Xiphophorus*, only species with the constitutively active oncogene X*mrk* develop melanomas, so not all species with macromelanophore patterns are expected to experience the melanoma fitness disadvantage (Anders and Anders, 1978; Weis and Schartl, 1998). Although, other undiscovered mutations that lead to melanoma formation in macromelanophores could potentially still exist (Fernandez and Bowser, 2008). Finally, macromelanophore patterns can potentially evolve repeatedly through mutation; if individuals with these mutations also repeatedly develop melanomas, natural selection would act to eventually include regulatory genes that prevent this deleterious side-effect, which have been found in multiple populations and species of *Xiphophorus* (Gordon and Gordon, 1957; Atz, 1962; Borowsky, 1973). Thus, the fitness disadvantage associated with melanomas is likely not a strong selective pressure on the melanic side-spotting pattern (Anders et al., 1973; Schartl, 2008). Melanomas also often develop later in life in both non-hybrid and hybrid *Xiphophorus*, weakening selection against it; selection delayed is selection denied (Gordon and Gordon, 1957; Williams, 1957; Mac Intyre, 1961; Anders et al., 1973; Schartl et al., 1995). Selective pressures that act upon the melanic side-spotting pattern before melanoma development would thus have stronger effects on the pattern's frequency in populations.

The risk of developing malignant melanomas was higher for homozygotes of *X. maculatus* with the "fuliginosus" pattern and of *X. variatus* with the "punctatus-2" pattern, indicating that the melanoma fitness disadvantage may be stronger in homozygotes than heterozygotes (Mac Intyre, 1961; Borowsky, 1973). If the spotted pattern has other fitness advantages, these positive selective pressures on heterozygotes may outweigh the negative effect of melanomas on homozygotes (Borowsky, 1973; Kazianis and Borowsky, 1995), and thus the pattern would be maintained in the population as a polymorphism. Higher fitness of heterozygotes for the melanic side-spotting pattern could potentially explain the evolution of sex-linkage in some poeciliid species, as only one of the sex chromosomes carries the pigmentation allele, thereby preventing melanoma (Borowsky, 1973). Alternatively, because of their correlation with heavier expression of pigmentation patterns, melanomas may be under positive selection if expression is correlated with a fitness advantage. For example, *X. cortezi* females in some populations preferred males with heavier expression of a macromelanophore-based tail spot pattern (spotted caudal) that is associated with an increased risk of melanoma (Fernandez and Morris, 2008). Melanomas enhance the preferred pattern's appearance, which could lead to its maintenance through sexual selection in some populations (Fernandez and Morris, 2008). The possible fitness advantages of melanomas have not yet been studied in relation to melanic side-spotting patterns. Other aspects of fish health have not been investigated for the melanic side-spotting pattern. Melanin has known anti-parasitic properties in poeciliids, so spotted morphs could potentially have greater resistance to parasites (Meyer et al., 2006; Horth et al., 2013). Differences in the immune system exist in other species with melanin-based pigmentation patterns (revs.: McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018), and thus should also be investigated for spotted and unspotted poeciliid morphs.

TABLE 2 | Summary of traits and selective pressures investigated for melanic side-spotting pattern in poeciliid species. We briefly describe the experiment and results. Direction of effect is listed as increased (+), decreased (−), and no difference (/) for the spotted morph compared to the unspotted morph.

Trait	Test	Result or Effect	Species	References
Molecular				
Macromelanophore-determining locus	Candidate genes linked to spotting pattern *Mdl*	*GIPC1*	*Gambusia holbrooki*	Kottler et al., 2020
Macromelanophores	Microscopy	Larger size, thicker and more dendritic arms, more melanosomes	*Gambusia holbrooki, Poecilia sphenops, Xiphophorus* hybrids, *Xiphophorus maculatus*	Gordon, 1931a; Regan, 1961; Vielkind, 1976; Anders et al., 1984; Kottler et al., 2020
	Skin regeneration and grafts	Inhibit differentiation and induce destruction of micromelanophores	*Xiphophorus* hybrids	Schmidt, 1978
	Adrenaline application to stimulate melanosome aggregation	No aggregation	*Gambusia holbrooki*	Kottler et al., 2020
Temperature-sensitivity mechanism	Tyrosinase activity	Activity increased with increasing temperatures; not the mechanism	*Poecilia latipinna*	Angus et al., 1999
Development				
	Crosses to determine genetic inheritance of spotting pattern Observations in the wild and in the lab	Born unspotted; pattern fully expressed after sexual maturity	*Gambusia holbrooki, Phalloceros caudimaculatus, Poecilia latipinna, Poecilia sphenops, Xiphophorus* hybrids, *Xiphophorus helleri*	Bellamy, 1924, 1928; Myers, 1925; Gordon, 1931b; Schröder, 1964; Angus, 1983; Martin, 1984; Gutiérrez and García, 2007
		Male expression > female expression	*Phalloceros caudimaculatus, Poecilia latipinna, Xiphophorus* hybrids, *Xiphophorus cortezi, Xiphophorus maculatus, Xiphophorus variatus*	Gordon, 1927, 1951; Häussler, 1928; Bellamy, 1936; Mac Intyre, 1961; Atz, 1962; Borowsky, 1973; Angus, 1983; McDowall, 1999
		Expression increases with age	*Gambusia holbrooki, Poecilia latipinna, Xiphophorus cortezi, Xiphophorus helleri, Xiphophorus variatus*	Myers, 1925; Atz, 1962; Kallman and Atz, 1966; Kallman, 1971; Angus, 1983; Angus et al., 1999; Meyer et al., 2006
Physiology				
Androgens	Methyl-testosterone on expression of pattern in adult females	/ (no difference)	*Gambusia holbrooki*	Angus, 1989; Horth, 2006
Glucocorticoids	Basal cortisol levels	+	*Gambusia holbrooki*	Humphrey, 2019
	Cortisol response to predator	/		Humphrey, 2019
Routine metabolic rate	Oxygen consumption rate in a respirometer	/	*Xiphophorus helleri*	Meyer et al., 2006
		/	*Xiphophorus variatus*	Culumber, 2016
Stress resistance	Activity during confinement test	+	*Xiphophorus variatus*	Culumber, 2016
Melanoma	Development in homozygotes compared to heterozygotes	+	*Xiphophorus maculatus*	Koßwig, 1938; Mac Intyre, 1961
		+	*Xiphophorus variatus*	Borowsky, 1973
Behavior				
Feeding	Juveniles raised in presence of phenotypically similar or different adults	/	*Gambusia holbrooki*	Culumber et al., 2018
Antipredator	Response to predator odorants	/	*Gambusia holbrooki*	Kraft, 2016
	Freezing behavior	/		Humphrey, 2019

(Continued)

TABLE 2 | Continued

Trait	Test	Result or Effect	Species	References
Dominance	Altered frequency of spotted morphs and density	+	*Gambusia holbrooki*	Martin, 1977
	Juveniles raised in presence of phenotypically similar or different adults	+		Kraft et al., 2018
	Paired male conflicts	/	*Xiphophorus helleri*	Franck et al., 2001
Aggression	Temperature-sensitive siblings in social group (same genotype, different phenotype)	/	*Gambusia holbrooki*	Horth, 2003
	Juveniles raised in presence of phenotypically similar or different adults	/		Kraft et al., 2018
Behavioral flexibility	Juveniles raised in presence of phenotypically similar or different adults	−	*Gambusia holbrooki*	Kraft et al., 2018
Boldness	Novel environment	−	*Xiphophorus variatus*	Culumber, 2016
Sexual activity	Altered frequency of spotted morphs and density	+ at low spotted frequency and high density − at equal frequencies	*Gambusia holbrooki*	Martin, 1977
	Female paired with both morphs with and without barriers	≠		Nelson and Planes, 1993
	Observed sexual behavior in the wild	/ or + depending on behavior		Karplus and Algom, 1996
	Temperature-sensitive siblings in social group (same genotype, different phenotype)	+		Horth, 2003
	Juveniles raised in presence of phenotypically similar or different adults	−		Kraft et al., 2018
	Social groups before and after predator exposure	+		Humphrey, 2019
	Morphs with different social experience (low or high frequency of spotted morph) grouped with females	+		Culumber et al., 2020
Female preference	Altered frequency of spotted morphs and density	−	*Gambusia holbrooki*	Martin, 1977
	Female had visual cues of both morphs	/		Martin, 1986
	Female paired with both morphs	/ with barriers − without barriers		Nelson and Planes, 1993
	Observed sexual behavior in the wild	+		Karplus and Algom, 1996
	Female had visual cues of both morphs	−		Taylor et al., 1996
	Two-model choice tests	+		Gould et al., 1999
	Females from different populations (spotted absent vs. spotted present) in 3-chamber choice test	− unspotted females + spotted females		Bisazza and Pilastro, 2000
	Females in 3-chamber choice test	−		Bisazza et al., 2001
	Temperature-sensitive siblings in social group (same genotype, different phenotype)	/		Horth, 2003
	Dichotomous choice test	− unspotted females from non-sulfidic spring / unspotted females from sulfidic spring + spotted females from sulfidic spring	*Poecilia mexicana*	Culumber et al., 2014
	School and dichotomous choice tests in clear conditions	− for schools and / for males by unspotted females + by spotted females	*Xiphophorus helleri*	Franck et al., 2001
Social group	Observations of social interactions in lab and wild	More social partners and higher association with females over males	*Gambusia holbrooki*	Kraft et al., 2016
	School choice test	Preferred phenotypically similar school	*Poecilia* hybrids	McRobert and Bradner, 1998; Bradner and McRobert, 2001b

(Continued)

TABLE 2 | Continued

Trait	Test	Result or Effect	Species	References
	School choice test based on experience	Preferred to school with familiar color pattern		Ledesma and McRobert, 2008
Life History Traits				
Gestation	Length of gestation for different female and male morphs	/	*Poecilia sphenops*	Schröder, 1964
Brood sex ratio	Crosses to determine genetic inheritance of spotting pattern	Usually equal sex ratios, although some male-biased	*Gambusia holbrooki*	Horth, 2006
Survival	Mesocosms with varying frequency	+ at low spotted frequency − at high spotted frequency	*Gambusia holbrooki*	Horth and Travis, 2002
	Field survival	/		Horth, 2004
	Crosses to determine genetic inheritance of spotting pattern	−	*Xiphophorus maculatus*	Mac Intyre, 1961
		−	*Xiphophorus* hybrids	Koßwig, 1927
Body size	Measured in nature and in the lab	+	*Gambusia holbrooki*	Martin, 1977; Horth et al., 2010
	Temperature-sensitive siblings (same genotype, different phenotype)	/		Horth, 2003
	Measured for same-age morphs in the lab	/	*Xiphoporus helleri*	Meyer et al., 2006
Body condition	Juveniles raised in presence of phenotypically similar or different adults	+	*Gambusia holbrooki*	Culumber et al., 2018
Environment				
Predation	Consumption by predator	−	*Gambusia holbrooki*	Martin, 1977; Bonner, 1980 in: Martin, 1986
		+ at low spotted frequency − at high spotted frequency		Horth, 2004
		/		Humphrey, 2019
		−	*Xiphophorus helleri*	Dürr, 1996 and Becker, 1997 in: Franck et al., 2001
	Hunting activities by predator	/	*Gambusia holbrooki*	Humphrey, 2019
		−	*Xiphophorus helleri*	Dürr, 1996 and Becker, 1997 in: Franck et al., 2001
Sulfidic springs	Dichotomous choice test (females from sulfidic spring)	/ unspotted females + spotted females	*Poecilia mexicana*	Culumber et al., 2014
Turbidity	School choice experiments in turbid conditions compared to clear conditions	+	*Xiphophorus helleri*	Franck et al., 2001
Temperature	Simulated population model	Seasonal changes around an evolutionarily stable mean	*Gambusia holbrooki*	Horth and Panayotova, 2012
	Simulated population model in response to climate change	−		Panayotova and Horth, 2018
	Penetrance of pattern expression	Higher in winter temperatures	*Gambusia holbrooki*	Angus, 1989; Horth, 2006
			Poecilia latipinna	Schröder, 1964; Angus, 1983; Angus et al., 1999

CONCLUSION

Melanin-based pigmentation patterns correlate with other traits across vertebrates through a variety of potential molecular and evolutionary mechanisms (revs.: Gray and McKinnon, 2007; Ducrest et al., 2008; Forsman et al., 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). Poeciliids have proven a tractable model for studying pigmentation-phenotype correlations, so we have presented here the melanic side-spotting pattern as a model for investigating the mechanisms underlying the evolution of similar color patterns and their correlations with other traits across populations, species, and genera. Specific results are summarized in **Table 2**. Although the development and inheritance of melanic side-spotting patterns are well known, we still do not understand the genetics of the pattern or all aspects of the pigment cells' biology. Most of the research on correlated traits focuses on a subset of populations from *Xiphophorus* species and Eastern Mosquitofish (*G. holbrooki*), so we recommend extending research into more poeciliid populations, species, and genera to determine whether the results reviewed here are generalizable trends. Little is known about physiological correlates and life history traits of spotted morphs. Spotted males have altered social conflict behavior and social groups compared to unspotted males. The melanic side-spotting pattern is generally uncommon in populations despite being an evolutionarily stable phenotype, so multiple positive and negative selective pressures are presumed to act upon it and its correlated traits. Predation appears to be an important negative frequency-dependent selective pressure, but social interactions may also function in the frequency-dependent selection that maintains the spotted morph at a low population frequency. Physical environmental factors are likely also important, but only a couple studies on water conditions exist. It is unclear whether sexual selection acts upon the melanic side-spotting pattern, and it may depend on a variety of factors such as population and species, morph frequency, and social and physical environment. Finally, whether melanic side-spotting patterns arose through homologous, convergent, and/or parallel evolution within and across Poeciliid species remains to be determined.

FUTURE STUDIES

Throughout this review, we identified what is still unknown within specific biological fields for the melanic side-spotting pattern in poeciliids. Here, we outline key research questions that may serve as a framework for future studies:

1. Which molecular mechanisms (sequential causation, co-expression, linkage disequilibrium, and pleiotropy) and/or evolutionary mechanisms (correlational selection, co-adaptive selection, co-dependence, and co-specialization) underlie the correlation between melanic side-spotting patterns and other phenotypic traits?
2. Which molecular signaling systems (e.g., peptide hormones, amino acid hormones, monoamines, sex steroids, and neurotransmitters) affect the expression of the melanic side-spotting pattern, or are differentially expressed between morphs?
3. What gene(s) determine the presence or expression of the melanic side-spotting pattern to create the variation seen among populations and species? Do identified gene(s) pleiotropically affect or link to other molecular signaling systems that affect other phenotypic traits, such as physiology, life history, or behavior?
4. Does manipulation of molecular signaling systems that affect life history, physiology, or behavior also affect the presence or expression of the melanic side-spotting pattern, or vice versa?
5. Does the melanic side-spotting pattern or a correlated trait have an effect that is more disadvantageous in females than males that could lead to sexually antagonistic selection (and thus higher expression of the pattern in males)?
6. Are the correlated physiological, behavioral, and life history traits identified in this review a general trend across poeciliid populations, species, and genera? How do these correlated traits affect fitness of the melanic side-spotting pattern, especially survival and reproduction?
7. What social and physical environmental factors drive or break correlations in physiological, behavioral, or life history traits with melanic side-spotting patterns?
8. How do social and physical environmental factors interact with the pattern and its correlated traits to affect morph fitness? How do these interactions affect the maintenance and frequency of the melanic side-spotting pattern in populations and species?
9. What is the evolutionary origin of the polymorphic melanic side-spotting pattern? Did these similar patterns arise through homologous, convergent, and/or parallel evolution within and across species?

AUTHOR CONTRIBUTIONS

TZ conducted the review and synthesis of the literature and created all figures and tables. Both authors wrote and contributed to the manuscript and approved the submitted version.

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A Novel Body Plan Alters Diversification of Body Shape and Genitalia in Live-Bearing Fish

R. Brian Langerhans[1]* *and Eduardo Rosa-Molinar*[2]

[1] Department of Biological Sciences and W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, United States, [2] Bi-Campus Neuroscience Graduate Program, Department of Pharmacology and Toxicology, University of Kansas, Lawrence, KS, United States

Major evolutionary innovations can greatly influence subsequent evolution. While many major transitions occurred in the deep past, male live-bearing fishes (family Poeciliidae) more recently evolved a novel body plan. This group possesses a three-region axial skeleton, with one region—the ano-urogenital region—representing a unique body region accommodating male genitalic structures (gonopodial complex). Here we evaluate several hypotheses for the evolution of diversity in this region and examine its role in the evolution of male body shape. Examining *Gambusia* fishes, we tested *a priori* predictions for (1) joint influence of gonopodial-complex traits on mating performance, (2) correlated evolution of gonopodial-complex traits at macro- and microevolutionary scales, and (3) predator-driven evolution of gonopodial-complex traits in a post-Pleistocene radiation of Bahamas mosquitofish. We found the length of the sperm-transfer organ (gonopodium) and its placement along the body (gonopodial anterior transposition) jointly influenced mating success, with correlational selection favoring particular trait combinations. Despite these two traits functionally interacting during mating, we found no evidence for their correlated evolution at macro- or microevolutionary scales. In contrast, we did uncover correlated evolution of modified vertebral hemal spines (part of the novel body region) and gonopodial anterior transposition at both evolutionary scales, matching predictions of developmental connections between these components. Developmental linkages in the ano-urogenital region apparently play key roles in evolutionary trajectories, but multiple selective agents likely act on gonopodium length and cause less predictable evolution. Within Bahamas mosquitofish, evolution of hemal-spine morphology, and gonopodial anterior transposition across predation regimes was quite predictable, with populations evolving under high predation risk showing more modified hemal spines with greater modifications and a more anteriorly positioned gonopodium. These changes in the ano-urogenital vertebral region have facilitated adaptive divergence in swimming abilities and body shape between predation regimes. Gonopodium surface area, but not length, evolved as predicted in Bahamas mosquitofish, consistent with a previously suggested tradeoff between natural and sexual selection on gonopodium size. These

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Bart Pollux,
Wageningen University and Research, Netherlands

Reviewed by:
Clint Kelly,
Université du Québec à Montréal, Canada
Alessandro Devigili,
Stockholm University, Sweden

***Correspondence:**
R. Brian Langerhans
langerhans@ncsu.edu

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results provide insight into how restructured body plans offer novel evolutionary solutions. Here, a novel body region—originally evolved to aid sperm transfer—was apparently co-opted to alter whole-organism performance, facilitating phenotypic diversification.

Keywords: adaptive radiation, body plan, exaptation, morphological evolution, genital evolution, novel traits, Poeciliidae, predation

INTRODUCTION

Evolutionary change is contingent on past evolutionary history (Gould and Vrba, 1982; Price et al., 2000; Gould, 2002; Losos, 2009; Barve and Wagner, 2013; Blount et al., 2018). Novel traits or body plans can lead to new evolutionary routes with far-reaching consequences. For instance, feathers initially evolved for reasons other than flight (Sumida and Brochu, 2000; Xu et al., 2009; Foth et al., 2014; Foth and Rauhut, 2020), while general body plans like bilateral symmetry strongly biased future trajectories of evolutionary diversification (Raff, 2000; Newman, 2016; Isaeva, 2018).

Major transitions in body plan clearly set the stage for much subsequent morphological evolution. But since the Cambrian explosion, very few novel body plans have appeared (Gould, 1989; Levinton, 1992; Erwin et al., 1997; Davidson and Erwin, 2006; Maxwell and Wilson, 2013). For fish, primary literature and textbooks in ichthyology and comparative vertebrate anatomy describe the teleost axial skeleton body plan and vertebral formulae as a two-part system: anterior trunk and posterior caudal vertebral regions [see Rivera-Rivera et al. (2010) and references therein]. However, recent work revealed male live-bearing fishes in the family Poeciliidae have altered this ancestral body plan with a novel three-part body plan composed of the two aforementioned regions plus a third region known as the ano-urogenital vertebral region (Rosa-Molinar et al., 1994, 1996; Rivera-Rivera et al., 2010). This body plan appears to have evolved at least 44 million years ago (Hrbek et al., 2007). Because of the relatively little research conducted on this novel body plan to date, and the emphasis on Poeciliidae so far, its origination and extent is not fully understood—this body plan could be more widespread throughout much of Atherinomorpha.

While many organisms have a general body region encompassing the anus, urinary tract, and reproductive organs, the "ano-urogenital vertebral region" is unique to male poeciliid fishes. This region reflects a marked remodeling of the axial and appendicular skeleton to accommodate development of male genitalia and associated structures. The novel region includes a highly modified anal fin used for sperm transfer (the gonopodium) along with its musculoskeletal components. This unique region comprises vertebrae with ribs and hemal spines (Rosa-Molinar et al., 1994, 1998) and is thought to reflect an adaptation for rapid and effective sperm transfer and internal fertilization in the group (Rivera-Rivera et al., 2010; Serrano-Velez et al., 2014). The ano-urogenital region is situated between the anterior trunk and posterior caudal regions, from vertebrae 11 to 16. Development of this region most notably involves the elongation, thickening, and anterior bending of hemal spines (typically of vertebrae 14–16; sometimes termed "gonapophyses") as well as the anterior transposition of the gonopodium and the gonopodial appendicular support (**Figure 1A**). As a consequence, poeciliid fishes exhibit striking sexual dimorphism in adults, with the male anal fin (i.e., gonopodium) positioned more anteriorly than the female anal fin (**Supplementary Figure 1**).

Because the evolution of novel body plans can have important consequences for further morphological and behavioral evolution, it is perhaps not surprising that poeciliid fishes exhibit great variability in the various features of the gonopodial complex, such as the number of modified hemal spines (typically between 1 and 3), their size and extent of modification (e.g., thickening, bending, uncinate processes), the degree of gonopodial anterior transposition (i.e., relative placement of the gonopodium along the body), and the size of the gonopodium (Rosen and Gordon, 1953; Rosen and Bailey, 1963; Chambers, 1987; Ghedotti, 2000; Langerhans, 2011). Yet, to date, explanations for the origins of such diversity and the potential consequences for the evolution of other body regions are lacking (Rosa-Molinar, 2005; Martinez-Rivera et al., 2010; Langerhans, 2011). When novel innovations evolve, can we predict their subsequent evolution based on our functional understanding of the structures, and how might novel traits influence adaptive diversification after their initial emergence?

Here we address several outstanding questions regarding the evolution of this understudied, unique region and its role in facilitating adaptive diversification in poeciliid fishes. We directly tackled three broad questions: (Q1) How are various components of the gonopodial complex developmentally or functionally integrated, and how might they evolve in a correlated manner? (Q2) What forms of selection shape diversity of this region? and (Q3) Has the novel ano-urogenital region been co-opted for adaptive diversification in lateral body shape? To address Q1 (blue region in **Figure 1**), we used a laboratory mating experiment to test whether correlational selection favors particular combinations of two gonopodial-complex traits (**Figure 1B**, see below) and used comparative analyses to test predictions of correlated evolution of particular traits in the ano-urogenital region due to developmental linkages and selection on copulatory performance (**Figure 1C**, see below). We addressed Q2 (yellow region in **Figure 1**) by testing among predatory environments specific predictions of how divergent selection on body shape and gonopodium size should drive evolutionary divergence of gonopodial-complex traits (**Figure 1D**, see below). Finally, we addressed Q3 (green region in **Figure 1**) by directly testing the co-optation prediction of correlated evolution between gonopodial anterior transposition and lateral body shape during predator-driven diversification in an adaptive radiation (**Figure 1E**, see below).

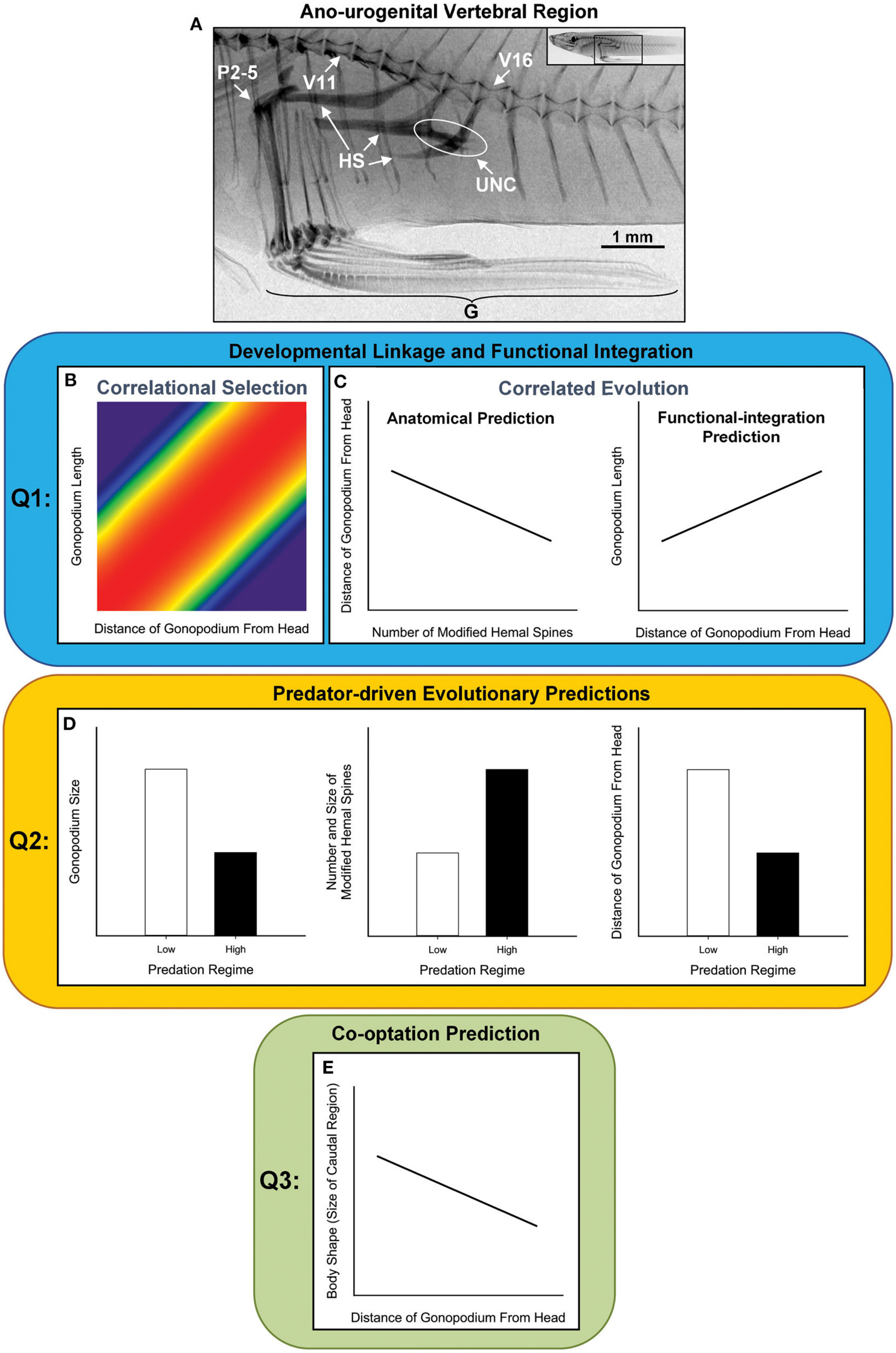

FIGURE 1 | Illustration of **(A)** the gonopodial complex in live-bearing fishes (*Gambusia affinis* depicted), and hypothetical depictions of our **(B)** hypothesis of correlational selection on two gonopodial-complex traits, **(C)** predictions of correlated evolution among traits, **(D)** predictions of trait divergence between predation

(Continued)

FIGURE 1 | regimes, and **(E)** prediction of co-optation of the ano-urogenital region for the evolution of different body forms (see text for details). Labels in **(A)** indicate key components of the ano-urogenital region: vertebrae 11 and 16 (V11, V16; demarcating the ano-urogenital vertebral region), the gonopodium (G), and its appendicular skeletal support [hemal spines (HS), the uncinate process on the 15th hemal spine (UNC), the columnar fusion of pterygiophores 2-5 (P2-5)]. Anterior/rostral is toward the left in **(A)**. Fitness in **(B)** ranges from low (cool colors) to high (warm colors). The three colored panels reflect the three core questions addressed in the study (Q1–Q3, see text).

MATERIALS AND METHODS

Overview of Hypotheses and Predictions

All hypotheses evaluated in this study derive from previous research, with testable evolutionary predictions being derived from the hypotheses. We first tested a copulatory-performance hypothesis of functional integration between gonopodial anterior transposition and gonopodium length. We specifically hypothesized that when the gonopodium is located closer to the head (greater gonopodial anterior transposition), high performance during copulation should be achieved by a shorter gonopodium, and vice versa. This hypothesis describes positive correlational selection on the two traits, with a ridge-shaped selection surface where trait combinations along the top of the ridge have similarly high mating performance values (**Figure 1B**). Copulation in most live-bearing fishes is very rapid, involving complex locomotor maneuvers, and selection favoring efficient sperm transfer may be strong in many species (Rosa-Molinar, 2005; Martinez-Rivera et al., 2010; Rivera-Rivera et al., 2010; Langerhans, 2011; Devigili et al., 2015; Head et al., 2015). As revealed in high-speed video (1000 Hz) of western mosquitofish (*Gambusia affinis*), the male circumducts the gonopodium, with the distal tip pointing anteriorly, and performs a rapid, complex torque-thrust behavior in an attempt to inseminate the female during copulation (Rosa-Molinar, 2005; Martinez-Rivera et al., 2010; Rivera-Rivera et al., 2010). When the gonopodium is positioned more posteriorly along the body (less transposition), a male may exhibit reduced maneuverability and acceleration [owing to a reduced caudal region (see below) and more posterior center of mass] and require a longer gonopodium to enhance insemination or fertilization success during copulation. Additionally, if males use visual cues for gonopodial placement during copulation (Langerhans, 2011), then an optimal position of the gonopodial tip relative to the eye would reinforce this functional integration. Thus, males' mating success should depend on the combination of the two traits, and we tested this prediction of correlational selection using a mating experiment.

We then examined two predictions regarding correlated evolution of genital traits among species (**Figure 1C**). First, we have an anatomical hypothesis that the magnitude of gonopodial anterior transposition is largely governed post-developmentally by variation in the extensive sexually dimorphic ligament system associated with the modified hemal spines (Rosa-Molinar et al., 1994). This anatomical hypothesis leads to the evolutionary prediction that species having a greater number of modified hemal spines will additionally exhibit greater gonopodial anterior transposition (gonopodium closer to the head, resulting in a smaller abdominal region). That is, an increased number of modified hemal spines should essentially push the gonopodium anteriorly to a greater extent through direct movement of the columnar fusion of pterygiophores 2–5 (see **Figure 1A**). Second, if our copulatory-performance hypothesis described above is accurate and captures the major aspects of selection on the traits (i.e., other aspects of natural and sexual selection are comparatively of little importance), this leads to an evolutionary prediction: correlational selection on gonopodial anterior transposition and gonopodium length will cause the two traits to exhibit correlated evolution among species/populations. Using phylogenetic comparative analysis of 10 closely related species in the genus *Gambusia* (mosquitofishes) known to exhibit variation in these genital traits, we tested the predictions that (1) a greater number of modified hemal spines is associated with greater gonopodial anterior transposition and that (2) a more posteriorly positioned gonopodium is associated with a longer gonopodium length (**Figure 1C**).

Next, we tested three general predictions regarding evolutionary divergence of genital traits between predatory environments (**Figure 1D**). Previous work has demonstrated the importance of two particular factors in generating diversity of gonopodium size and general body form in live-bearing fishes: (1) sexual selection via female mating preferences and (2) natural selection via predation from piscivorous fish [both reviewed in Langerhans (2010), Langerhans (2011)]. Specifically, females of some poeciliid species exhibit mating preferences for males with particular body shapes and larger gonopodia (Langerhans et al., 2005, 2007; Kahn et al., 2010; Langerhans and Makowicz, 2013), while natural selection in the presence of fish predators favors larger caudal regions and smaller gonopodia due to their positive effects on locomotor escape behaviors (Langerhans et al., 2005; Langerhans, 2009a, 2010, 2011). This has led to divergence in these traits between environments with and without piscivorous fish. For example, male poeciliids of a number of species exhibit smaller gonopodia and larger caudal regions in localities with higher intensities of fish predation [reviewed in Langerhans (2010), Langerhans (2011), Langerhans (2018)]. To date, no study has investigated the role the gonopodial complex might play in these observed patterns of morphological divergence.

Divergent selection between environments with and without predatory fish arising from this interplay of natural and sexual selection leads to three general predictions of phenotypic divergence in the gonopodial complex: males in high-predation environments should exhibit (1) smaller gonopodia, (2) more modified hemal spines that are larger and more strongly modified, and (3) a greater magnitude of gonopodial anterior transposition compared to males in low-predation localities (**Figure 1D**). The first prediction derives from previous work demonstrating both female preference for larger gonopodia and detrimental effects of gonopodium size on escape performance and survivorship with a predatory fish. The latter two predictions derive from the functional and developmental hypotheses that increasing gonopodial anterior transposition should facilitate both shorter gonopodia and larger caudal regions. If selection on general body shape has driven evolution in the ano-urogenital region, we should see correlated divergence of gonopodial anterior transposition and lateral body shape across populations (**Figure 1E**). Here we test these predictions using the model system of the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting inland blue holes.

Experimental Test of Functional Integration of Gonopodial-Complex Traits

Using laboratory-born Bahamas mosquitofish from a single population on Andros Island, The Bahamas (Cousteau's), we conducted 39 mating trials to test for correlational selection via copulatory performance on two male traits: gonopodial anterior transposition and gonopodium length. All fish were raised under common laboratory conditions in 10-L aquaria within a recirculating system (providing biological, mechanical and UV-filtration) at ∼25°C in a temperature-controlled room and fed a varied diet of live brine shrimp, freeze-dried daphnia and bloodworms, and TetraMin Pro flakes. Each trial comprised one virgin female placed with one virgin male in a 2.5-L polycarbonate aquarium (22 × 9.5 × 12 cm, L × W × H) with no structure for 1 h. The first 30 min were video recorded from the side with a Sony DCR-SR68 camera (Sony, Tokyo, Japan). From the video, we counted the number of copulation attempts and the number of apparently successful copulations where appropriate genital contact seemed to occur. We watched videos frame-by-frame during copulation attempts, and recorded genital contact when unambiguous physical contact was made between the male gonopodium tip and the female body in the immediate vicinity of the urogenital opening. Fish were euthanized immediately following the trial, photographed for morphological measurements (standard length and the two aforementioned traits), and preserved in 95% ethanol. Using tpsDig2 (Rohlf, 2017), we measured gonopodial anterior transposition as the distance from the anterodorsal tip of anal fin ray 1 to the center of the eye orbit, and gonopodium length as the distance from the anterodorsal tip of anal fin ray 1 to the distal tip of the gonopodium. We estimated mating success in three ways, as it was not obvious which measure might be most appropriate: genital contact success (0 or 1), number of genital contacts, and genital contact efficiency (number of genital contacts divided by number of copulation attempts). Using sperm retrieval, we confirmed that these estimates provided adequate surrogates for insemination success (see **Supplementary Material**).

To test for positive correlational selection on gonopodium length and gonopodial anterior transposition, we employed three statistical approaches, one for each estimate of mating success. Prior to analysis, we regressed each $\log_{10}$-transformed trait on $\log_{10}$-transformed standard length and saved residuals to examine size-independent variables. Note that results are virtually identical if we measured relative gonopodial anterior transposition as its proportion of standard length, as described below (correlation among the two trait estimates: $r = 0.999$, $P < 0.0001$). In each case, we tested for significance of the interaction between the two traits on variation in mating success, and calculated the standardized correlational selection gradient (γ) using multiple regression of standardized trait values (mean = 0, standard deviation = 1) on relative fitness (mating success score divided by average mating success score) (Lande and Arnold, 1983). To accurately and thoroughly characterize selection on these traits, we additionally tested for directional selection (linear selection acting directly on each trait) and quadratic selection (i.e., stabilizing/disruptive selection on each trait). Note that the hypothesized ridge-shaped selection surface implies both positive correlational selection on the traits, as well as stabilizing selection acting on both traits. For significance testing, we conducted a logistic regression for genital contact success, a generalized linear model with a Poisson distribution and log link for the number of genital contacts, and a multiple regression for genital contact efficiency. We arcsin square-root transformed genital contact efficiency, as this transformation of the proportional values increased normality of model residuals. In each model, gonopodium length, gonopodial anterior transposition, their squared terms, and their interaction served as independent variables. The significance of the interaction term from these models provided the relevant tests of correlational selection. To interpret any observed correlational selection, we visualized the quadratic selection surface.

Correlated Evolution of Gonopodial-Complex Traits Among Species

To test predictions of correlated evolution among species, we chose to focus on the *Gambusia* genus, as this is the most species-rich poeciliid genus and has experienced the greatest scrutiny to date in understanding the development and anatomy of the gonopodial complex (Langerhans, 2011). We generated phylogenetic hypotheses for 10 *Gambusia* species using mitochondrial and nuclear gene sequences, measured the relevant traits using radiograph images, and calculated standardized phylogenetic independent contrasts. First, we used PCR to amplify a 975-bp fragment of the NADH subunit 2 mitochondrial gene (ND2) (following Langerhans et al., 2012), a 402-bp fragment of the cytochrome *b* mitochondrial gene (cyt *b*) (following Lydeard et al., 1995), and a 760-bp fragment of the first intron of the S7 ribosomal protein nuclear gene (S7) (following Langerhans et al., 2012) for each of 10 species of *Gambusia*, as well as two outgroup species, *Heterophallus rachovii* (*Gambusia rachovii*) and *Belonesox belizanus*. We sequenced a single specimen for most species (outgroups, *G. clarkhubbsi*, *G. gaigei*, *G. geiseri*, *G. heterochir*, *G. hurtadoi*, *G. speciosa*) but included multiple samples (each from geographically distinct populations) for four species (*G. affinis*: 4, *G. aurata*: 3, *G. holbrooki*: 3, *G. quadruncus*: 4). Sequences were aligned by eye and deposited in GenBank (**Supplementary Table 1**). We constructed phylogenetic trees using two separate Bayesian analyses with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), one for concatenated mitochondrial genes and one for the nuclear gene, as the different datasets revealed significantly different evolutionary histories (partition homogeneity test, $P = 0.02$). For additional details, see the **Supplementary Material**.

We obtained radiograph images of 4–50 adult males for each species to obtain estimates of the number of modified hemal spines on vertebrae 14–16, the magnitude of gonopodial anterior transposition, and the relative length of the gonopodium. The number of modified hemal spines, defined as hemal spines exhibiting obvious anterior bending, uncinate processes, or both, was counted on each radiograph. Although most species exhibit a fixed number of modified hemal spines (Rosen and Bailey, 1963; Rauchenberger, 1989), some species are polymorphic. Of the 10 species examined in the interspecific component of this study, two species (*G. clarkhubbsi*, *G. holbrooki*) were found to exhibit variation in modified hemal spine number (either 2 or 3) and were classified as 2.5 for the analyses performed here. We measured the magnitude of gonopodial anterior transposition as the relative placement of the columnar fusion of pteryogiophores 2–5 along the body: distance from the anterodorsal tip of the second pterygiophore to the center of the eye orbit divided by standard length (larger ratio values indicate relatively farther distances from the eye, and thus *less* transposition). We employed this ratio metric as our estimate of relative gonopodial anterior transposition because we were specifically interested in the relative placement of the gonopodium on the body (results are qualitatively similar using residuals of log-log regression on standard length instead, as the two estimates of relative gonopodial anterior transposition were highly correlated, $r = 0.99$, $P < 0.0001$). Gonopodium length was measured as the distance from the anterodorsal tip of anal fin ray 1 to the distal tip of the gonopodium. To control for the possible confounding effects of body size, we tested for associations between the three traits described above and standard length using linear regression and statistically removed effects of size where necessary. Relative gonopodial anterior transposition was not correlated with body size, and thus no additional size correction was performed for this variable. Correlation with body size was evident for the other two traits. We conducted phylogenetic size correction (following Revell, 2009) for both modified hemal-spine number and gonopodium length to eliminate size effects, i.e., calculated residuals from linear regression of hemal spine number and $\log_{10}$-transformed gonopodium length on $\log_{10}$-transformed standard length, controlling for non-independence due to phylogenetic history.

We calculated standardized phylogenetic independent contrasts using the *ape* package (Paradis et al., 2004) in the R statistical program (R Core Team, 2019) for both the mtDNA and nDNA phylogenetic hypotheses (after pruning the tree so that each species was represented by a single tip). Because normality of residuals was evident in all cases, we used linear regression through the origin to test for correlated evolution among (1) the number of modified hemal spines and gonopodial anterior transposition and (2) gonopodial anterior transposition and relative gonopodium length. For analyses using the nDNA phylogeny, we adjusted degrees of freedom based on the number of polytomies (Purvis and Garland, 1993; Garland and Diaz-Uriarte, 1999).

Predator-Driven Divergence in Gonopodial-Complex Traits in Bahamas Blue Holes

To evaluate the predictability of evolution in the gonopodial complex between divergent predation regimes, we investigated morphological divergence among male Bahamas mosquitofish inhabiting blue holes on Andros Island, The Bahamas. Bahamas mosquitofish colonized these relatively isolated blue holes (water-filled vertical caves) during the past ∼15,000 years, with some blue holes being additionally colonized during this period by a larger predatory fish (bigmouth sleeper, *Gobiomorus dormitor*). Because no environmental factor is known to co-vary with the presence of the fish predator (e.g., salinity, water transparency, surface diameter, depth, resource availability; Langerhans et al., 2007; Heinen et al., 2013; Riesch et al., 2013), this system represents an ideal "natural experiment" with which to test the effects of predation on evolutionary diversification (e.g., Langerhans et al., 2007; Langerhans, 2010, 2018).

Based on hypothesized divergent selection between predation regimes, it is predicted that Bahamas mosquitofish should evolve larger caudal regions and smaller gonopodia in high-predation blue holes [see Langerhans (2010) for details], changes that might be facilitated by phenotypic modifications of the gonopodial complex (see **Figure 1D**). Prior work revealed that fish inhabiting blue holes with predatory fish have indeed evolved larger caudal regions, facilitating increased locomotor escape performance and survivorship during predator encounters (Langerhans, 2009a, 2010, 2018). Using the males from 18 populations examined in this study (see below), we confirmed prior studies' results regarding this pattern of morphological divergence using a partially overlapping dataset (see **Supplementary Figure 2**). Previous work also demonstrated a pattern of smaller gonopodia in high-predation localities of Bahamas mosquitofish across a range of habitat types (as measured by the gonopodium lateral surface area; Langerhans et al., 2005), but no direct test of differences in gonopodium size has yet been performed for inland blue hole populations. Here we perform such a test. It is important to note that although the hypothesis of divergent selection on gonopodium size focuses on effects of its *surface area* on fitness components (i.e., mating preferences for lateral projection area, frictional drag incurred by gonopodia during locomotion), the hypothesis of correlational selection on gonopodial anterior transposition and gonopodium size centers on its *length*. Thus, here we examine both gonopodium length (measured as described above) and its lateral surface area (measured as area inside the gonopodium's outer boundaries, comprising anal-fin rays 1–5) in our assessment of divergence in gonopodium size among predation regimes. The two aspects of gonopodium size are not very tightly correlated with one another ($r = 0.34$, $P < 0.001$). We test whether changes in the ano-urogenital region might be partially responsible for mediating responses to selection on lateral body shape and gonopodium size by comparing trait values among predation regimes (see **Figure 1D**).

We obtained radiograph images of 272 males from 18 blue holes: nine populations that evolved in the absence of fish predators and nine that evolved in the presence of predatory fish (**Supplementary Figure 3**). The following nine traits were measured on each image: number of modified hemal spines, length of the 14th hemal spine, length of the 15th hemal spine, length of the uncinate process on the 15th hemal spine, length of the 16th hemal spine, angle of the 16th hemal spine, magnitude of gonopodial anterior transposition, length of the gonopodium, and lateral surface area of the gonopodium. We selected these traits due to their hypothesized effects on gonopodial anterior transposition, and thus body shape, as well as the observation that these traits exhibited considerable variation in these fish. Length of hemal spines was measured using straight-line distances between 2 and 3 landmarks on each bone, depending on the presence of bone curvature. Angle of the 16th hemal spine (degrees) was measured from the angle formed by lines connecting each end of the bone with the vertex at the most posterior edge along the length of the bone (smaller angles depict greater curvature, with stronger anterior bending of the bone). Gonopodial anterior transposition was again measured as the distance from the second pterygiophore to the center of the eye orbit divided by standard length, to capture the relative position of the gonopodium along the body; however all results were again virtually identical if we used log-log residuals of the distance regressed on standard length rather than the ratio. Other traits were measured as previously described. We made all measurements using tpsDig2 software (Rohlf, 2017). Because prior work has shown that blue hole populations are relatively isolated and that fish from similar predation regimes are not more closely related to one another than to populations in different predation regimes (Schug et al., 1998; Langerhans et al., 2007; Heinen-Kay and Langerhans, 2013; Riesch et al., 2013), populations were treated as independent replicates in statistical analyses.

To test for differences among predation regimes, we first conducted a mixed-model nested multivariate analysis of covariance (MANCOVA) using all traits as dependent variables [e.g., see Riesch et al. (2013)], predation regime and population nested within predation regime (random factor) as independent variables, and $\log_{10}$-transformed standard length as a covariate to control for effects of body size. We found no evidence for heterogeneity of slopes among predation regimes ($P = 0.55$). This analysis provided the overall test for differences in gonopodial-complex traits between predation regimes. To inspect trait-specific patterns of among-population variation, we additionally conducted mixed-model nested analysis of covariance (using restricted maximum likelihood estimation) separately for each trait, mirroring the model structure of the MANCOVA described above. Body size did not differ among predation regimes (mixed-model nested ANOVA, $P = 0.95$), indicating that predation regime was not confounded with variation in body size. Prior to analysis, the length and area measurements were $\log_{10}$-transformed to meet assumptions of normality of residuals; the count data for hemal spines, the ratio metric for gonopodial anterior transposition, and the angle of the 16th hemal spine were left untransformed. For the univariate model examining variation in the number of modified hemal spines, we employed a generalized linear mixed model with a Poisson distribution and log link.

To more directly test the role of the ano-urogenital vertebral region in divergence of body shape and gonopodium length among populations, we calculated population means of our measurements of hemal-spine morphology (6 hemal-spine variables), gonopodial anterior transposition, gonopodium length, and the morphological divergence vector (**d**, describing multivariate lateral body shape variation, see **Supplementary Material**). Mean values represent least-squares means, statistically adjusted for effects of body size. We tested our anatomical prediction that hemal-spine morphology underlies divergence in gonopodial anterior transposition using canonical correlation analysis. We tested for an association between the six hemal-spine variables and gonopodial anterior transposition, and visualized the relationship using the canonical axis derived from the statistical model. Using linear regression between multivariate body shape (**d**) and gonopodial anterior transposition, we then tested our co-optation prediction that gonopodial anterior transposition partially underlies this primary axis of divergence in lateral body shape among populations, reflecting a novel mechanism for adaptive diversification of body form. Finally, we used linear regression to test the functional-integration prediction that populations with greater gonopodial anterior transposition will also exhibit shorter gonopodium length to enhance copulatory performance. We used the statistical program JMP (v. 14.2, 2018, SAS Institute Inc.) for most analyses, but used SAS (v. 7.15, 2017, SAS Institute Inc.) for the mixed-model MANCOVA, and the R package *lme4* (Bates et al., 2011) for the generalized linear mixed model. In all cases, one-tailed P values are employed when we have *a priori* directional hypotheses (Rice and Gaines, 1994).

Because variation among populations in gonopodial-complex traits could reflect either genetically-based differentiation, phenotypic plasticity, or both, we tested for a genetic basis to trait divergence using a common-garden experiment. We raised lab-born offspring ($n = 158$) from eight populations (four without predators, four with predators) to adulthood under common laboratory conditions in a large, recirculating aquarium system comprising 72 115-L aquaria with biological, mechanical, and ultraviolet filtration. Water was maintained at ∼25°C, a conductivity of 2,850 μS/cm, and a pH of 8.3, with a 14-h light/10-h dark photoperiod. Fish were fed daily with a mixture of TetraPro Tropical Crisps, Fluval Bug Bites for Tropical Fish, and Hikari freeze-dried *Daphnia*, blood worm, and brine shrimp. We collected the parental generation (F0) from the wild as newborns (in an attempt to minimize maternal and environmental effects), transported them to the laboratory at North Carolina State University, raised them to adulthood, and mated each female with one unique male from the same population (∼19 mated pairs per population). Offspring (F1) were then raised to adulthood, with sexes separated as they matured (males raised in 4–5 separate tanks per population; 5–15 males per tank).

For F1 fish, we measured all traits as described above for wild-caught fish. We then performed statistical analyses using a dataset that included for these eight populations both wild-caught fish that had been immediately euthanized and preserved after field collection and F1 lab-born fish that had been euthanized and preserved at ∼225 days of age. We conducted MANCOVA using all traits as dependent variables and ANCOVAs separately for each trait. In all models, $\log_{10}$-transformed standard length served as a covariate; population (testing for genetic differences), rearing environment (lab-born or wild-caught), and their interaction served as independent variables. Of particular interest in this study is whether fish born and raised in a common laboratory environment retained trait differences between populations consistent with those observed in wild-caught individuals (population term of model), indicating evolutionary divergence in gonopodial-complex traits among populations. In all cases, we did not adjust P values for multiple tests; however all significant results would remain significant when adjusted to maintain a false discovery rate of 5% (Benjamini and Hochberg, 1995).

RESULTS

Experimental Test of Functional Integration of Gonopodial-Complex Traits

We found significant positive correlational selection on gonopodium length and gonopodial anterior transposition when using either genital contact success or efficiency as our estimate of mating success, but not when using the number of genital contacts as the mating-success surrogate (**Supplementary Table 2**). We found a saddle-shaped selection surface rather than the predicted ridge-shaped selection surface (**Figure 2**). This shape arose from the presence of correlational selection and absence of stabilizing selection (**Supplementary Table 2**). Consistent with expectations, males with a long gonopodium and a long distance between their gonopodium and eye had relatively high mating success, as did males with a short gonopodium and a short distance between their gonopodium and eye. But males with intermediate values of both traits had slightly reduced mating success, while males with mismatched trait values (long gonopodia with short distance to eye or short gonopodia with long distance to eye) had especially low mating success (**Figure 2**). This latter effect was particularly pronounced for the combination of short gonopodia and a far distance from the eye.

Correlated Evolution of Gonopodial-Complex Traits Among Species

Bayesian analysis of mtDNA gene sequences produced a well-supported phylogeny for the 10 *Gambusia* species examined (**Figure 3A**), while analysis of nDNA gene sequences yielded a less resolved phylogeny, differing in topology primarily with regard to the placement of *G. aurata*, *G. speciosa*, and *G. holbrooki* (suggesting possible historical mtDNA introgression; **Figure 3B**).

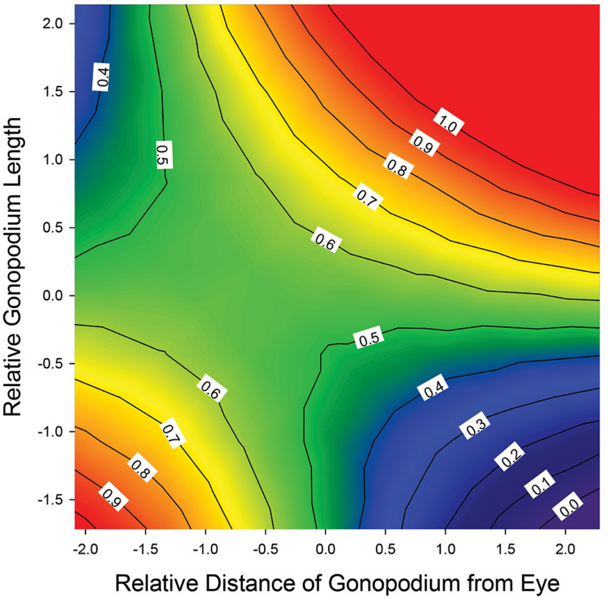

FIGURE 2 | Quadratic selection surface for relative gonopodium length and gonopodial anterior transposition, depicting the predicted mating success of males across the range of trait values. Traits are mean-centered and in units of standard deviation. Genital contact success depicted on the Z axis (elevation), but results are similar for genital contact efficiency (see **Supplementary Table 2**). Surface constructed using all selection gradients (linear, quadratic, correlational).

Species were found to vary in the number of modified hemal spines present, with five species exhibiting two such spines (*G. aurata*, *G. gaigei*, *G. geiseri*, *G. heterochir*, *G. hurtadoi*), three species having three (*G. affinis*, *G. speciosa*, G. *quadruncus*), and two species having variable spine numbers (see above). Using phylogenetic independent contrasts, we found a consistent trend for correlated evolution of modified hemal spines and gonopodial anterior transposition (mtDNA: $F_{(1, 8)} = 4.46$, one-tailed $P = 0.0338$; nDNA: $F_{(1, 5)} = 3.87$, one-tailed $P = 0.0532$). As predicted from previous anatomical work, species with more modified hemal spines tended to exhibit greater gonopodial anterior transposition (**Figures 3C,D**). On the other hand, phylogenetic independent contrasts did not uncover a strong relationship between gonopodial anterior transposition and gonopodium length (mtDNA: $F_{(1, 8)} = 1.41$, one-tailed $P = 0.13$; nDNA: $F_{(1, 5)} = 0.01$, one-tailed $P = 0.47$).

Predator-Driven Divergence in Gonopodial-Complex Traits in Bahamas Blue Holes

In Bahamas mosquitofish, we found considerable among-population variation in male genital traits, with males from divergent predation regimes differing in mean trait values for most measured traits (**Table 1**). Consistent with predictions, male *G. hubbsi* inhabiting blue holes with predatory fish had a greater number of modified hemal spines (**Figure 4A**), a longer 14th, 15th, and 16th hemal spine

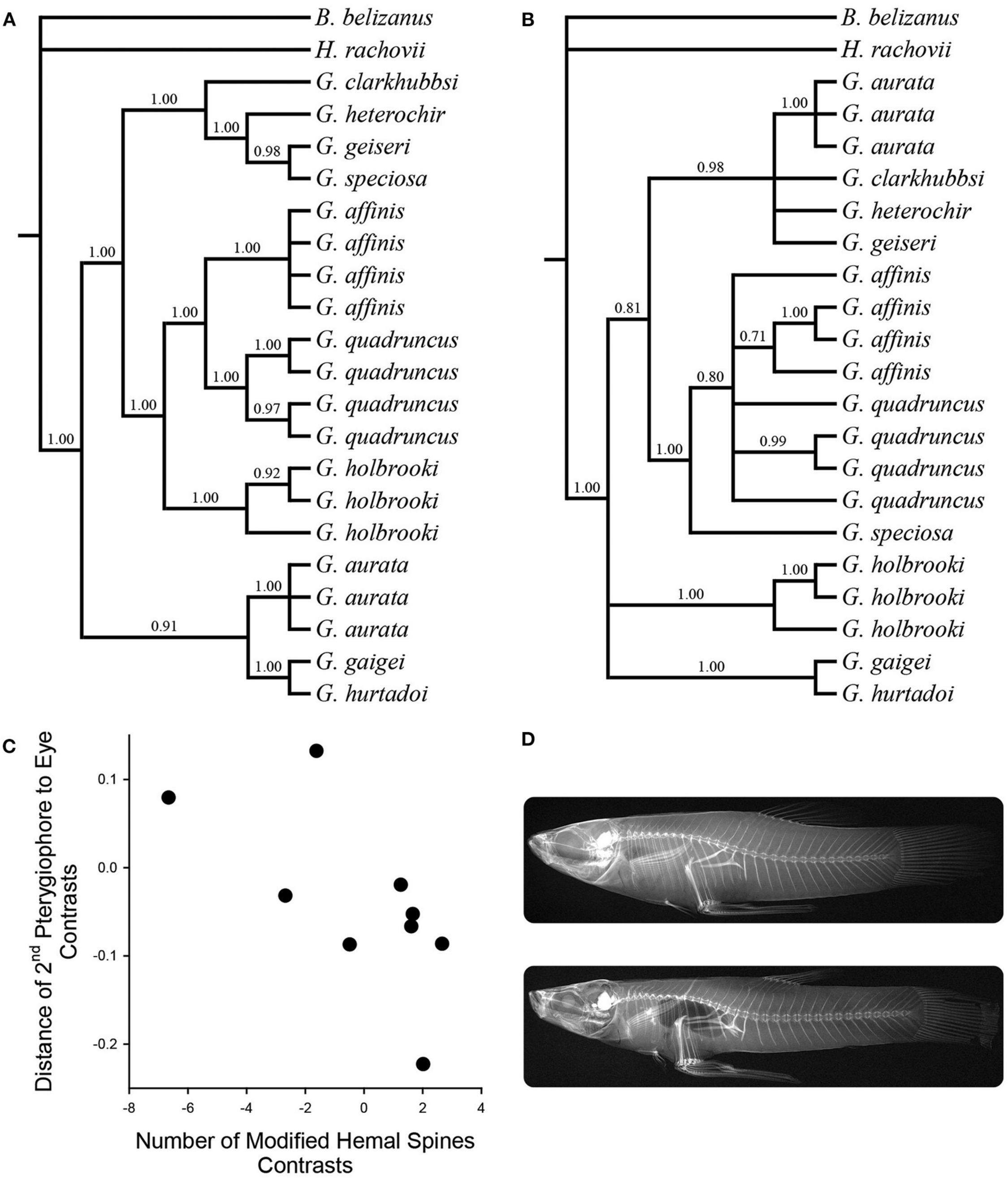

FIGURE 3 | Bayesian phylogenetic hypothesis using **(A)** concatenated mtDNA gene sequences and **(B)** the nuclear encoded S7 intron. Topologies represent 50% majority-rule consensus trees, and numbers represent Bayesian posterior probabilities of nodes. **(C)** Phylogenetically independent contrasts based on the S7 phylogeny, depicting correlated evolution of the relative number of modified hemal spines and gonopodial anterior transposition. **(D)** Representative radiographs of *G. aurata* (top; two modified hemal spines, weak gonopodial anterior transposition) and *G. affinis* (bottom; three modified hemal spines, strong gonopodial anterior transposition).

(**Supplementary Figures 4A–C**), a longer uncinate process on the 15th hemal spine (**Supplementary Figure 4D**), a smaller angle (greater anterior bending) of the 16th hemal spine (**Supplementary Figure 4E**), greater gonopodial anterior transposition (**Figure 4B**), and a smaller lateral surface area of the gonopodium (**Figure 4C**) than did males from blue holes without piscivorous fish. In contrast with our prediction, populations in blue holes with predatory fish did not exhibit relatively shorter gonopodia than those in predator-free blue holes (**Supplementary Figure 4F**).

TABLE 1 | Statistical results examining variation in gonopodial-complex traits across predation regimes in Bahamas mosquitofish (see statistical details in text).

	Predation regime		Log_{10} standard length	
Dependent Variable	**Test statistic**	**1-tailed *P***	**Test statistic**	***P***
All Traits (MANCOVA)	$F_{(8,\ 899)} = 29.21$	<0.0001	$F_{(9,220)} = 308.34$	<0.0001
Number of modified hemal spines	$z = 3.70$	0.0001	$z = 1.88$	0.0600
Length of 14th hemal spine	$F_{(1,\ 16.0)} = 5.26$	0.0179	$F_{(1,\ 263.1)} = 961.54$	<0.0001
Length of 15th hemal spine	$F_{(1,\ 16.2)} = 12.04$	0.0016	$F_{(1,\ 243.1)} = 1031.29$	<0.0001
Length of uncinate process on 15th hemal spine	$F_{(1,\ 15.0)} = 14.41$	0.0009	$F_{(1,\ 259.1)} = 193.58$	<0.0001
Length of 16th hemal spine	$F_{(1,\ 16.6)} = 24.98$	<0.0001	$F_{(1,\ 214.8)} = 370.91$	<0.0001
Angle of 16th hemal spine	$F_{(1,17.0)} = 19.44$	0.0002	$F_{(1,\ 171.2)} = 5.81$	0.0170
Distance of 2nd pterygiophore to eye	$F_{(1,14.1)} = 7.36$	0.0084	$F_{(1,195.6)} = 5.12$	0.0247
Gonopodium lateral surface area	$F_{(1,\ 14.9)} = 9.75$	0.0035	$F_{(1,\ 204.4)} = 496.52$	<0.0001
Gonopodium length	$F_{(1,\ 17.0)} = 2.42$	0.9308	$F_{(1,\ 244.5)} = 1819.53$	<0.0001

Using population means, we found support for our anatomical and co-optation predictions, but not for our functional-integration prediction. Hemal-spine morphology was indeed significantly associated with gonopodial anterior transposition ($F_{(6,\ 11)} = 4.61$, one-tailed $P = 0.0070$): populations with a greater number of modified hemal spines, longer hemal spines, a longer uncinate process on the 15th hemal spine, and greater curvature of the 16th hemal spine exhibited a gonopodium positioned more anteriorly (**Figure 5A**, **Supplementary Table 3**). Populations exhibiting greater gonopodial anterior transposition also had relatively larger caudal regions (one-tailed $P = 0.0003$, **Figure 5B**), matching predictions. However, populations with greater gonopodial anterior transposition did not have shorter gonopodia (one-tailed $P = 0.93$).

Results from the common-garden experiment suggested that divergence in gonopodial-complex traits among populations, at least partially, reflects genetic differentiation. Our multivariate model revealed highly significant effects for all model terms, indicating that although genetically-based differences were evident, some traits for some populations exhibited differences between wild and laboratory conditions (**Supplementary Table 4**). Examining each trait separately revealed that some traits showed clear, genetically-based differences among populations with little-to-no effects of a laboratory environment on their expression, while others showed both genetic differences and influences of laboratory rearing (**Supplementary Table 4**). For instance, we found no evidence for consistent differences between wild and lab-raised fish for the length of the 15th hemal spine, length of the uncinate process of the 15th hemal spine, or gonopodial anterior transposition, but found strong effects of laboratory rearing on gonopodium size and number of hemal spines (smaller gonopodia and more hemal spines in lab-raised fish). In most cases, we found significant evidence of population-specific environmental effects (i.e., G × E), although its strength was always weaker than overall effects of population (see **Supplemental Material**). Thus, environmental influences on observed trait values in the wild likely exist for some traits, but genetically-based differentiation in gonopodial-complex traits was evident.

DISCUSSION

The evolution of novel traits and body plans can dramatically alter subsequent evolutionary trajectories (see Introduction). Here we uncovered several important insights into the evolutionary causes and consequences of an understudied novel body region, the ano-urogenital vertebral region of male live-bearing fish in the family Poeciliidae. Our findings revealed that developmental linkages among components of this gonopodial complex underlie correlated evolution among populations and species, but that correlational selection for functional integration of traits has not driven correlated evolution. We further discovered that predator-associated natural and sexual selection has driven evolutionary diversification of this region: novel traits intimately related to copulation have been co-opted to facilitate evolutionary changes in locomotor performance and body form. This work supports the notion that major evolutionary innovations fundamentally alter future evolutionary patterns, and one need not look into the deep past to find such examples.

In the mating experiment, we uncovered significant evidence for functional integration of two key gonopodial-complex traits. We predicted a ridge-shaped selection surface, where a combination of stabilizing selection and correlational selection would favor a positive association between relative gonopodium length and its distance from the eye—i.e., successful sperm insemination would benefit from longer gonopodia when positioned more posteriorly and from shorter gonopodia when positioned more anteriorly. The saddle-shaped correlational selection surface we observed is only partially consistent with this expectation. Our finding of positive correlational selection indeed suggests that insemination is highest for fish with the appropriate combination of gonopodium length and gonopodial anterior transposition, and particularly low for mismatched combinations of the traits; however males with intermediate values of both traits suffered slightly reduced fitness. This dip in fitness for intermediate trait values arose because of the lack of stabilizing selection on the traits—it is unclear why this fitness valley might occur. Moreover, when using the number of genital contacts as our estimate of mating success, we found no evidence

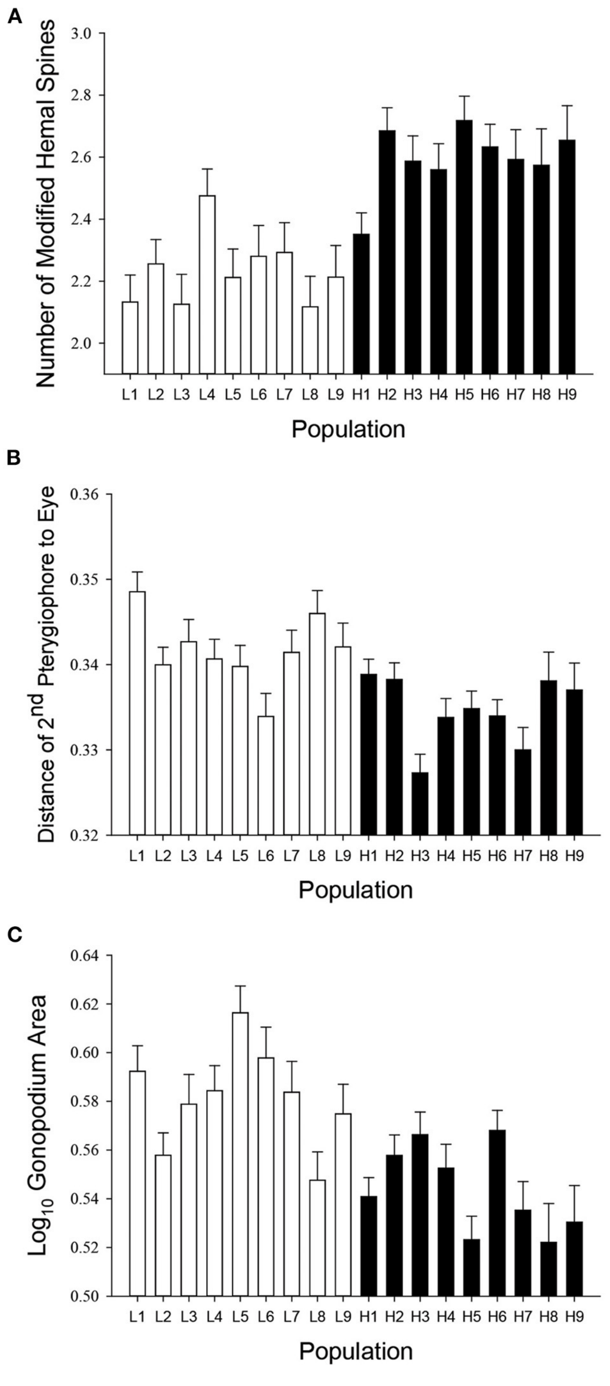

FIGURE 4 | Differences between low-predation (open bars) and high-predation (filled bars) populations of Bahamas mosquitofish in **(A)** number of modified hemal spines, **(B)** gonopodial anterior transposition, and **(C)** gonopodium lateral surface area (see **Table 1** for statistical details). Least-squares means ± one standard error depicted.

for correlational selection. This implies that this fitness estimate captures something different than the others (i.e., genital contact success and efficiency), perhaps reflecting male behavior, rather than mating success, more so than the others. For instance, males with more genital contacts might simply attempt more copulations or have larger sperm reserves, and not necessarily reflect sperm insemination the same way the other estimates do. Indeed, the number of genital contacts observed here was highly correlated with the number of coerced copulation attempts ($P < 0.0001$), while genital contact efficiency was not ($P = 0.52$). The functional integration of gonopodial anterior transposition and gonopodium length during mating is apparently more related to the ability and efficiency of achieving mating success than the overall number of times that a successful copulation might occur during a given mating encounter.

Although a number of studies have examined effects of gonopodium length and distal-tip elements on mating success in poeciliid fishes (Evans et al., 2011; Gasparini et al., 2011; Kwan et al., 2013; Devigili et al., 2015; Head et al., 2015, 2017; Hernandez-Jimenez and Rios-Cardenas, 2017; Chung et al., 2020), no prior study has investigated the relationship examined here. Our findings add to the evidence that complex selection surfaces may characterize insemination and fertilization success in *Gambusia* fishes (Devigili et al., 2015; Head et al., 2015) and suggest we still have much to learn. That said, further work in additional populations and species, with larger sample sizes, will help us understand the generality and robustness of these findings. Overall, our results indicate that gonopodium length and its position along the body are functionally related to one another, and if this copulatory function captures the primary link between these traits and fitness, then this predicts correlated evolution of the two traits across populations and species.

Examining 10 closely related *Gambusia* species, and investigating 18 populations of the post-Pleistocene radiation of Bahamas mosquitofish, we did not find this predicted correlated evolution—we found no evidence that gonopodium length and gonopodial anterior transposition coevolve in the expected manner. The evolution of a more anteriorly positioned gonopodium apparently does not necessarily lead to the evolution of a shorter gonopodium, even though these trait combinations seem to enhance mating success. One possible explanation for this finding is that correlational selection might vary over time or among populations/species (e.g., Siepielski et al., 2009, 2013; Weese et al., 2010). Further work is needed to evaluate this putative cause. However, we suggest a likely explanation for this finding is these traits experience complex forms of selection from multiple agents other than their co-dependency during copulation and their genetic basis probably involves different genes (i.e., little-to-no pleiotropy). One possible complicating factor is selection on testes size (e.g., via sperm competition), which could result in reduced gonopodial anterior transposition to accommodate larger testes. However, we have found no evidence supporting this notion (see **Supplementary Material**). Still, considering the disparate forms of selection, such as natural selection on locomotor performance and sexual selection via female preference, that might act on gonopodium length and gonopodial anterior transposition, it is perhaps not surprising that their evolutionary trajectories are not tightly correlated.

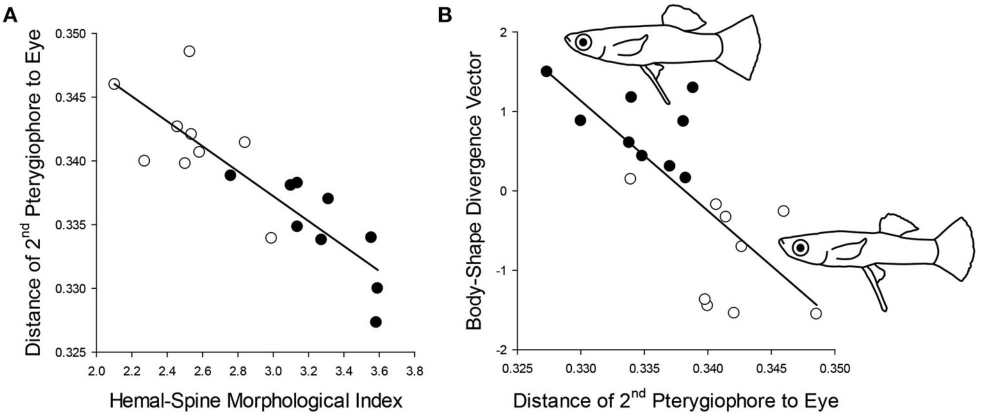

FIGURE 5 | Bahamas mosquitofish populations with **(A)** more numerous, larger, and more curved modified hemal spines (positive scores on hemal-spine morphological index) exhibit greater gonopodial anterior transposition (shorter distance between the second pterygiophore and the eye), and consequently **(B)** these populations also exhibit bodies with larger caudal regions (relatively larger trunk/caudal region and smaller head, positive scores on body shape divergence vector; see **Supplementary material** for details). Population means depicted; open symbols: blue holes without predatory fish; filled symbols: blue holes with predatory fish; line drawings illustrate representative body shapes of fish from blue holes with and without predatory fish (dotted line depicts outline of anal-fin ray 5, distinguishing the gonopodium from the rest of the anal fin).

On the other hand, at both micro- and macroevolutionary scales, we revealed correlated evolution of hemal-spine morphology and the magnitude of gonopodial anterior transposition, matching our *a priori* anatomical prediction. As *Gambusia* species evolve more modified hemal spines, they additionally evolve a positioning of the gonopodium closer to the head. Similarly, populations of Bahamas mosquitofish with more numerous, larger, and more curved modified hemal spines also show a more anteriorly positioned gonopodium. The anatomical/developmental linkage between growth of the modified hemal spines and the anterior transposition of the gonopodium has so far been described in only one species (*G. affinis*; Rosa-Molinar et al., 1994, 1998), but the findings here suggest this may represent an evolutionarily conserved mechanism of gonopodial transposition in poeciliid fishes. Thus, one of the most obvious external differences in anatomy between the sexes in the family Poeciliidae may be largely explained by the modified hemal spines (and their associated ligament system) present in males.

In the post-Pleistocene radiation of Bahamas mosquitofish inhabiting blue holes, we uncovered clear evidence for predator-driven change in the gonopodial complex, pointing to ecological agents partly responsible for the diversity of morphological variation associated with the novel ano-urogenital vertebral region in poeciliid fishes. Findings largely corresponded to our *a priori* predictions. Specifically, male Bahamas mosquitofish evolved predictable differences between predation regimes in hemal-spine structure, gonopodial anterior transposition, and lateral surface area of the gonopodium. We argue that these differences largely resulted from divergent natural and sexual selection on locomotor performance, body shape, and gonopodium size.

First, we found that diversification of male body shape in Bahamas mosquitofish—a well-known example of predator-driven parallel evolution—appears to be partially governed by changes in the ano-urogenital vertebral region. Natural selection favoring more energetically efficient steady-swimming abilities in low-predation environments where resource competition is particularly high has resulted in more streamlined bodies in these populations, while selection favoring greater acceleration during fast-start escape behaviors in the presence of predatory fish has resulted in bodies with larger mid-body caudal regions in high-predation populations (Langerhans et al., 2007; Langerhans, 2009a,b; Langerhans, 2018; Riesch et al., 2020; this study). Moreover, females show mating preferences within populations based on male body shape, with low-predation females preferring more streamlined males and high-predation females preferring males with larger caudal regions (Langerhans and Makowicz, 2013). We hypothesized that one way to partially develop these divergent morphologies in males involves modification of the hemal-spine morphology and transposition of the gonopodium: a more anteriorly positioned gonopodium creates a larger caudal region, while a more posteriorly positioned gonopodium creates a larger anterior region and may delay the separation of the boundary layer during steady swimming (Langerhans, 2010). Consistent with this notion, we found here that high-predation populations exhibited a suite of differences in hemal-spine morphology that apparently resulted in greater anterior transposition of the gonopodium compared to low-predation populations. This highlights the role of predation regime in driving variation in this novel body region and suggests the region was co-opted to facilitate diversification of body shape after its initial evolution for genital support and mating functions.

The strongest evidence in support of this co-optation hypothesis was the observation that among populations there was a clear relationship between gonopodial anterior transposition and lateral body shape. The most parsimonious explanation for our results is that the ano-urogenital vertebral region, originally evolved for effective sperm transfer, served as an exaptation for body form and locomotor performance, and then evolved in response to selection on these attributes, reflecting secondary adaptation for a new function. That is, once the novel body region had evolved as an adaptation for rapid and effective sperm transfer, it inadvertently affected other attributes which selection later acted upon, leading to greater diversification in the region and novel evolutionary solutions. In this light, re-examination of past work on predator-driven morphological divergence in poeciliid fishes reveals that males often exhibit greater gonopodial anterior transposition in high-predation populations: e.g., *G. affinis*, *G. caymanensis*, *Poecilia reticulata*, *B. rhabdophora* (Langerhans and DeWitt, 2004; Langerhans et al., 2004; Langerhans and Makowicz, 2009; Langerhans, 2010; Ingley et al., 2014). Meanwhile, female poeciliids also often exhibit morphological differences between predation regimes, but not a shift in the position of the anal fin (e.g., Langerhans, 2009b; Langerhans and Makowicz, 2009; Ingley et al., 2014), perhaps because they lack a readily available means of anteriorly transposing their anal fin. Combined with the widespread pattern of habitat-associated lateral morphology in poeciliid fishes (e.g., Hendry et al., 2006; Gomes and Monteiro, 2008; Tobler et al., 2008; Langerhans and Reznick, 2010; Torres-Mejia, 2011; Hassell et al., 2012; Ingley et al., 2014; Landy and Travis, 2015; Riesch et al., 2016, 2018; Langerhans, 2018; Moody and Lozano-Vilano, 2018), it seems that the co-optation of the novel ano-urogenital region could represent a common, but previously unrecognized, means of male body shape evolution in the family.

Prior work suggests females of several poeciliid fishes, including *G. hubbsi*, prefer males with larger gonopodia, but larger gonopodia increase drag and thus reduce fast-start escape performance (e.g., Langerhans et al., 2005; Kahn et al., 2010; Langerhans, 2011). Consistent with our prediction, and with empirical work in other habitat types and species, we found that low-predation populations exhibited a larger lateral surface area of the gonopodium. The evidence that a tradeoff between natural and sexual selection underlies this divergence was further strengthened by the fact that we did not find divergence in gonopodium length between predation regimes. This suggests that the results for the two estimates of gonopodium size were not confounded, and divergent selection acts on the surface area of the organ (via mating preference of lateral projection area and locomotor effects of frictional drag), not on its length. This further means that the changes observed between predation regimes in hemal-spine morphology and gonopodial anterior transposition cannot be explained as accommodations for changes in gonopodium length. Some prior studies have found longer gonopodia in high-predation environments [Kelly et al., 2000; Jennions and Kelly, 2002; but see Evans et al. (2011) for results similar to this study], perhaps explained by positive effects of length on insemination during coercive matings in some species (Evans et al., 2011; Devigili et al., 2015; Head et al., 2015, 2017). However, patterns of gonopodium length divergence between predation regimes appear inconsistent. The marked variation in gonopodium length across the family Poeciliidae has long attracted considerable research attention. Our findings add to this body of research, but suggest the primary causes of this variation are neither functional integration with gonopodial anterior transposition, nor the influence of either female mating preferences or natural selection via predation. Because most prior studies examining size of the gonopodium have only measured length, we caution researchers to consider whether in certain cases surface area might provide a more relevant measure.

We found that a combination of both genetically based differences and environmental influences appear to explain among-population variation in components of the gonopodial complex. Depending on the trait, we found moderate to strong population differences persisted in a common laboratory environment, suggesting evolutionary differentiation has occurred in all traits examined. Yet, the laboratory environment altered the expression of most traits, in at least some populations—the exception being gonopodial anterior transposition. Consistent with work in other poeciliid fishes (*G. affinis*: Langerhans, 2009b; *Heterandria formosa*: Landy and Travis, 2015), this particular trait showed strong genetically based population differences with little-to-no effects of laboratory rearing. On the other hand, the laboratory environment clearly had the greatest influence on gonopodium size, consistent with recent work showing that higher food levels result in shorter gonopodia relative to body size (Broder et al., 2020)—we observed relatively smaller gonopodia in the lab compared to the wild, and food was very likely in higher quality and quantity in the laboratory. But still, even gonopodium size showed genetically-based differences as well, matching prior work on heritability of gonopodium size within populations (Evans et al., 2013; Booksmythe et al., 2016) and genetically-based differences among populations in gonopodium morphology (Langerhans et al., 2005; Heinen-Kay and Langerhans, 2013; Broder et al., 2020). Future work can more carefully disentangle the roles of genetic divergence, plasticity, and population-specific plasticity in these traits, but it is clear all sources of variation may prove important for different traits and evolutionary changes in these traits have occurred among populations.

CONCLUSION

Among vertebrates, poeciliid fishes have evolved a unique body plan, and the ano-urogenital region appears to provide novel means of diversification. Subsequent to the evolution of the ano-urogenital region in poeciliids, the region has apparently been co-opted as a means of responding to natural and sexual selection on locomotor performance and lateral body shape, but the length of the gonopodium appears to evolve somewhat independently of the rest of the gonopodial complex. Changes in gonopodial anterior transposition may represent a common means of responding to selection on body shape in male live-bearing fishes, but peripheral consequences of such changes include a restricted trunk region which could lead to reshaping and repositioning of organs, decreased organ sizes, altered diets, and even changes in habitat use and foraging behaviors. These potential effects represent new hypotheses pointing to future research directions. Population divergence in body shape and genitalia can potentially influence reproductive compatibility, suggesting that evolution of diversity in the novel body region could play an important role in speciation. Evolution of the ano-urogenital region made new evolutionary solutions possible in poeciliid males. Poeciliid fishes exhibit marked variation in mating systems and habitat types, and future investigations at intraspecific and interspecific scales should yield important insights into the influence of the ano-urogenital region in diversification patterns in the family.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. GenBank accession numbers are provided in the article/**Supplementary Material**, and other datasets are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.fbg79cnt8 (Langerhans and Rosa-Molinar, 2021).

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of North Carolina State University (protocols 13-101-O, 16-193-O).

AUTHOR CONTRIBUTIONS

RL and ER-M conceived the study and wrote the manuscript. RL collected and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.619232/full#supplementary-material

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Phenotypic Variation in an Asexual-Sexual Fish System: Visual Lateralization

Allison D. Connelly and Michael J. Ryan*

Department of Integrative Biology, The University of Texas at Austin, Austin, TX, United States

Sexual reproduction is nearly ubiquitous in the vertebrate world, yet its evolution and maintenance remain a conundrum due to the cost of males. Conversely, asexually reproducing species should enjoy a twofold population increase and thus replace sexual species all else being equal, but the prevalence of asexual species is rare. However, stable coexistence between asexuals and sexuals does occur and can shed light on the mechanisms asexuals may use in order to persist in this sex-dominated world. The asexual Amazon molly (*Poecilia formosa*) is required to live in sympatry with one of its sexual sperm hosts –sailfin molly (*Poecilia latipinna*) and Atlantic molly (*Poecilia mexicana*)—and are ecological equivalents to their host species in nearly every way except for reproductive method. Here, we compare the visual lateralization between Amazon mollies and sailfin mollies from San Marcos, Texas. Neither Amazon mollies nor sailfin mollies exhibited a significant eye bias. Additionally, Amazon mollies exhibited similar levels of variation in visual lateralization compared to the sailfin molly. Further investigation into the source of this variation –clonal lineages or plasticity—is needed to better understand the coexistence of this asexual-sexual system.

Keywords: clones, mirror image response, *Poecilia formosa*, *Poecilia latipinna*, sexual reproduction

Edited by:
Andrea S. Aspbury,
Texas State University, United States

Reviewed by:
Jonathan Mee,
Mount Royal University, Canada
Caitlin R. Gabor,
Texas State University, United States

****Correspondence:***
Allison D. Connelly
acdavis@utexas.edu

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INTRODUCTION

Sexual reproduction is practiced by 99.9% of species—either exclusively or at one point in their life—despite the 2-fold cost of sex (Avise, 2008). Historical arguments suggest that this cost is more than offset by the benefit to sex that accrues from greater genetic variance (Williams, 1975; Maynard Smith, 1978; Maynard Smith and Szathmary, 1999). Recent studies further highlight the importance of breaking apart genetic associations during recombination; separating deleterious alleles from beneficial alleles, as well as spreading beneficial alleles across a wide genomic background increases the efficiency of selection and adaptation to a capricious environment (Barton and Charlesworth, 1998; Otto, 2009; Sharp and Otto, 2016; McDonald et al., 2016). Indeed, empirical studies now document the long-term, evolutionary benefit held by sexually reproducing populations compared to asexually reproducing populations (Poon and Chao, 2004; Cooper, 2007; Becks et al., 2012; Gray and Goddard, 2012; McDonald et al., 2016). Despite this, some asexual species persist (Bi and Bogart, 2010; Fradin et al., 2017; Warren et al., 2018), even to the point of being named “ancient” (Heethoff et al., 2009; Schön et al., 2009). Of particular interest are the forms of asexual reproduction—gynogenesis and hybridogenesis—that require the asexual and sexual species to coexist along much of their range (reviewed in Avise, 2008; gynogenetic: *Poecilia formosa*, *Menidia clarkhubbsi*, *Phoxinus eos-neogaeus*, *Cobitis elongatoides-taenia*, *Fundulus heeroclitus*; hybridogenetic: *Poeciliopsis monacha-lucida*, *Rana esculenta*). The aforementioned studies investigated asexuals and sexuals in isolation, however, further investigations are needed into how asexual species can persist alongside sexual species in light of the benefits of sex.

Coexisting asexual and sexual species often mediate direct competition through niche partitioning, in which asexuals occupy a narrow portion of the habitat or resource range of their host species (MacArthur and Pianka, 1966; Vrijenhoek, 1978; Fussey and Sutton, 1981; Schenck and Vrijenhoek, 1986; Case, 1990; Rist et al., 1997; Martins et al., 1998; Negovetic et al., 2001). However, not all coexisting species exhibit niche separations, so their coexistence proposes even more of a dilemma. One such species complex is the Amazon and sailfin molly system. The Amazon molly (*Poecilia formosa*) is an asexual fish originating from a single hybridization event between an Atlantic molly female (*P. mexicana*) and sailfin molly male (*P. latipinna)* over 100,000 years ago in Tampico, Mexico (Schartl et al., 1995b; Stöck et al., 2010; Warren et al., 2018 but see Alberici da Barbiano et al., 2013). Amazon mollies reproduce via gynogenesis –females usually require the sperm of a closely related species to trigger embryogenesis, but the paternal genome is usually excluded (Schlupp, 2005; occasional paternal introgression discussed in Schartl et al., 1995a). This type of reproduction requires that they occupy the range of one of their host species, thus placing them in direct competition. Indeed, the Amazon molly now covers a broad range of habitats from Rio Tuxpan, Mexico to the Nueces River, Texas USA –as well as introduced locations in central Texas, always sympatric with one of its sexual hosts (Schlupp et al., 2002). Previous investigations into this asexual-sexual system found few significant differences among morphological traits, physiological traits, and ecological traits (**Table 1**). Their continued coexistence with their sexual host may rely on genetic and phenotypic variation. This variation may occur at the population level, with genetic variation partitioned amongst different clonal lineages (Lampert et al., 2006; Stöck et al., 2010; Alberici da Barbiano et al., 2013; Warren et al., 2018). Phenotypic variation can also result from behavioral plasticity. Cognitive behaviors are often the most plastic phenotype (Bell et al., 2009). Plasticity in asexual species may provide them with a broad range of responses to environmental change and thus assist in persistence.

In this study we focus on cognitive behaviors –which is lacking in previous comparisons—to determine if average performance or variation in performance could shed light on the coexistence of the asexual Amazon molly with its sexual host. We used a well-studied cognitive task—visual lateralization (Clayton, 1993; McGrew and Marchant, 1999; Pascual et al., 2004; Rogers et al., 2004; Vallortigara and Rogers, 2005; Rogers and Vallortigara, 2008). Visual lateralization is known to vary based on the stimulus (Bisazza et al., 1997a,b, 1998, 1999; De Santi et al., 2001; Sovrano et al., 2001; Fuss et al., 2019), environmental pressures such as predation (Brown et al., 2004; Ferrari et al., 2015), and density dependent selection (Ghirlanda and Vallortigara, 2004; Nakajima et al., 2004). Visual lateralization is present in many poeciliid species in response to some but not all stimuli, including the two species used in this study: *Girardinus falcatus, Gambusia holbrooki, G. nicaraguensis, Brachyrhaphis roseni* (Bisazza et al., 1997b), *Poecilia latipinna, P. formosa, P. mexicana, P. reticulata* (Fuss et al., 2019). Additionally, eye bias is heritable for one poeciliid species (Bisazza et al., 2000). We chose to examine lateralization using a mirror image scrutiny test (Sovrano et al., 1999; De Santi et al., 2001). The mirror image scrutiny test assesses the preference for left- or right-eye use when examining a reflection. Here we compare eye use between the asexual Amazon molly and sexual sailfin molly.

MATERIALS AND METHODS

Sampling was approved by Texas State University (permit 04-523-691) and all animal procedures were approved by the University of Texas Institutional Animal Care and Use Committees (AUP-2018-00089).

Specimen

We collected sailfin and Amazon mollies from San Marcos, Texas (29.89, −97.93) in August 2018 and 2019 and brought them back to the lab at the University of Texas in Austin. We separated species into 37-liter tanks with approximately eight individuals to a tank; male sailfin mollies were included in this total and the ratio of males to females was balanced for both species. Each tank contained gravel and air filtration and set to a 14:10 light dark schedule. All fish were fed twice daily *ad libitum* with commercial fish flakes (Tetramin, Germany). We injected unique elastomer tags into fish at least 72 h prior to experimentation, to track identity throughout experimentation. We tested 25 female sailfin mollies and 27 Amazon mollies in June–August 2019 (2018 collection specimen, both species) and June 2020 (2019 collection specimen, both species). Prior to experiments, we removed fish from communal tanks and placed them individually in 3-L isolation tanks for 24 h.

Apparatus

The experiment consisted of two identical tanks (length × width × height: 41 × 21 × 26 cm) to control for any tank effect. Tanks contained 20 cm high Acrylic mirrors on the two longer walls and white corrugated plastic board completely covering the two shorter walls. Black corrugated poster board covered the exterior of the long walls to prevent visuals of the room. Blue filter (Marineland magnum bonded pad filter media) covered the bottom of the tank and each tank was filled with 15 cm of treated water, aerated prior to trial. A camera (Microsoft lifecam cinema, 720p HD) attached to the center of an aquarium light (NICREW deluxe LED aquarium light, full spectrum 18-Watt, 1,200 LM, 7,500 K) was positioned above each tank using a desk clamp mount; the clamp mount was visible from the tank so we positioned it on the right for one tank and on the left for the other tank to account for any side bias. An isolation zone created

TABLE 1 | Summary table of all traits previously compared between the Amazon molly and its sexual host.

Trait	Species compared	Results	Source
Fecundity	Amazon molly, sailfin molly, Atlantic molly	Equivalent fecundities among all species.	Schlupp et al., 2010
Juvenile survival	Amazon molly, sailfin molly	Equivalent survival rates.	Hubbs and Schlupp, 2008
Salinity tolerance	Amazon molly	Equivalent salinity tolerance to those seen in sailfin mollies.	Schlupp et al., 2002
Thermal tolerance	Amazon molly vs. sailfin molly	Amazon mollies had higher minimum critical temperatures*.	Fischer and Schlupp, 2009
Morphology (dentition)	Amazon molly vs. sailfin molly vs. Atlantic molly	Number of upper and lower teeth differs significantly between all species*.	Lewis et al., 1999
Morphology (visual sensitivity)	Amazon molly vs. sailfin molly vs. Atlantic molly	Amazon mollies are heterozygous at all opsin loci, with an expanded sensitivity compared to either parental species*.	Sandkam et al., 2013
Parasite load	Amazon molly vs. Atlantic molly	Equivalent parasite loads between species.	Gösser et al., 2019
	Amazon molly vs. sailfin molly	Equivalent parasite loads between species.	Tobler et al., 2005
Foraging efficiency	Amazon molly vs. sailfin molly	Equivalent foraging efficiencies; Sailfin mollies with lower body condition in intraspecific treatments*.	Alberici da Barbiano et al., 2014
	Amazon molly vs. sailfin molly	Equivalent foraging rates in summer treatments; Amazon mollies with higher foraging rates in winter treatments*.	Fischer and Schlupp, 2010
	Amazon molly vs. sailfin molly	Equivalent foraging efficiencies in conspecific treatments; Amazon mollies with lower foraging efficiency in heterospecific treatments*.	Alberici Da Barbiano et al., 2010
	Amazon molly vs. sailfin molly vs. Atlantic molly	Equivalent foraging efficiencies.	Scharnweber et al., 2011b
Foraging preferences	Amazon molly vs. sailfin molly vs. Atlantic molly	Equivalent dietary resource use.	Scharnweber et al., 2011
Food stress	Amazon molly vs. sailfin molly	Neonate survival unaffected by variable temperatures; Amazon mollies with lower neonate survival under high food stress*.	Tobler and Schlupp, 2010
Boldness	Amazon molly vs. sailfin molly	Equivalent performances in exploration of a new environment, latency to feed in a new environment, and recovery time after a simulated predation event.	Scharnweber et al., 2011a

Significant differences between species denoted by an asterisk (*).

from gray PVC (diameter 10 cm) affixed with a plastic lid was used for each trial.

Procedure

This procedure follows De Santi et al. (2001). Each fish was randomly assigned an experimental tank order. We removed the fish from isolation using a small fish net and placed into a beaker with approximately 300 mL of water. We then gently poured the fish into the isolation zone positioned in the center of the tank for a 5 min acclimation period. Recording (Debut video capture, NCH software) started at the end of acclimation after removing the isolation zone and lasted for a 10 min period. A second trial immediately followed in the other tank. We measured standard length prior to returning the fish to the isolation tank. We subsequently drained, cleaned, and refilled both testing tanks with treated water in preparation for the next test subject.

Videos were scored using BORIS (version 7.6). The scoring code consisted of a left ocular use, right ocular use, or no direct ocular use. See **Figure 1** for how ocular use was determined. The video was transformed into a series of frames occurring every 0.1 s. Scoring began at the 10 s mark and continued every 2 s until the 10 min mark. We saved the results for each individual tank as well as combined both tank results for total ocular use (referred to as "combined" in subsequent analyses).

Analyses were performed in R studio 1.1.456. We calculated each fish's laterality index score for tank A, tank B, and combined video results following Bisazza et al. (1997b): (left-eye use—right-eye use)/(left-eye use + right-eye use) * 100; positive values would indicate a preference for left-eye use and negative values would indicate preference for right-eye use. We performed a paired *t*-test test to determine if the laterality index score differed significantly between tanks, thus detecting any tank effects. A two-tailed one-sample *t*-test determined within species eye bias with the null assumption of a zero index score. Additionally, we calculated Pearson's correlation coefficients to determine the repeatability of eye use across trials in both species. We used a *t*-test to determine differences in mean eye bias between the species as well as a *t*-test examining overall laterality; overall laterality is based on the absolute value of the index scores to indicate the degree to which each species is

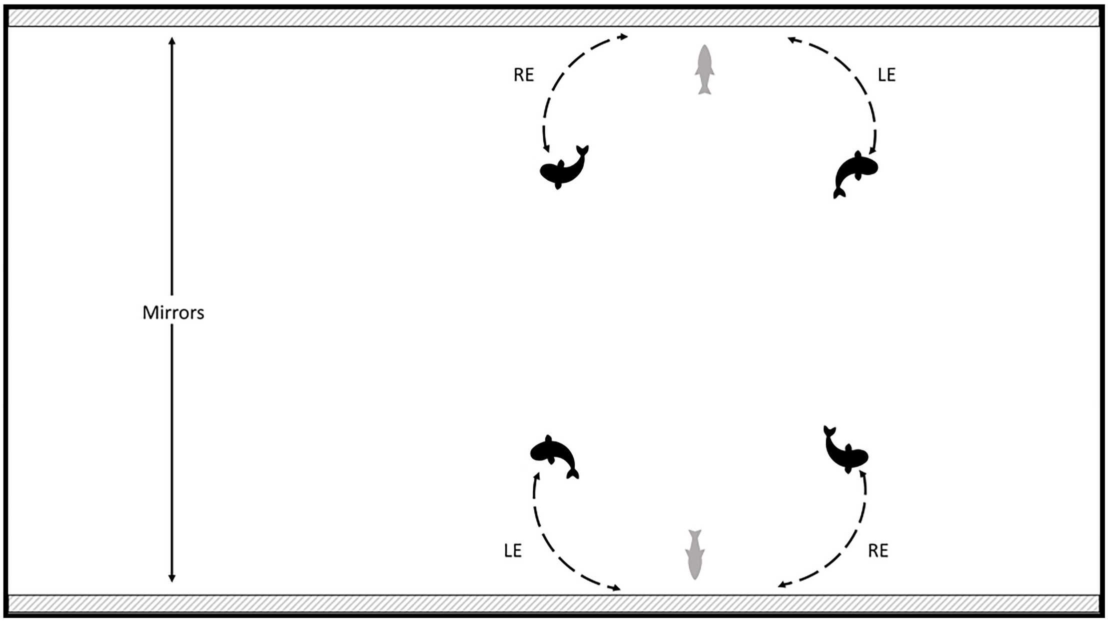

FIGURE 1 | Qualifications for calculating laterality index based on ocular use with closest mirror. LE is left eye use, and RE is right eye use. The gray fish in the middle indicates no specific eye use.

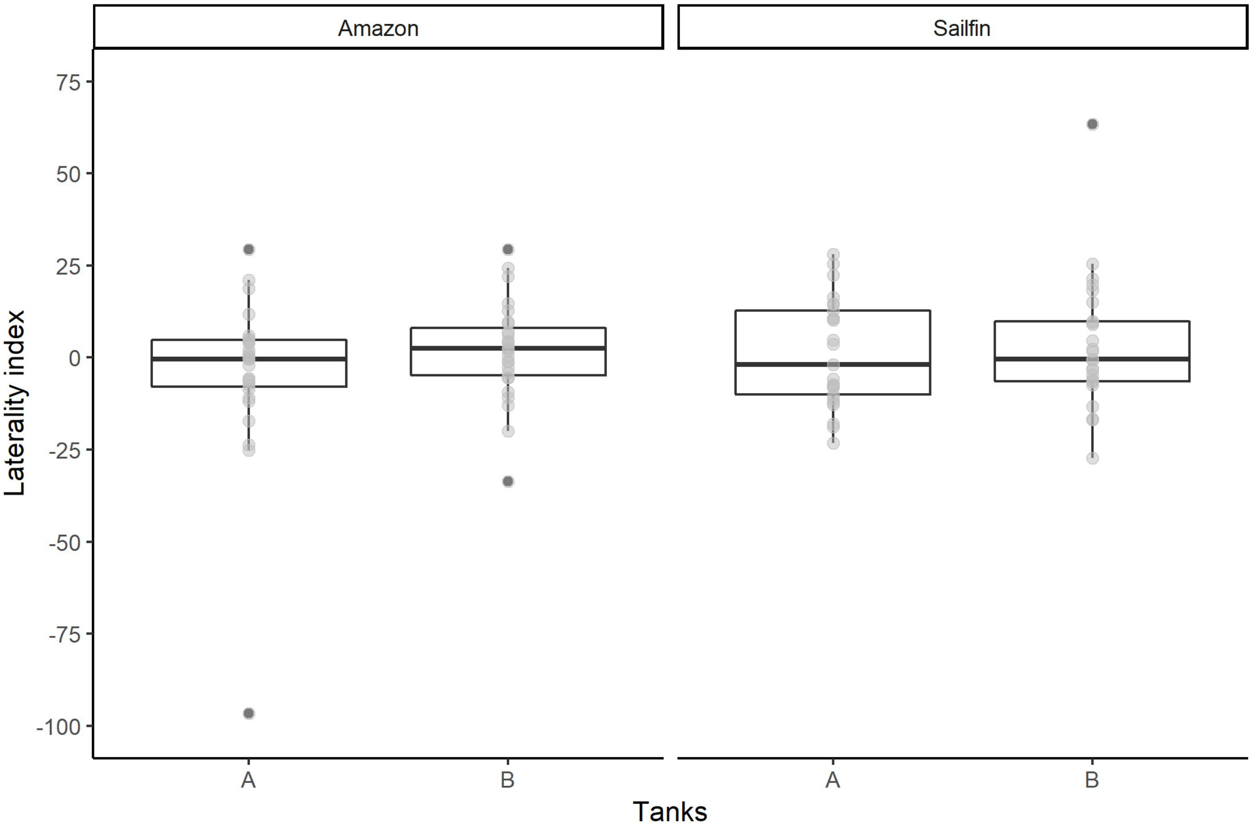

FIGURE 2 | Boxplots (— , median; □ , 25–75th percentile; outliers in dark gray; raw data in light gray) of laterality index scores across tanks for both species. Eye use did not significantly differ between tank A and B for either Amazon mollies or sailfin mollies (paired *t*-tests).

lateralized, regardless of direction. Lastly, we performed a two-tailed *F*-test on index scores to determine if variation in eye preference for sailfin mollies was greater or less than variation in Amazon mollies.

RESULTS

All raw data can be found in the **Supplementary Material**. Normality was confirmed using a Kolmogorov-Smirnov test for both species (sailfin: D = 0.19, *p* = 0.28; Amazon: D = 0.23, *p* = 0.10). We found no clear difference in eye laterality between tanks A and B for each species (paired *t*-test: sailfin: df = 24, *p* = 0.61, 95% CI -11.67—7.00; Amazon: df = 26, *p* = 0.24, 95% CI -16.24—4.23; **Figure 2**). Therefore, we used the laterality index score calculated from combined A and B video results for all subsequent analyses (excluding Pearson's correlation).

Ocular preference within species was determined as a significant deviation from a zero laterality index score. Neither sailfin mollies nor Amazon mollies showed a significant eye bias during mirror image scrutiny (one-sample two-tailed *t*-test: sailfin: *p* = 0.33, mean = 2.40, 95% CI −2.62—7.42; Amazon: *p* = 0.63, mean = −1.21, 95% CI −6.34—3.92; **Figure 3**). There was also no significant correlation between eye use in tank A and tank B for either species (Pearson's correlation coefficient: sailfin: *R* = 0.05, *p* = 0.81; Amazon: *R* = -0.005, *p* = 0.98). Sailfin mollies did not clearly differ in mean eye preference when compared to Amazon mollies (two-tailed *t*-test: df = 49.99, *p* = 0.30, 95% CI −3.39—10.61). Additionally, there was no clear difference in the degree of lateralization between Amazon and sailfin mollies (two-sided *t*-test: df = 49.70, *p* = 0.81, 95% CI −4.55—5.80). Lastly, we did not find a significant difference in variation between the species (*F*-test: *F* = 0.88, *p* = 0.76; **Figure 4**).

DISCUSSION

Asexual species are often touted as evolutionary dead ends due to mutation accumulation and the lack of recombination (Muller, 1964; Maynard Smith, 1978; Lynch et al., 1993; Lynch and Gabriel, 2006), yet some asexual species persist despite these costs (Heethoff et al., 2009; Schön et al., 2009; Bi and Bogart, 2010; Fradin et al., 2017; Warren et al., 2018). The asexual Amazon molly lives in direct competition with its sexual counterpart, the sailfin molly and they appear to be ecological equivalents in morphology, physiology, and ecology (**Table 1**). Our investigation into cognitive behavior, specifically visual lateralization, shows no significant difference in average

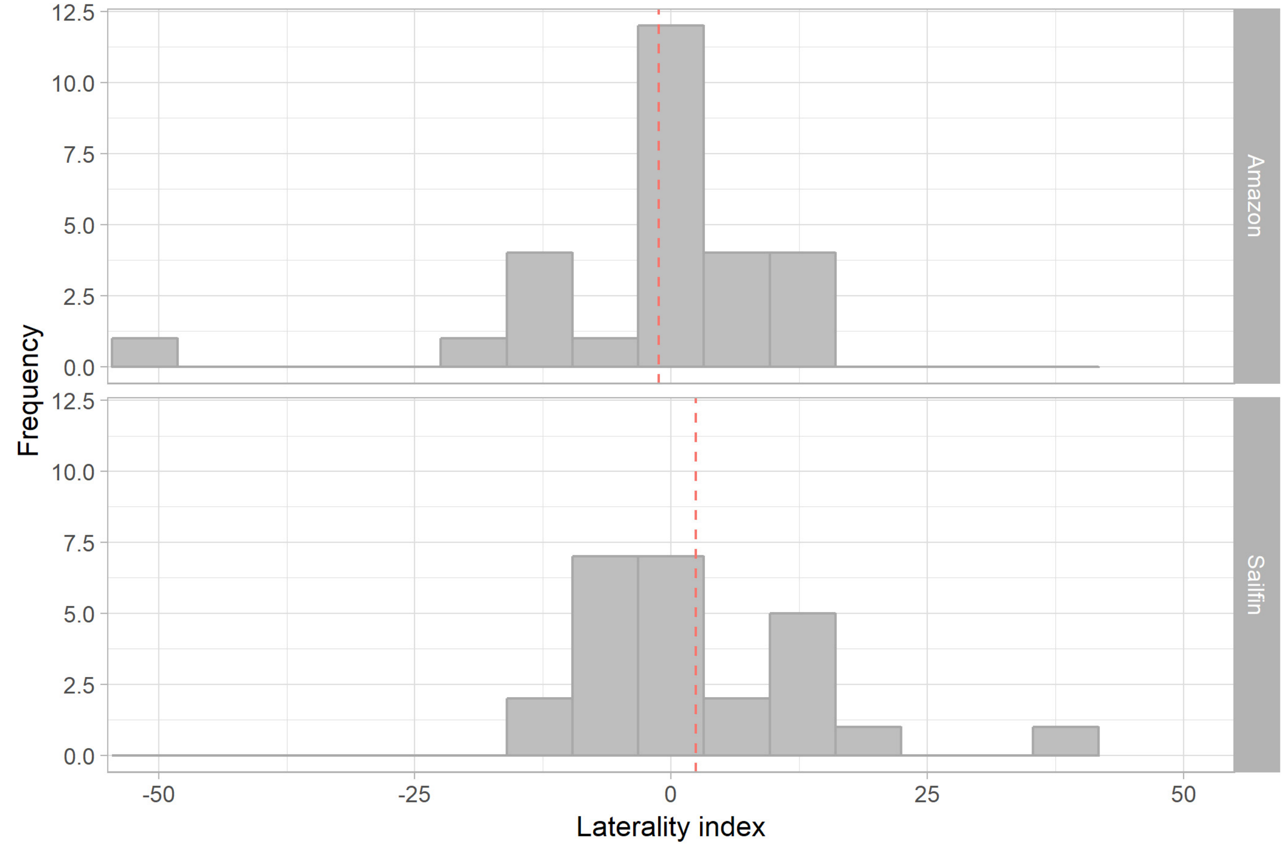

FIGURE 3 | Frequency of laterality index scores among Amazon and sailfin mollies. The red dashed line represents the group mean. Variation in laterality index scores was not clearly different between Amazon and sailfin mollies.

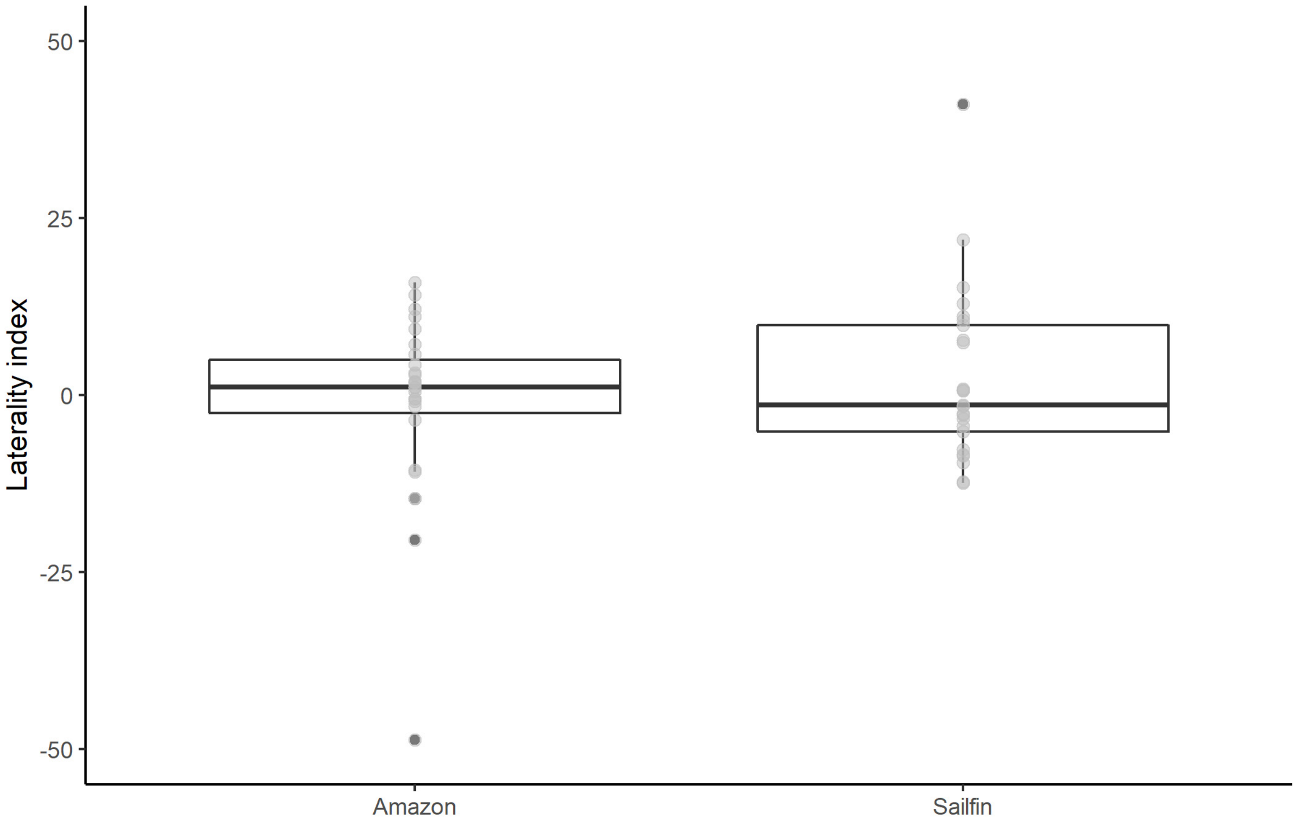

FIGURE 4 | Boxplots (, median; □, 25–75th percentile; outliers in dark gray; raw data in light gray) of laterality index scores for Amazon and sailfin mollies. Neither species exhibited a significant eye bias (one-sample *t*-tests), nor did the species clearly differ in their average laterality index score (two-sample *t*-tests).

performance or variation between the Amazon molly and sailfin molly. While our results showing similar average performance are in line with previous comparative studies between these species, our variation results highlight a potential mechanism for the persistence of the asexual Amazon molly.

Neither species clearly expressed visual lateralization in this study. Organisms with laterally positioned eyes discriminate conflicting stimuli through separate but parallel processing (Rogers, 2000), increasing their ability to distinguish between two stimuli [food vs. non-food (Vallortigara et al., 1998; Güntürkün et al., 2000) and food vs. predator (Güntürkün et al., 2000; Rogers, 2000; Rogers et al., 2004)]. Furthermore, mixed visual lateralization in schooling fish determine the fishes' position within the school and overall school cohesion (Dadda and Bisazza, 2006; Bibost and Brown, 2013). A number of studies using poeciliid species found a clear eye bias, with female poeciliids commonly exhibiting a left-eye bias for conspecific stimuli and a right-eye bias for predator or male stimuli (Bisazza et al., 1997a,b, 1998, 1999; De Santi et al., 2001; Fuss et al., 2019). Therefore, the type of stimulus used, particularly the social stimulus, can affect lateralization. Fuss et al. (2019) found a statistically significant left-eye bias for female sailfin mollies and Amazon mollies when viewing a female group stimulus, although no such bias when viewing a single female. While most fish are not thought to recognize their own reflection (Kohda et al., 2019), the lack of visual bias and similar variation in both species of our study could indicate a lack of motivation to perform lateralization for the mirror reflection meant to represent a single female. The lack of motivation to a mirror image may be specific to the species used here, as previous studies using the mirror image scrutiny setup obtained lateralized responses from other fish species (Sovrano et al., 1999; De Santi et al., 2001). Further testing with different stimuli might show the degree to which the social environment influences visual lateralization for both species. Predation can also influence the strength and direction of lateralization: high-predation environments induced a strong lateralized response to novel and predator stimuli in the poeciliid *Brachyraphis episcopi* but no clear lateralization appeared for *B. episcopi* from low-predation environments (Brown et al., 2004). While predatory species are present at our San Marcos site (personal observation), the degree of predation and its influences on visual lateralization in this population are unknown.

Previous comparative studies, including one on visual lateralization (Fuss et al., 2019), focus on the average performance between the Amazon molly and sailfin molly. Here we also focus on the variation in behavior; variation in asexual populations may indicate a potential mechanism to circumvent the classic costs of this reproductive style and thus perpetuate their coexistence with a sexual species. The Amazon and sailfin mollies of our study exhibited similar levels of variation in the visual lateralization task. One way to obtain variation in an asexual population is through clonal lineages (Schartl et al., 1995b; Stöck et al., 2010; Alberici da Barbiano et al., 2013; Warren et al., 2018). Populations of Amazon mollies may contain multiple clonal lineages (Lampert et al., 2006; Stöck et al., 2010; Gösser et al., 2019); it is currently unknown how many clonal lineages existed or developed since the introduction of Amazon mollies to our study site in the 1950s (Schlupp et al., 2002). However, it is possible that our sample contains individuals from multiple clonal lineages thus leading to higher-than-expected levels of variation. Two invertebrate studies investigating morphological variation similarly found equal or higher levels of variation in clonal populations compared to the sexual populations (Oliver and Herrin, 1976; Atchley, 1977). However, neither study addressed the effects of multiple clonal lineages on the variation seen within these wild populations. Studies that account for clonal type or lineage find that the asexual species contain less phenotypic variation than the sexual species, and variation within the asexual species is partitioned among clonal lines (Parker, 1979; Vrijenhoek, 1984; Cullum, 2000; however see Doeringsfeld et al., 2004 and discussion below). Further investigation into the clonal lineages potentially present in our sample is underway.

Asexual populations can also achieve variation via plastic responses to environmental characteristics. Doeringsfeld et al. (2004) found similar levels of morphological variation in one clonal lineage (i.e., one genotype) of asexual dace (*Phoxinus eos-neogaeus*) as the sexual host species. They attributed this finding to the plasticity of the asexual genome, thus allowing the asexual species to cohabit the broad range of their hosts. Indeed, further work on this asexual complex showing epigenetic variation associated with pH tolerance rather than genetic variation emphasizes the role of environmentally induced plasticity in the maintenance of asexual-sexual systems (Massicotte and Angers, 2012). The similar levels of variation and lack of repeatability between trials may be the result of plastic responses to the rearing or testing environment; a previous study with Amazon mollies highlights the potential of the rearing environment to invoke plasticity in behavior (Bierbach et al., 2017). It is clear future work investigating the coexistence of the Amazon molly with its sexual host must include the variance brought about by clonal lineages and environmentally induced plasticity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Texas Institutional Animal Care and Use Committees.

AUTHOR CONTRIBUTIONS

AC and MR conceived and designed the study and drafted the manuscript. AC collected field specimen, executed experiments, and performed the data analysis. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.605943/full#supplementary-material

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Artificial Light at Night Alters the Physiology and Behavior of Western Mosquitofish (*Gambusia affinis*)

Krystie A. Miner[1], Mar Huertas[1], Andrea S. Aspbury[1]* and Caitlin R. Gabor[1,2]

[1] Population and Conservation Biology Group, Department of Biology, Texas State University, San Marcos, TX, United States, [2] The Xiphophorus Genetic Stock Center, Texas State University, San Marcos, TX, United States

Human population growth and its associated effects on the environment contribute to the rapid decrease of biodiversity worldwide. Artificial light at night (ALAN) is an anthropogenic pollutant that is increasing with the spread of urbanization and may contribute to biodiversity declines. ALAN alters the migration patterns of birds, communication in frogs, and impacts reproduction, behavior, and physiology of multiple other taxa. However, most of the studies on ALAN are based on terrestrial systems, and overall, the effects of ALAN on freshwater organisms are poorly understood. We investigated how ALAN affects the physiology, behavior, and reproduction of a widespread, tolerant species of freshwater fish. *Gambusia affinis* are small livebearing fish often found in urban streams. We exposed groups of female *G. affinis* to either a natural light cycle or a constant 24-h light cycle (ALAN) in the laboratory for 60 days. In another experiment, we exposed female *G. affinis* to the same treatments in outdoor mesocosms for 32 days. We found that exposure to ALAN lowered glucose levels in the brain and decreased swimming activity, but had no effect on cortisol release rates, reproduction, survival, or growth. This research is strengthened by measuring multiple metrics in response to ALAN and by incorporating both a field and laboratory component which confirm similar results. These results suggest that this tolerant species of fish may behaviorally adjust to ALAN rather than modulate their endocrine stress response.

Keywords: *Gambusia affinis*, artificial light at night, corticosterone, urban stream syndrome, poecilidae, fish physiology

Edited by:
Jordi Figuerola,
Estación Biológica de Doñana (EBD),
Spain

Reviewed by:
Emily Fobert,
The University of Melbourne, Australia
Michael J. Pauers,
Milwaukee Public Museum,
United States

***Correspondence:**
Andrea S. Aspbury
aspbury@txstate.edu

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INTRODUCTION

Anthropogenic disturbances contribute to habitat loss and alteration, climate change, and increased exploitation of natural resources by humans (Dudgeon et al., 2006; Vörösmarty et al., 2010; Ellis, 2011; Helm et al., 2013). These disturbances are associated with shifts in water quality, water flow, seasonal timing, sound, and light pollution (Jenkins, 2003; Allan, 2004; Barbier, 2012; Davies et al., 2014; Swaddle et al., 2015; Shannon et al., 2016; Buxton et al., 2017; Sordello et al., 2019). Artificial light at night (ALAN) is one form of anthropogenic pollution that alters the natural light and dark cycle in an ecosystem (Swaddle et al., 2015). Light plays a key role in the ecology of organisms as a source of energy and information, a regulator of circadian rhythms, and as a cue for communication, navigation, and orientation (Gaston et al., 2012, 2017). As worldwide urbanization increases, ALAN is becoming so widespread that 83% of the global human population lives under light-polluted skies, and 40% lives in areas that are continually illuminated due to ALAN (Cinzano et al., 2001; Swaddle et al., 2015; Falchi et al., 2016). Hölker et al. (2010) showed that ALAN is increasing alongside urbanization at an average annual rate of 6%.

Global freshwater systems support 9.5% of all extant described species, and these species' populations are declining at rates exceeding those of tropical rainforests, primarily due to anthropogenic stressors (Ricciardi and Rasmussen, 1999; Xenopoulos et al., 2005; Dudgeon et al., 2006; Balian et al., 2008). Moreover, 90% of the human population lives within 10 km of a freshwater body and 50% within 3 km, making freshwater areas the most impacted by anthropogenic disturbances, such as ALAN (Dudgeon et al., 2006; Kummu et al., 2011; Venohr et al., 2018). Aquatic organisms are affected by ALAN because they are influenced by photoperiod across life history stages, including reproduction, growth, development, and activity (Downing and Litvak, 2002; Mehner, 2012). ALAN has detrimental effects on behavior, reproduction, foraging, orientation, predation, physiology, and migration in various taxa (Longcore and Rich, 2004; Navara and Nelson, 2007; Hölker et al., 2010; Ouyang et al., 2011; Gaston et al., 2013, 2014, 2015; Davies et al., 2014), yet most studies of ALAN focus on terrestrial taxa. Of the limited studies of ALAN on aquatic organisms, there is a knowledge gap regarding the consequences of ALAN on behavior, physiology, and reproduction in aquatic species (Depledge et al., 2010; Perkin et al., 2011; Jechow and Holker, 2019).

Because ALAN can affect circadian and circannual rhythms, physiology and behavior can be altered by exposure to ALAN. Individuals of some fish species increase their activity, modify their shoaling behavior, and spend more time in open (riskier) areas under ALAN which can alter foraging and increase their risk of predation (Becker et al., 2013; Foster et al., 2016; Kurvers et al., 2018; Sanders and Gaston, 2018; Czarnecka et al., 2019). ALAN is also associated with increased blood glucose in goldfish, *Carassius aurarus* (Ryu et al., 2019), and impaired melatonin rhythms in European perch, *Perca fluviatilis*. Further, reproductive hormones (17β-estradiol and 11-ketotestosterone), along with mRNA expression of gonadotropins, were reduced in fish exposed to ALAN (Brüning et al., 2018). Together, these changes may alter survival and reproduction of fish.

Given these effects of ALAN on fish behavior and physiology, it is likely that ALAN can also affect the fish stress response. Organismal response to stressors, such as ALAN, can be quantified by measuring cortisol release rates, the primary glucocorticoid (GC) in fishes (Idler and Truscott, 1972) that is released in response to a potential stressor (Hopkins et al., 1997; Dickens and Romero, 2013; King et al., 2016**;** Gabor et al., 2018). When a fish encounters a stressful event, its hypothalamic-pituitary-interrenal (HPI) axis is activated, resulting in a release of cortisol into the bloodstream that induces a variable response in target organs, thus altering individual behavior and physiology to maintain homeostasis (Wendelaar Bonga, 1997; Romero, 2004). When faced with an acute stressor, this mechanism is adaptive, but prolonged exposure to a stressor can have harmful, long-term, and even fatal effects (Sapolsky et al., 2000; Romero, 2004). Elevated GCs are linked to lower survival, reproduction, and dysregulation of immune responses (Bonier et al., 2009); however, stress may have a bidirectional effect and can also enhance immunity, growth, and reproductive output (Dhabhar et al., 1995; Dhabhar and McEwen, 1996; Dhabhar and Viswanathan, 2005; Viswanathan et al., 2005; Thawley and Kolbe, 2020). Because GCs are also involved in altering other physiological processes (Sapolsky et al., 2000; Le et al., 2005; Dhabhar, 2009), if ALAN causes changes to cortisol release rates, it can indirectly affect downstream traits such as behavior, growth, and reproduction. Alternatively, organisms may behaviorally adjust to the perturbation of ALAN rather than modulate their regulation of the HPI axis.

Here, we propose to quantify the consequences of ALAN on physiology, behavior, and reproduction of Western mosquitofish (*Gambusia affinis*). These are small, livebearing fish native to eastern North America, but introduced globally, and found in a wide variety of environments, including urban streams (Lloyd et al., 1986; Hubbs, 2000; Pyke, 2005; Page and Burr, 2011). This species is generally found in shallow waters and forages near the surface primarily at morning and dusk, though sometimes during the day (Hess and Tarzwell, 1942; Belk and Lydeard, 1994). Unlike egg-laying species of fish, ALAN may affect gravid females and their offspring while *in utero*, which could alter offspring survival. Mosquitofish are considered tolerant due to their success as invaders and their ability to live in adverse conditions which could suggest that they will be less adversely affected by ALAN than other less tolerant species (Cherry et al., 1976; Lloyd et al., 1986; Pyke, 2008). We used laboratory and mesocosm experimental approaches to test the hypotheses that ALAN alters the physiology, behavior, and reproduction of female *G. affinis*. We performed the mesocosm experiment second to validate our laboratory experimental findings and test these questions in an ecological context.

EXPERIMENT 1: CONSEQUENCES OF EXPOSURE TO ALAN IN AQUARIA ON PHYSIOLOGICAL STRESS, BEHAVIOR, AND FITNESS CORRELATES OF FEMALE MOSQUITOFISH

Materials and Methods

Fish Collection and Maintenance

We collected *Gambusia affinis* from the Blanco River in Hays County, Texas during the breeding season (13 April 2018 and 13 June 2018) with a seine and transported them to the laboratory. We placed lux meters (Dr. Meter, model LX1330B) at the level of the water at the collection site at night and found that it was not exposed to ALAN. We placed fish in 38 L tanks and fed them *ad libitum* daily with ISO flake food (TetraMin®) and supplemented them three times a week with brine shrimp. One week after collection, we haphazardly placed 80 mature females individually into semi-clear 0.95 L cylindrical plastic containers fitted (stacked) into another 0.35 L

FIGURE 1 | Example of container setup for ALAN and control groups in the laboratory. Each container held one female *Gambusia affinis* in the top portion and a bottom container into which offspring could swim to escape cannibalism. Smaller holes in the top and bottom allowed for exchange of chemical cues between female focal fish.

cylindrical plastic container (**Figure 1**). We made small holes at the bottom of the upper container to allow any offspring that were born to move into the lower container (but not the adults) and avoid maternal cannibalism (following Cazan and Klerks, 2015). Each of the upper containers also had small holes on the sides to facilitate flow of conspecific cues and reduce stress of solitary confinement (personal observation) while simultaneously allowing for individual sampling. Eight of the containers were placed together in a 38 L tank filled with 28.5 L of dechlorinated tap water and gravel across the bottom.

ALAN Exposure Design and Reproduction

We conducted the experiment starting on three separate dates (21 April, 23 June, and 5 September) in the same space and using the same design each time. For each of these date "blocks" we exposed five tanks (each with the eight containers; $N = 40$ females), to a control treatment of 14:10 h light: dark cycle and five other tanks ($N = 40$ females) to the experimental treatment (ALAN) of 24:0 h light: dark cycle (14 h simulated daylight: 10 h ALAN) for 50–60 days, for a total of 15 replicates per treatment. We simulated daylight with a full spectrum white fluorescent light (MingDak) at 880 lux and ALAN using white LED lights (Utilitech) at 120 lux. Full daylight levels can reach up to 25,000 lux with illuminances up to 100,000 lux in direct sunlight (Blume et al., 2019). Light levels at night in Hays County, Texas ranged from 16 lux at dim streetlamps to 230 lux at flood lights, therefore this nighttime lux level was ecologically relevant. We hung all lights 51 cm above the tanks. After the first block, we realized that some measurements of ALAN in Hays County were higher than the lux values used in our original ALAN treatment. Consequently, we increased the daylight to 2,380 lux and ALAN to 246 lux for the next two blocks. Our measures represent the level of light reaching the water where organisms can be found because lights from bridges, roads, boardwalks, and homes next to water sources are common. Indeed, organisms in close proximity to the light source can experience light intensities greater than 100 lux (Bolton et al., 2017).

We monitored the containers daily for offspring. Females generally give live birth to all offspring in a brood within a short period as this species do not show superfetation (Turner, 1937), therefore when present, we recorded the date, number of offspring, and offspring survival (alive vs. dead) in each brood. We changed the tank water by siphoning out 3/4 of the water from the bottom (to remove feces) and replacing it with dechlorinated tap water every 2 weeks.

Water-Borne Cortisol Collection and Growth

For the first two time blocks, we collected water-borne hormones 2 days after the females had been placed in their experimental container (but light exposure had not yet begun) and thus had an opportunity to acclimate to the experimental set-up (day 0), then again on day 7, 30, and the last day of the block. The standardized methods we used for hormone collection followed Blake et al. (2014). We placed each fish in a LDPE plastic insert in a 250 ml glass beaker with 60 ml of dechlorinated water for 30 min. After that time we recorded mass (g) and standard length (SL: mm) of each fish, then returned the fish to its original container. Each hormone collection event began at 0900 h to control for natural diel fluctuations of cortisol release rates. We cleaned beakers and inserts with 95% ethanol and rinsed them with deionized water before use and handled them with non-powdered gloves to prevent contamination. Scott et al. (2008) tested this non-invasive method for establishing cortisol release rates from fish and Blake et al. (2014) validated this method of analyzing cortisol release rates from water-borne hormones using *Gambusia geiseri,* a close relative of *G. affinis.* After the last cortisol release sample was taken on the last day of the block, we assessed whether the fish were chronically stressed (as indicated by responsiveness of the HPI axis) by agitating the fish while collecting their hormones. This allowed us to measure cortisol release rates in response to an acute stressor. For this test, we placed fish into the same set-up as above and shook them for 1 min every other minute for a total of 30 min.

Shoaling Behavior

We removed $N = 22$ fish from both treatments after day 60 and randomly placed them into plastic containers (33.02 cm × 20.32 cm × 11.43 cm) in groups of 4, a shoal size used in previous studies with Poeciliidae (Tobler and Schlupp, 2008). We filled containers (lined on the outside with white paper towels to create contrast and track the fish easier) with 5 L of dechlorinated tap water. Groups acclimated in the containers for 30 min. We then recorded the groups under their respective treatment for ∼24 h with webcams mounted above each container using ManyCam software. Videos were taken over 2 days for logistical purposes. We analyzed videos using EthoVision XT (Noldus) and recorded the average distance moved (cm) among all four fish, time resting, and shoaling (time spent within 2 cm of each other) during day (14 h) and night hours (10 h).

Hiding Behavior

For this experiment we tested $N = 29$ fish which were randomly selected from both treatments from the second block of the experiment. We set up 18 L tanks with one opaque half cylinder of PVC (as a hiding place) and one clear half cylinder of PVC (which controlled for the preference to hide in a smaller place vs. out of view) which were randomly assigned to each side of the tank. We filled tanks with 1.3 L of treated tap water (just enough to cover the half cylinders). At night, we removed fish from the ALAN treatment (after day 50) and individually placed them into the testing tank located under an ALAN light and allowed the fish to acclimate under a clear, plastic 1 L container for 10 min. We covered the sides of the tanks with black paper in case the fish could still be distracted from their surroundings at night. Following acclimation, we removed the clear container and began recording with ManyCam software for 10 min. We analyzed videos using EthoVision XT (Noldus) to estimate time (s) spent "hiding" under either half cylinder and time (s) spent resting.

Cortisol Extraction, Reconstitution, and Enzyme Immunoassays

We stored water-borne hormone samples at –20°C until thawed for extractions following methods of Gabor et al. (2016). We pulled water samples through C18 solid phase extraction (SPE) columns (SepPak Vac3 cc/500 mg; Waters, Inc., Milford, MA, United States) using Tygon tubing under vacuum pressure. SPE columns were primed with 4 ml of methanol followed by 4 ml of distilled water. We then eluted columns with 4 ml methanol into borosilicate vials then evaporated the methanol by placing the vials in a 37°C water bath while under nitrogen gas. We resuspended the residue in 5% ethanol (95% lab grade) and 95% enzyme immunoassays (EIA) buffer (Cayman Chemical, Inc) to a total volume of 720 μl based on dilutions from Blake et al. (2014).

We measured cortisol release rates in duplicate for all samples using EIA kits (No. 500360, Cayman Chemical Company, Inc.). Sample absorbances were read on a spectrophotometer plate reader at 405 nm (BioTek 800XS). To obtain cortisol release rates, we multiplied cortisol concentrations (pg/ml) by the final resuspension volume (0.720 ml), divided by the SL (mm) of the individual, and 0.5 h for a final unit of pg/mm/h. Inter-plate variation was 12.35% for the laboratory experiment (five plates) while intra-plate variation ranged from 0.39 to 14.88%. For the mesocosm experiment (six plates), inter-plate variation was 11.53% and intra-plate variation ranged from 0.45 to 6.95%.

Statistical Analyses

In the laboratory experiment, we used a generalized linear mixed model (GLMM) with natural log transformed cortisol release rates (standardized for standard length −pg/mm/h) as the dependent variable, with treatment and day as the fixed effects and ID as the random effect to account for repeated measures. We did not include the blocking effect of increasing lux, as adding this variable did not significantly affect the model results. When there were significant ($p < 0.05$) fixed effects differences, we used *post hoc* Tukey's HSD comparisons. To explore effects of ALAN on offspring survival we used a chi-square test. We used GLMM to analyze changes in mass and SL over time with treatment and time as fixed effects and individual as the random effect to account for repeated measures. To determine the effects of ALAN on shoaling behavior, we used a repeated measure ANOVA. Due to the quality of videos recorded, we were unable to analyze each shoaling video we recorded, resulting in an uneven sample size. For all other behavioral analyses, we used GLMM with SL and treatment as model effects. We used JMP Pro 14.0.0 (SAS Institute, Inc.) for all analyses.

Results

Reproduction, Growth, and Cortisol

Offspring survival did not differ significantly between treatments ($\chi^2_1 = 3.07, N = 32, p = 0.08$). Because so few fish had offspring, we did not statistically compare offspring number but the mean for the control was ($N = 17$, Mean ± SE = 13 ± 1.65) and for ALAN was ($N = 15$, Mean ± SE = 11.33 ± 1.26).

In the laboratory experiment, we found no significant differences in cortisol release rates between fish in the control vs. ALAN treatment (GLMM: treatment × day: $F_{4,155} = 1.44$, $p = 0.224$; **Table 1** and **Figure 2A**). Fish had significantly higher agitation cortisol release rates compared to baseline after 60 days irrespective of treatment (day: $F_{4,155} = 103.32$, $p < 0.0001$; **Figure 2A**), indicating that the fish from both treatments could mount a stress response. There was no significant effect of ALAN on mass (GLMM: treatment × time: $F_{3,460} = 0.30, p = 0.824$) or SL ($F_{3,460} = 0.25, p = 0.86$) over the duration of the experiment. There were no significant random effects of individual fish (Wald p-value $= 0.78$).

Shoaling Behavior

After being in the laboratory experiment for 59 days, female *G. affinis* in the ALAN treatment spent significantly less time shoaling (s) during the day than the control treatment (repeated measures ANOVA: time × treatment: $F_{1,18} = 5.92$, $p = 0.026$; **Figure 3**). Fish from both treatments moved a significantly greater distance (cm) during the day than at night (time: $F_{1,18} = 8.05, p = 0.011$).

TABLE 1 | Parameter estimates ± SE from the generalized linear mixed model for effects of ALAN on cortisol release rates of female *G. affinis* in the laboratory.

	Mean ± SE	*t*-value	*df*	*p*-value	Lower 95% CI	Upper 95% CI
Intercept	3.69 ± 0.06	60.58	99.42	**<0.0001**	3.56	3.81
Control	0.03 ± 0.06	0.42	99.42	0.68	−0.10	0.15
Day 7	−0.64 ± 0.10	−6.10	99.42	**<0.0001**	−0.85	−0.43
Day 30	−0.88 ± 0.10	−8.30	99.42	**<0.0001**	−1.08	−0.67
Day 60	−**0**.54 ± 0.10	−5.12	99.42	**<0.0001**	−0.75	−0.33
Control*day 7	−0.13 ± 0.10	−1.26	99.42	0.21	−0.34	0.08
Control*day 30	−0.13 ± 0.10	−1.26	99.42	0.21	−0.34	0.08
Control*day 60	0.14 ± 0.10	1.34	99.42	0.18	−0.07	0.35

*"Treatment" and "day" were included as fixed effects and fish "ID" was included as the random effect. Statistically significant values are in bold. *indicates interaction effects.*

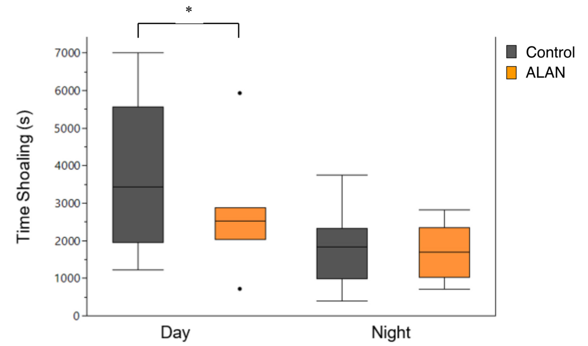

FIGURE 3 | Time spent shoaling (s) by female *G. affinis* in the control ($N = 15$) and ALAN ($N = 7$) treatments in the day and night in the laboratory. Box plots indicate median, range, and first and third quartiles. Dots indicate outliers. Asterisk indicates a significant difference ($p < 0.05$).

Hiding Behavior

In the laboratory hiding experiment, after exposure to ALAN, fish spent significantly more time resting (LM: treatment: $F_{1,22} = 6.93$, $p = 0.015$; **Table 2** and **Figure 4**) than fish in the control treatment. There was no significant effect of SL on time resting. There was no significant effect of ALAN on time spent hiding at night ($F_{1,22} = 0.02$, $p = 0.896$).

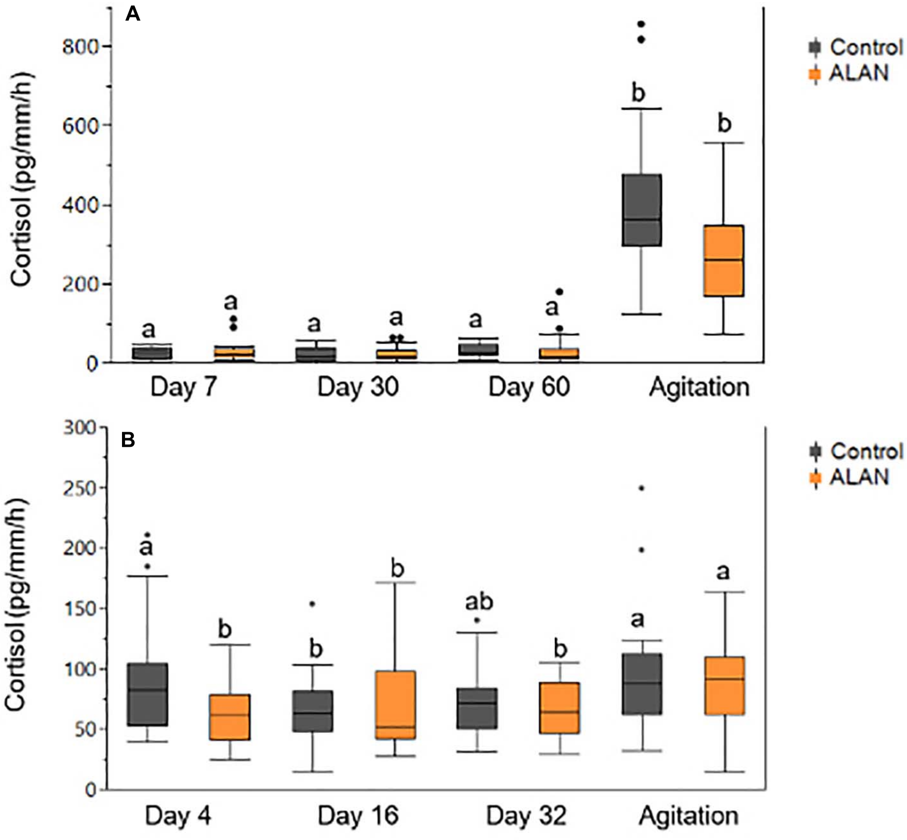

FIGURE 2 | Cortisol release rates (pg/mm/h) obtained from female *G. affinis* after 30 min of baseline and agitation collection in the **(A)** laboratory and **(B)** mesocosm experiment. Agitation measures were obtained on day 60 in the lab and day 32 in mesocosms. One high agitation value was excluded from the laboratory ALAN group to increase the spread of the figure. Different lowercase letters indicate significant differences ($p < 0.05$) among treatment groups from Tukey's HSD comparisons. Box plots indicate median, range, and first and third quartiles. Dots indicate outliers.

TABLE 2 | Results from the general linear model testing the effects of ALAN on behavior of female *G. affinis* in the laboratory.

		Estimate ± SE	*t*-value	*df*	*P*-value	Lower 95% CI	Upper 95% CI
Resting	Intercept	246.19 ± 167.56	1.47	22	0.16	−101.30	593.68
	SL (mm)	0.03 ± 4.84	0.01	22	0.99	−10.00	10.06
	Control	−52.17 ± 19.82	−2.63	22	**0.02**	−93.27	−11.07
	SL*control	−4.68 ± 4.84	−0.97	22	0.34	−14.71	5.34
Hiding	Intercept	−50.34 ± 140.14	−0.36	22	0.72	−340.98	240.30
	SL (mm)	2.86 ± 4.04	0.71	22	0.49	−5.52	11.25
	Control	2.19 ± 16.58	0.13	22	0.90	−32.18	36.57
	SL*control	−0.39 ± 4.04	−0.10	22	0.92	−8.78	7.99

*"Treatment" and "SL" were included as fixed effects. *Indicates interaction effects. Statistically significant values are in bold.*

EXPERIMENT 2: CONSEQUENCES OF ALAN IN MESOCOSMS ON PHYSIOLOGICAL STRESS AND BEHAVIOR OF FEMALE MOSQUITOFISH

Materials and Methods

Mesocosms set-up

We constructed 16 mesocosms using 62.45 L clear, #5 (polypropylene) plastic containers with six 5 cm holes drilled in the sides toward the top and covered with mesh for overflow water drainage while preventing fish from escaping. We cut out the center plastic of the lids and replaced it with mesh to allow light to pass through. We then placed the mesocosms outdoors underneath 60% shade cloth. On 25 June 2019, we added 48 L of water to each mesocosm. The following day we added 4 L of sediment collected from the Blanco River in Hays County, Texas, and 1 L of pond water. We added 1 L aliquots of zooplankton to each mesocosm on 29 June 2019 and 2 July 2019. We also added 16 pieces of ceramic bio media (11 BrightWater Bio Media® and five Fluval BioMax®) to each mesocosm to provide a substrate for microorganisms. Additionally, we added one sponge filter to each mesocosm to prevent the buildup of ammonia and nitrates. We added two artificial breeder plants (Penn Plax Aquarium Breeding Grass) to each mesocosm for habitat cover and to provide refuge for any offspring produced. We recorded water quality parameters (ammonia, nitrites, nitrates, total dissolved solids, temperature, conductivity, pH, and salinity) from each mesocosm twice a week. We added dechlorinated tap water to the mesocosms when it was lower than the drainage holes.

ALAN Experimental Design

From 19 June–21 June 2019 we collected 240 *G. affinis* from the Blanco River in Hays County, Texas using dipnets and seines, transported them to the lab, and fed them ISO flake food (TetraMin) daily. On 27 June 2019 we marked 64 females with white, red, orange, or yellow elastomer tags ($N = 16$ per color). On 3 July 2019, after mesocosms were established, we haphazardly selected five tagged females (for repeated measures of water-borne hormones (see below), eight non-tagged females (to provide adequate numbers of conspecifics since individuals would be removed throughout the experiment), and two males (totaling 15 fish) for placement into each mesocosm. We hung two artificial night lights (Onforu 35 W LED Flood Lights; 3,300 lumens 5,000 K) 52 cm above half of the mesocosms (below the shade cloth). Five days after placing fish into mesocosms, we turned on the artificial night lights on the experimental (ALAN) side (day 0) exposing eight mesocosms ($N = 120$ fish) to 24 h of light. These lights were on from 2000–0600 h and ranged from 260–280 lux at the top of the mesocosms and 155–175 lux at the surface of the water. We measured lux with a digital lux meter (Dr. Meter, model LX1330B). To prevent light from reaching the control mesocosms at night we hung a black plastic curtain in between treatment blocks. Ammonia and nitrites were not detected at any point in the mesocosms.

Water-Borne Cortisol Collection, Growth, Survival, and Glucose

We collected water samples to measure cortisol release rates from each tagged female on day 4 to capture the potential effect of treatment while giving enough time to acclimate to the mesocosms. Beginning at 0900 h we placed each fish in a LDPE plastic insert in a 250 ml glass beaker with 60 ml of spring water for 30 min (following methods above). After 30 min we collected the water sample then measured and recorded the mass (g) and standard length (SL; mm) of each fish, then returned the fish

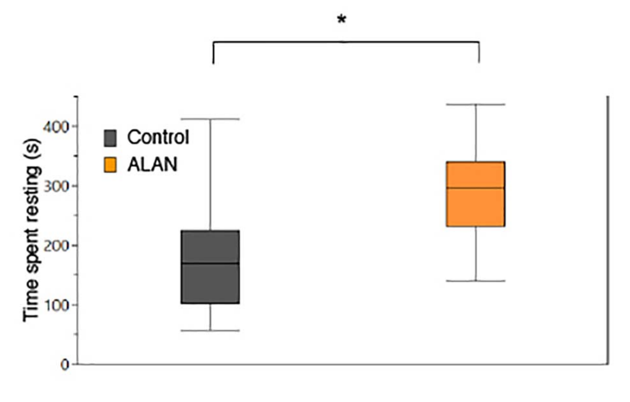

FIGURE 4 | Mean time spent resting (s) by female *G. affinis* ($N = 29$) in the laboratory. Asterisk indicates a significant difference ($p < 0.05$). Box plots indicate median, range, and first and third quartiles.

into its original mesocosm. Using the same methods, we collected baseline cortisol release rates from the same tagged individuals on days 16 and 32. Immediately after baseline cortisol release rate collection on day 32, we collected water samples to measure cortisol release rates when under an agitation treatment to test for an acute stress response (following the lab protocol above). We extracted and analyzed cortisol release rates following the same protocol as in experiment 1. On day 32 we euthanized fish in an ice slurry for 30 min then dissected 5–6 non-tagged females per mesocosm to obtain the brain, muscle, and liver tissues which were frozen until glucose analysis.

Hiding Behavior

We repeated the hiding behavioral experiment following the methods from experiment 1 (above) using tagged females from each mesocosm but performed the experiment during the day and with a 5 min acclimation period. Data were collected during the day instead of at night to examine if ALAN had effects that could be observed in daytime hours.

Glucose Extraction, Reconstitution, and Colorimetric Assays

For each mesocosm, we combined common tissue types (brain, muscle, and liver) from six fish and weighed the tissues (average weight ± SE) before adding them 400 μl of 100% ethyl alcohol. Samples where homogenized with an IKA Ultra-Turrax T25 and then centrifuged for 10 min at 10,000 rpm at 4°C. We removed the supernatant of each sample and transferred it to a new Eppendorf tube which was then placed in a vacufuge overnight (Eppendorf Vacufuge plus). We reconstituted the samples using 100 μl of 1 M phosphate buffer saline (PBS) and stored at −80°C.

We measured glucose levels following the colorimetric protocol for microplate from Bethke and Busse (2008). Duplicate samples were diluted to half by adding 25 μl of 1 M PBS per 25 μl of sample. Then 25 μl of 10 mM sodium acetate trihydrate (pH 5) and 10 μl of 150 mM PBS was added to all wells. We mixed plates then incubated them for 1 h at 40°C. After incubation, 25 μl of 150 mM PBS (pH 7.4) was added to each well, followed by 25 μl of enzyme mix. The enzyme mix was an aqueous solution containing ampliflu red, horseradish peroxidase, glucose oxidase, and 150 mM sodium phosphate buffer. Samples were measured at 560 nm in a spectrophotometer (accuSkan FC) after incubation at room temperature for 30 min every 5 min for a 20 min interval. Inter-plate variation was 10.22% and intra-plate variation ranged from 6.93 to 12.19%.

Statistical Analyses

To examine effects of ALAN on cortisol release rates in mesocosms, we performed a repeated measure generalized linear mixed model (GLMM) with natural log-transformed cortisol release rates standardized by standard length (pg/mm/h) with treatment as the fixed effect and individual nested in mesocosm as the random effect. When there were significant treatment effects, we used *post hoc* Tukey's HSD comparisons. To assess effects of ALAN on *G. affinis* glucose levels, we used a GLMM with treatment as the fixed effect and mesocosm as the random effect. We used a GLMM to analyze changes in mass and SL over time with treatment and day as fixed effects and individual nested in mesocosm as the random effect. For behavior analyses, we used generalized linear models (LM) with SL and treatment as fixed effects and individual nested in mesocosm as the random effect. To explore differences in survival we ran a Log-Rank survival analysis. We used JMP Pro 14.0.0 (SAS Institute, Inc.) for all analyses.

Protocols and housing were approved by the Institutional Animal Care and Use Committee of Texas State University (IACUC # 83).

Results

Growth, Survival, Cortisol, and Glucose

In the mesocosm experiment, we found no significant effect of ALAN on SL (GLMM: treatment × day: $F_{3,138} = 0.60, p = 0.619$) or mass ($F_{3,138} = 0.95, p = 0.42$), however, fish in both treatments increased in SL (day: $F_{3,138} = 16.17$, $p < 0.0001$) and lost mass ($F_{3,138} = 78.26, p < 0.0001$) over the duration of the experiment. There were no differences in survival between treatments (Log-Rank Survival Analysis: $\chi^{2}{}_{1} = 0.89$, $p = 0.35$). We found no significant differences in cortisol release rates between treatments across days (GLMM: treatment × day: $F_{3,138} = 2.10$, $p = 0.10$; **Table 3** and **Figure 2B**). As in the lab study, cortisol release rates were higher after agitation compared to baseline for both treatments (day: $F_{3,138} = 4.99, p = 0.003$; **Figure 2B**), indicating the fish could mount a stress response. The random effects of mesocosm were not significant (Wald *p*-value = 0.12). There were significantly lower glucose levels in the brain tissues of fish from the ALAN treatment than fish from the control treatment (GLMM: treatment: $F_{1,13} = 8.04, p = 0.014$; **Figure 5**), but there was no effect of treatment on the glucose levels in any other tissue type (all $p > 0.05$; **Table 4**).

Hiding Behavior

In the mesocosm experiment, we found no significant differences in time resting, or time spent hiding (all $p > 0.05$; **Table 5**) between treatments.

TABLE 3 | Results from the generalized linear mixed model testing the effects of ALAN on cortisol release rates of female *G. affinis* in mesocosms.

	Mean ± SE	*t*-value	*df*	*p*-value	Lower 95% CI	Upper 95% CI
Intercept	4.22 ± 0.04	105.60	46	**<0.001**	4.14	4.31
Control	0.05 ± 0.04	1.14	46	0.26	−0.03	0.13
Day 4	0.01 ± 0.06	0.24	138	0.81	−0.10	0.12
Day 16	−0.15 ± 0.06	−2.60	138	**0.01**	−0.026	−0.03
Day 32	−0.06 ± 0.06	−1.10	138	0.27	−0.17	0.05
Control*Day 4	0.13 ± 0.06	2.34	138	**0.02**	0.02	0.24
Control*day 16	−0.08 ± 0.06	−1.50	138	0.13	−0.20	0.03
Control*day 32	0.00 ± 0.06	−0.04	138	0.97	−0.11	0.11

*"Treatment" and "day" were included as fixed effects and "ID" nested in "mesocosm" was included as the random effect. Statistically significant values are in bold. *indicates interaction effects.*

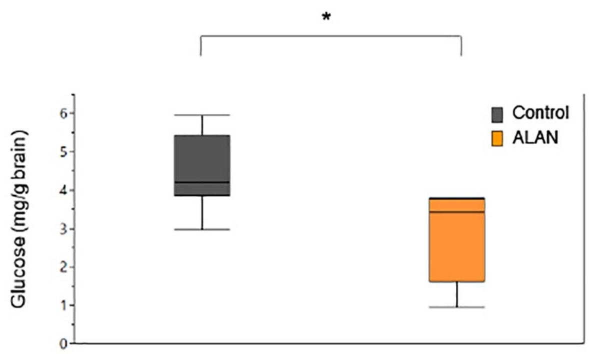

FIGURE 5 | Brain glucose levels (mg/g brain) of female *G. affinis*. Asterisk indicates a significant difference ($p < 0.05$). Box plots indicate median, range and first and third quartiles.

DISCUSSION

With the spread of urbanization, light pollution increases world-wide (Hölker et al., 2010; Davies et al., 2014; Falchi et al., 2016) and has the demonstrated potential to negatively impact exposed organisms by affecting their physiology, behavior, and reproduction. We found that the western mosquitofish, *G. affinis,* a tolerant and widespread species, responds to ALAN by decreasing their activity (both individually and around conspecifics during the day) and exhibiting reduced brain glucose levels compared to those exposed to normal light dark cycles. We did not, however, observe a change in cortisol release rates, growth, survival, or reproduction in response to ALAN. These results suggest that *G. affinis* may behaviorally adjust to the perturbation of ALAN rather than modulate their regulation of the HPI axis.

The lack of a cortisol response by *G. affinis* to ALAN may play a role in their success as invaders and establishing populations in water with varying physical properties as they may not experience the negative effects of altered cortisol release rates (Lloyd et al., 1986; Hubbs, 2000). Indeed, *G. affinis* copes with urban streams by flexibly altering their GC profile by reducing stress responsiveness followed by rapid negative feedback (recovery) (Kolonin et al., unpublished data). When fish from both ALAN and control groups were exposed to an agitation treatment, cortisol release rates were significantly higher than baseline levels, indicating they were capable of mounting a stress response. Therefore, the lack of a significant effect of ALAN on cortisol release rates under laboratory conditions and in mesocosms was not due to a dysregulated HPI axis, signifying that they were not chronically stressed.

In the laboratory experiment, we also measured cortisol release rates on day 2 and found that they were significantly elevated in both treatments as compared to days 0 and 7, suggesting that fish were not acclimated to the experimental setup. By day 7, cortisol release rates returned to baseline in both treatments. In the mesocosm study, we tested the hypothesis that there was a transient cortisol response to ALAN sometime between days 2 and 7 by collecting cortisol release rates on day 4; however, we still did not detect a change in cortisol release rates in response to ALAN. Our results are in concordance with other studies that found a lack of GC response to ALAN using comparable or greater lux levels. For example, Szekeres et al. (2017) found that juvenile bonefish (*Albula vulpes*) exhibit increased blood glucose in response to ALAN but there was no effect of ALAN on whole body

TABLE 4 | Results from the generalized linear mixed model testing the effects of ALAN on glucose levels of female *G. affinis* tissues in mesocosms.

Tissue		Mean ± SE	*t*-value	*df*	*P*-value	Lower 95% CI	Upper 95% CI
Muscle	Intercept	0.22 ± 0.02	10.98	14	**<0.0001**	0.18	0.27
	Control	0.02 ± 0.02	1.01	14	0.33	−0.02	0.06
Liver	Intercept	7.01 ± 0.81	8.64	13	**<0.0001**	5.26	8.77
	Control	0.32 ± 0.81	−0.39	13	0.70	−2.07	1.44
Brain	Intercept	3.65 ± 0.27	13.29	13	**<0.0001**	3.05	4.24
	Control	0.78 ± 0.27	2.84	13	**0.01**	0.19	1.37

“Treatment” was included as a fixed effect and “mesocosm” was included as a random effect. Statistically significant values are in bold.

TABLE 5 | Results from the generalized linear mixed model testing the effects of ALAN on behavior of female *G. affinis* in mesocosms.

		Estimate ± SE	*t*-value	*df*	*p*-value	Lower 95% CI	Upper 95% CI
Resting	Intercept	−0.07 ± 80.78	−0.00	38	1.00	−163.59	163.46
	SL (mm)	4.61 ± 2.58	1.79	38	0.08	−0.61	9.82
	Control	−2.76 ± 11.23	−0.25	38	0.81	−25.50	19.98
	SL*control	−2.51 ± 2.58	−0.97	38	0.34	−7.72	2.71
Hiding	Intercept	28.87 ± 94.44	0.31	38	0.76	−162.33	220.06
	SL (mm)	1.70 ± 3.01	0.57	38	0.57	−4.39	7.80
	Control	3.17 ± 13.13	0.24	38	0.81	−23.42	29.76
	SL*control	−2.01 ± 3.01	−0.67	38	0.51	−8.11	4.09

*“Treatment” and “SL” were included as fixed effects and “ID” nested in “mesocosm” was included as a random effect. *indicates interaction effects.*

cortisol. Additionally, European perch (*Perca fluviatilis*) exposed to ALAN had decreased melatonin production compared to the control group, but there were no differences in cortisol release rates between treatments (Brüning et al., 2015). There were no differences in corticosterone levels between control and ALAN-exposed treatments in brown anoles (*Anolis sagre;* Thawley and Kolbe, 2020) or wood frogs (*Lithobates sylvaticus*; May et al., 2019). These results suggest there are other mechanisms of coping with stressors (Ouyang et al., 2018) or ALAN may simply not elicit a GC response during the breeding season in many species (Grunst et al., 2019), including *G. affinis*. The species tested thus far may be more tolerant species as they are abundant, therefore testing less tolerant species might be necessary to fully understand the implications of exposure to ALAN.

Female *G. affinis* from mesocosms exposed to ALAN had lower glucose levels in their brain tissue compared to fish kept under control light conditions. Similarly, fish showed lower activity and daytime shoaling behavior when exposed to ALAN. Glucose provides precursors for neurotransmitter synthesis as well as ATP production in the brain, therefore the decreased levels of glucose in the brain may be associated with insufficient glucose for the required energetic demands to move (Mergenthaler et al., 2013). Lowered brain glucose could indicate that *G. affinis* may have diminished neuronal activity when exposed to ALAN. Experiments on the cognitive ability of fish could elucidate whether there are differences in brain function between fish exposed to ALAN and those kept under a natural light cycle.

The activity of *G. affinis* was overall reduced both individually at night and in shoaling during the day after exposure to ALAN. Fish moved less often at night after 50 days of exposure to ALAN in the laboratory. Because they did not spend more time hiding at night, this could put them at greater risk of predation. This may contribute to a better understanding of the mechanism behind the prior finding that fish exposed to ALAN experienced higher predation rates (O'Connor et al., 2019). This result opposes several other studies which found an increase in activity in fish (Becker et al., 2013; Foster et al., 2016; Kurvers et al., 2018; Czarnecka et al., 2019), American toads (*Anaxyrus americanus*), zebra finches (*Taeniopygia guttata*), and anoles (*Anolis leachii* and *A. wattsi*: Dananay and Benard, 2018; Batra et al., 2019; Maurer et al., 2019) after exposure to ALAN. May et al. (2019), however, found that *Lithobates sylvaticus* tadpoles were also less active in the day and night after exposure to ALAN and Buchanan (1993) found that gray treefrogs, *Hyla chrysoscelis,* reduced foraging activity at night under ALAN. These results suggest that the effects of ALAN vary by species. Female *G. affinis* also spent less time shoaling during the day after the same exposure to ALAN in the laboratory. Shoaling is beneficial as a defense against predation and generally results in more efficient foraging, therefore a lack of shoaling during the day could leave them more susceptible to predation and affect their ability to find food (Pitcher, 1986; Laland and Williams, 1997). In the control group, there was a clear display of diel shoaling activity where fish were more active and swam in closer proximity during the day than at night. This diel activity pattern disappeared after exposure to ALAN as fish in the treatment group shoaled the same amount during the day (and less than the control) and night. However, we cannot discount the alternative hypothesis that the treatment and control groups differ because the control group fish were in complete dark at night, without their regular lunar cycles. These results align with studies of fish where shoaling was decreased after exposure to other disturbances such as psychotropic drugs and parasites (Tobler and Schlupp, 2008; Green et al., 2012). Overall, in the laboratory, *G. affinis* exposed to ALAN moved less often at night and shoaled less during the day, which could leave them more susceptible to predation at all times.

Because these fish are found in highly developed urban settings, we chose to additionally run the experiment with fish exposed to slightly higher levels of light than we found in Hays County (230 vs. 246 lux). *Gambusia affinis* tend to be found in shallow, slow moving water (Hess and Tarzwell, 1942; Casterlin and Reynolds, 1977; Belk and Lydeard, 1994) indicating that they will experience high light levels much of the time. Further, we found that *G. affinis* move less at night after ALAN exposure and were not hiding, indicating that the fish are unlikely to swim away from or hide when exposed to light at night. Nonetheless, cortisol release rates were not affected by ALAN exposure.

Offspring number and survival did not differ significantly between treatments. Most females did not experience parturition during the experiment and consequently our sample size was low. Offspring counts could have been affected by cannibalism and this species can resorb embryos under suboptimal conditions (Meffe and Vrijenhoek, 1981). Offspring number and survival was not measured in mesocosms because we did not observe any offspring in either treatment during the duration of the experiment. In previous studies, continuous light cycles resulted in earlier hatching and smaller larvae size of haddock, *Melanogrammus aeglefinus*, embryos, complete failure of embryo hatching in the common clownfish, *Amphiprion ocellaris*, under a much lower lux level (26 lux), and early-stage pregnancy termination in female white rats, *Rattus norvegicus* Wistar (Downing and Litvak, 2002; Berbets et al., 2019; Fobert et al., 2019).

One caveat of our experiment is that we were not able to randomize the distribution of the treatments. The blocked treatments and associated spatial differences cannot be ruled out as factors in affecting our results. However, we measured several variables to ensure that there was no "location effect" including water quality measures and fish behavior. While conditions still could have differed in other non-measured variables, we did not anticipate this as all replicates were held in the same room under the same conditions and were handled similarly.

Pervasive effects of ALAN on fish behavior and physiology have previously been reported and here we show ALAN has some of these effects on a tolerant, invasive fish species. Since urbanization is on the rise mitigation efforts are necessary to minimize these impacts (Falchi et al., 2011). In areas where such efforts have already taken place, night light is successfully conserved without compromising human safety or security (Kyba et al., 2015, 2017; Steinbach et al., 2015). Additionally, few experiments have examined how various light color effects different taxa, which could potentially become a mitigation strategy. For example, melatonin rhythm was the most suppressed in European perch by green and red light and less so by blue light (Brüning et al., 2016). Expansion on studies evaluating the consequences of different spectral quality needs to be conducted to combat the negative consequences of increasing light pollution. ALAN did not compromise every variable we measured; however, the reduction of activity and brain glucose in *G. affinis* could have consequences for organismal fitness and in less tolerant species ALAN could have additional consequences.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Texas State University (IACUC # 83).

AUTHOR CONTRIBUTIONS

CG, AA, and KM contributed to the conception and design of the study. KM conducted experimental testing and wrote the first draft of the manuscript. MH contributed to experimental design and analysis of glucose. All authors contributed to manuscript revision, read, and approved the final version.

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Predator Environment Does Not Predict Life History in the Morphologically Constrained Fish *Alfaro cultratus* (Cyprinodontiformes: Poeciliidae)

Kaitlyn B. Golden[1]*, Mark C. Belk[1] and Jerald B. Johnson[1,2]

[1] Department of Biology, Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT, United States, [2] Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT, United States

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***Correspondence:**
Kaitlyn B. Golden
kaitlynbgolden@gmail.com

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Predation is known to have a significant effect on life history diversification in a variety of species. However, physical constraints of body shape and size can sometimes limit life history divergence. We test this idea in the Costa Rican livebearing fish *Alfaro cultratus*. Individuals in this species have a narrow body and keeled ventral surface, and females do not develop a distended abdomen when pregnant like other livebearing fishes. Here, we describe the life history of *A. cultratus* from 20 different populations across both high-predation and low-predation environments. We found significantly lower reproductive allotment in females from high-predation environments than in females from low-predation environments, but no significant difference in female or male size at maturity, number of offspring produced by females, or size of offspring. We found that *A. cultratus* exhibit isometric patterns of allocation for clutch dry mass in relation to female dry mass in high-predation and low-predation environments. Our results suggest that body shape constraints in this species limit the life history divergence we typically see between populations from high-predation and low-predation environments in other species.

Keywords: Poeciliidae, life history, *Alfaro cultratus*, allometry, constraints, predation, terminal investment hypothesis

INTRODUCTION

A life history strategy defines how an organism utilizes and optimizes energy to survive and reproduce (Fisher, 1930; Williams, 1966; Stearns, 1977; Roff, 1992). The optimal strategy can be influenced by extrinsic factors such as mortality rate (Strauss, 1990; Jennions et al., 2006; Riesch et al., 2013; Mukherjee et al., 2014; Olinger et al., 2016), resource availability (Reznick et al., 1992; Riesch et al., 2013; Moore et al., 2016; Zandonà et al., 2017), population density (Bronikowski et al., 2002; Schrader and Travis, 2012), and environmental conditions (e.g., salinity, gradient, elevation, etc.) (Zúñiga-Vega et al., 2007; Jourdan et al., 2016; Rius et al., 2019). Predator environments have often been used to study the effects of mortality rate on life history strategies (Law, 1979; Reznick and Endler, 1982; Johnson and Belk, 1999; Gosline and Rodd, 2008) and have been found to affect a wide variety of taxa, including fish, anurans, and insects. Among other things, the presence of a predator can influence timing and size of maturation and changes among developmental stages (Chivers et al., 2001; Johnson, 2001; Hilton et al., 2002; Stoks et al., 2006; Peterson et al., 2019), growth rate (Lardner, 2000; Altwegg, 2002; Šupina et al., 2016; Brown et al., 2018; DeWitt et al., 2019), and investment in offspring (Johnson and Belk, 2001; Gorini-Pacheco et al., 2017). Previous work consistently finds divergent life history patterns in high-predation and low-predation environments. For example, in the family Poeciliidae (livebearing fishes) many studies have shown a divergent pattern of smaller size at maturity, higher fecundity, smaller offspring, and greater reproductive allotment in populations in high-predation environments relative to low-predation or no-predator environments (Reznick, 1990; Johnson, 2001; Jennions and Telford, 2002; Walsh and Reznick, 2009; Moore et al., 2016). Similarly, in anurans the timing and size of metamorphosis (Laurila et al., 1998; Lardner, 2000) and timing of hatching (Laurila et al., 2002; Capellán and Nicieza, 2007) change in response to the presence of a predator. Therefore, we expect to see patterns of life history divergence in response to predator environments in additional species.

There are limits, however, to divergent evolution in predator environments. Divergent evolution requires that populations are able to adapt to different selective pressures. That said, there are genetic, phylogenetic, morphological, and physiological constraints that can all limit adaptive evolution (Gould, 1980). Morphological constraints are particularly important in life history evolution as they can affect the internal body space available for reproduction. This has been frequently studied in turtles (Clark et al., 2001; Ryan and Lindeman, 2007; Rollinson and Brooks, 2008; Macip-Ríos et al., 2012). For example, the small African tortoise *Homopus signatus*, produces single-egg clutches. Although producing one large egg is best for the fitness and survival of the offspring, *H. signatus* is constrained by a small body size and pelvic canal limiting how large the egg can be (Hofmeyr et al., 2005). Similar patterns were found in other species of turtles where the pelvic girdle (also influenced by evolutionary pressures on locomotion) limited egg size, especially in small individuals (Congdon and Gibbons, 1987). Thus, morphology can constrain a life history trait due to internal space, size, and shape of an organism.

Morphology is also important in survival; tradeoffs between the optimal morphology for survival and the optimal morphology for reproduction may be present in some species. Size and shape can be very important in predator avoidance. For example, the humpback chub, *Gila cypha*, has a large dorsal cranial hump that increases the depth of their body and therefore protects against gape limited predators (Portz and Tyus, 2004). Tradeoffs occur because certain morphologies may be optimal to some selective pressures, but not to others. In the family Poeciliidae, fishes invest more in offspring when predators are present than when they are absent; however, this investment comes at a cost of decreased swimming performance (Ghalambor et al., 2004). Thus the optimal morphology for swimming performance and the optimal morphology for reproduction can be in conflict (Zúñiga-Vega et al., 2007; Wesner et al., 2011; Hassell et al., 2012; Ingley et al., 2016; Quicazan-Rubio et al., 2019). Selective pressures acting on morphology can limit the optimal adaptation in life history or vice versa in a given environment. However, we don't know how morphological adaptations limit life history adaptations in predation environments.

An additional question is how predation and morphological constraints influence lifetime reproductive allocation. The terminal investment hypothesis predicts that organisms will invest more in reproduction as they age, as chances for future reproduction decrease (Williams, 1966). Specifically, in environments that experience high mortality (such as high-predation environments) individuals may allocate energy to current reproduction over future reproduction; however, in low mortality environments (such as low-predation environments) individuals may allocate more to future reproduction than current reproduction, consistent with the terminal investment hypothesis (Law, 1979; Michod, 1979; Billing et al., 2007; Belk et al., 2011; Billman et al., 2014; Nickley et al., 2016). Thus, reproductive allocation can change in response to mortality pressures presented in predation environments as high mortality limits the chance of survival and opportunities for future reproduction. Morphological constraints can also influence within-lifetime reproductive allocation. In *Brachyrhaphis parismina* (a poeciliid fish), populations showed isometric allocation of reproductive allotment to female body mass with age (Belk et al., 2011). This is possibly due to a narrow-bodied shape that might constrain reproductive allocation from being greater than proportionate to body size. Thus, mortality rates and morphological constraints can influence patterns of reproductive investment.

In this study, we test the ideas that: 1) divergence in life history traits among populations in different predator environments may be limited when there are strong morphological constraints; and 2) within lifetime reproductive allocation, consistent with the terminal investment hypothesis, may be limited within predation environments due to morphological constraints. If true, we expect to find isometric allocation rather than hyper-allometric allocation in morphologically constrained species. To test these ideas, we used the fish *Alfaro cultratus* (Regan, 1908) from the family Poeciliidae. Poeciliids provide an optimal study system as they are livebearers, have a short generation time, and are found in many different selective environments (Reznick and Endler, 1982). *Alfaro cultratus* is an ideal species for our study as it is an extremely narrow-bodied poeciliid with a keeled ventral surface (**Figure 1**). Additionally, *A. cultratus* do not develop a distended abdomen during pregnancy. The body morphology of this fish is likely a constraint for reproduction as it does not allow additional space via abdominal expansion during pregnancy as exemplified in other poeciliids.

MATERIALS AND METHODS

Study Sites, Collections, and Characterizing Predation Environments

We collected fish from eight different sites in Costa Rica during February and May 2006, and May 2007. Additionally, we collected *A. cultratus* from 12 different sites in northeast

FIGURE 1 | Photos of the lateral and dorsal view of a female *Alfaro cultratus*. Dorsal view shows laterally compressed body shape.

Costa Rica during April 2019 (**Figure 2**). We collected fish under Brigham Young University IACUC committee approval (Protocol #15-0404). All fish were collected with permission and corresponding permits from the Sistema Nacional De Áreas De Conservación in Costa Rica (011-2006-SINAC, 015-2007-SINAC, R-SINAC-PNI-ACAHN-011-2019). We collected samples with a handheld seine (1.3 × 5 m; 8 mm mesh size). We tried to collect approximately 100 females (**Table 1**) from each site to ensure that we had enough mature and immature individuals for analysis without taking more than a fraction of the local population. We euthanized all fish in the field with an overdose of 3- amenobenzoic acid ethyl ester (MS-222), preserved samples in the field in 95% ethanol, and then transported them to the laboratory for analysis where they were stored in 70% ethanol.

We identified high-predation sites as locations where the piscivorous species *Parachromis dovii* (Johnson and Belk, 2001) and/or *Parachromis managuensis* were found during seining. At each location we made multiple seine hauls (10 or more). Low-predation sites were identified as locations where *A. cultratus* was found alone or only with non-piscivorous fishes. Here, we analyze 11 high-predation sites (one from 2006, three from 2007, and seven from 2019) and nine low-predation sites (two from 2006, two from 2007, and five from 2019). We term these sites as "high predation environments" or "low-predation environments," respectively. High-predation and low-predation environments are expected to vary in predation risk but also may be confounded with other environmental factors such as resource availability, temperature, elevation, flow, and density (Johnson, 2002; Jourdan et al., 2016; Olinger et al., 2016). Thus, predation environments are characterized by the presence or absence of a predator, but they are called "environments" to encompass the many different factors that may be causally or incidentally correlated with the presence or absence of a predator (see Johnson, 2002). In addition to piscivorous predators, other factors can contribute to morality rates, including avian and invertebrate predators. Previous work on the fish *Brachyrhaphis rhabdophora* suggests that categorizing locations this way into high and low-predation environments does accurately predict mortality rates and divergent life history traits (Johnson and Belk, 2001; Johnson, 2002; Johnson and Zúñiga-Vega, 2009). Therefore, although we have not measured mortality rates in this system, we use the presence/absence of a predator as a predictor of mortality (Johnson, 2001; Johnson and Belk, 2001; Belk et al., 2011; Wesner et al., 2011).

Life History

We measured five life history traits: 1) male size at maturity; 2) female size at maturity; 3) number of offspring; 4) size of offspring; and 5) reproductive allotment. All traits were measured from alcohol-preserved specimens. We recognize that this preservation technique results in the extraction of fats from

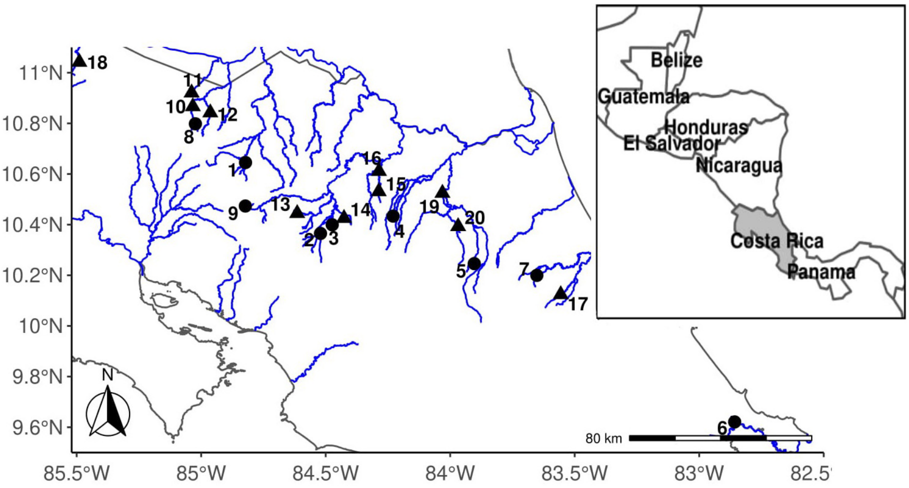

FIGURE 2 | Map of Costa Rica collection locations. Localities are numbered according to the site number found in **Table 1**. High-predation sites are triangles; low-predation sites are circles.

TABLE 1 | Descriptive statistics for life history characteristics of *Alfaro cultratus* for 20 populations.

	Location	Year	Site Number	Number of Males	Mean length of adult males (mm)	Size range of adult males (min–max)	Number of Females	Minimum size of gravid females (mm)	Brood Dry Mass (mg)	Number of Offspring	Offspring Size (mg)	Mean Female Dry Mass (mg)
Low-predation Sites	Rio Queque	2019	1	6	34.14	30–42.8	33/54	32	0.009	9.714	0.00101	0.115
	Rio Balsa Tributary	2019	2	40	34.4	28.1–41.7	56/156	34	0.008	7.491	0.00114	0.142
	Quebrada Serena	2019	3	48	33.33	28.2–43	65/94	34	0.019	18.145	0.00110	0.136
	Quebrada Sahino	2019	4	14	33.86	30–42	49/90	32	0.009	6.200	0.00159	0.078
	Rio Sucio	2019	5	13	34.78	28.2–42.6	32/50	34	0.011	13.397	0.00083	0.098
	Trib. To Rio Sixaola	2007	6	14	26.486	21.7–38.1	24/33	30	0.008	7.431	0.00123	0.099
	Trib. to Rio Parismina	2007	7	11	36.291	28.9–41.5	20/21	30	0.009	7.765	0.00124	0.247
	Rio Salto	2006	8	21	31.433	25.3–52.7	27/82	28	0.012	11.862	0.00151	0.100
	Quebrada Perez	2006	9	7	39.643	30–46.5	12/57	42	0.022	27.744	0.00069	0.184
High-predation Sites	Rio Zapote (Side Channel)	2019	10	18	33.35	25.9–47.6	32/96	34	0.019	17.888	0.00107	0.128
	Quebrada Las Latas	2019	11	57	30.34	23–43.2	59/108	28	0.009	9.001	0.00108	0.131
	Rio Ricardo	2019	12	22	37.65	31.9–43.8	43/76	34	0.011	10.840	0.00106	0.125
	Quebrada Piedra	2019	13	20	35.28	23.9–44.6	60/106	34	0.011	10.243	0.00113	0.115
	Rio San Rafael Tributary	2019	14	35	32.37	26.8–41.4	55/114	30	0.008	6.395	0.00127	0.130
	Quebrada Huevo	2019	15	26	33.37	27.7–41.5	46/130	32	0.013	9.412	0.00147	0.124
	Rio Saino	2019	16	24	30.58	25.5–38.3	43/98	34	0.014	12.121	0.00119	0.097
	Rio Herediana	2007	17	13	40.523	35.5–46.8	27/104	40	0.012	13.657	0.00092	0.220
	Rio Sabalo	2007	18	17	35.465	25.7–47.3	17/90	42	0.006	6.921	0.00086	0.244
	Trib. to Rio Sarapiqui	2007	19	21	34.814	26.4–45.8	49/92	32	0.010	10.993	0.00100	0.179
	Isla Grande	2006	20	13	30.339	25.3–41.6	19/73	32	0.004	9.254	0.00086	0.096

Brood size, number of offspring, and size of offspring are least squares means that come from the linear models reported in the text. Brood size and number of offspring least squares means have been back transformed to represent true numeric values. Number of females for populations in 2006 and 2007 are reported only as the number that were mature out of the number dissected; for 2019 populations these include all females collected.

the specimens, an approach that has been applied widely across life history studies, including in our previous work (Johnson and Belk, 2001; Belk et al., 2011; Brown et al., 2018; Molina-Moctezuma et al., 2020), thus allowing us to compare findings here with previous work. We collected life history data using methods described in Johnson and Belk (2001). In brief, we did this as follows. We first measured the length of each adult female fish. We then dissected each specimen on the left lateral side where we removed stomachs and embryos. We counted and staged each embryo. To score female size at maturity for each population, we first divided females into 2 mm size classes. We identified size at maturity as the size class where at least half of the females were mature with developing embryos. Developing embryos were classified using Haynes (1995) classification method (stages 1–11). Stage 1 and 2 are immature and unfertilized eggs, and stage 3 and above are developing embryos. Stage 3 is a fully yolked and fertilized egg, and stage 11 is a mature embryo with the yolk sac entirely, or almost entirely, absorbed (Haynes, 1995). In cases where population samples of mature females were small, the actual value may be slightly smaller or larger than reported because we lacked adequate sampling. We counted number of offspring as the number of developing embryos contained in each mature female. We determined size of offspring as the dry mass of the brood divided by the number of offspring in each brood. We measured reproductive allotment as the dry mass of the brood. Female dry mass (digestive tract removed) and brood dry mass were measured after they were separated and dried for 24 h in a 55°C desiccating oven. We determined male size at maturity as the mean standard length of all mature males (male poeciliids grow little, if at all, after maturation) (Turner, 1942; Johnson and Belk, 2001; Belk et al., 2011). We identified mature males by the presence of a fully developed modified anal fin (gonopodium).

Allometry Analysis

We built two models of reproductive allotment as the relationship between the natural log of clutch dry mass and the natural log of female dry mass in both high-predation and low-predation environments. We used the slopes of these models as allometric coefficients (**Table 2**). We included developmental stage of offspring as a covariate and collection location as a random effect in the models. We determined patterns of allometry using ordinary least squares regression (Kilmer and Rodríguez, 2017). When the slope was equal to one, this indicated isometry and not terminal investment. A slope greater than one is consistent with terminal investment (Billman et al., 2014), where the mass of the clutch is proportionately larger than predicted by body size. Females exhibit indeterminate growth; thus, we used size of females as a surrogate of age. All analyses were done using R version 3.5.2 (R Project for Statistical Computing, RRID:SCR_001905).

Life History Trait Analysis

We ran general linear models for each life history trait to assess the effect of predation. We included covariates for the life history models as described in Johnson and Belk (2001). In brief, when analyzing number of offspring, we included female dry mass as a covariate. When analyzing offspring size and reproductive allotment, we used female dry mass and developmental stage of embryos as covariates. We did not include any covariates for male or female size at maturity. Brood dry mass was our measure of reproductive allotment. We log transformed reproductive allotment and number of offspring in the analysis to satisfy assumptions of the linear model. All output data for reproductive allotment and number of offspring were back-transformed to the original scale before being included in graphs or tables. We included location in each model as a random effect. We calculated population least squares means for reproductive allotment, number of offspring, and size of offspring for comparable estimates (**Table 1**). Additionally, we ran the analysis for reproductive allotment with and without the population from Quebrada Serena (a possible outlier) to determine the significance of this population. We found that with the removal of this site, predation no longer significantly affected reproductive allotment. All analyses were done using R version 3.5.2 (R Project for Statistical Computing, RRID:SCR_001905).

TABLE 2 | Allometric coefficients for *Alfaro cultratus* in high-predation and low-predation environments.

Predator	Allometric Coefficient (AC)	SE	95% CI	AC > 1	Intercept
High	1.052	0.061	0.932–1.172	No	−3.161
Low	1.083	0.081	0.924–1.243	No	−2.753

Isometry is seen in high-predation and low-predation environments as evidenced by confidence intervals that span a slope of 1.

RESULTS

Life history traits in *A. cultratus* did not differ significantly between high-predation versus low-predation environments except reproductive allotment (**Table 3**). Females from high-predation environments had significantly lower values of reproductive allotment than those from low-predation environments (ANCOVA, $F = 5.7$, $df = 1$, $P = 0.017$, slope = −0.15, $R^2 = 0.46$). The statistical significance of this relationship is entirely due to one population with high

TABLE 3 | Statistical tests for effect of predation environments on the five life history traits.

Life History Trait	*F*	*df*	*P*-value	Slope	R^2	Intercept
Reproductive Allotment	5.730	1	0.017	−0.1500	0.455	−5.702
Number of Offspring	1.118	1	0.291	−0.0700	0.401	1.659
Offspring Size	2.975	1	0.085	−0.0001	0.089	0.001
Female Size at Maturity	1.011	1	0.315	0.3170	0.002	32.284
Mean Male Size at Maturity	1.266	1	0.261	0.2860	0.003	33.308

Female dry mass and development stage are covariates for reproductive allotment and offspring size. Development stage is a covariate for number of offspring. Location is included as a random effect in each model.

brood dry mass in the low-predation category (Quebrada Serena) (see S1, **Supplementary Material**). Size of offspring, number of offspring, and size at maturity for males and females did not differ significantly in high-predation versus low-predation environments (**Table 3** and **Figures 3**, **4**).

Similarly, the allometric coefficients for reproductive allotment did not differ between high-predation and low-predation environments. Individuals in both environments displayed isometric reproductive allocation with age, inconsistent with the terminal investment hypothesis (**Table 2** and **Figure 5**).

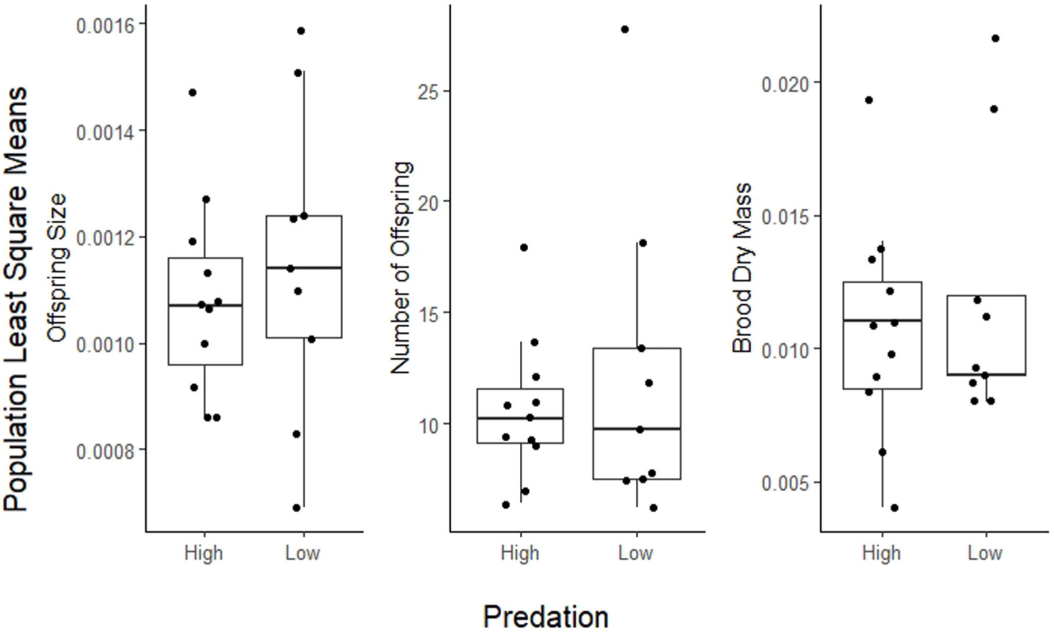

FIGURE 3 | Plot of population least squares means for reproductive allotment (brood dry mass), number of offspring, and size of offspring in high-predation and low-predation environments. Population means (dots) are jittered for better visualization.

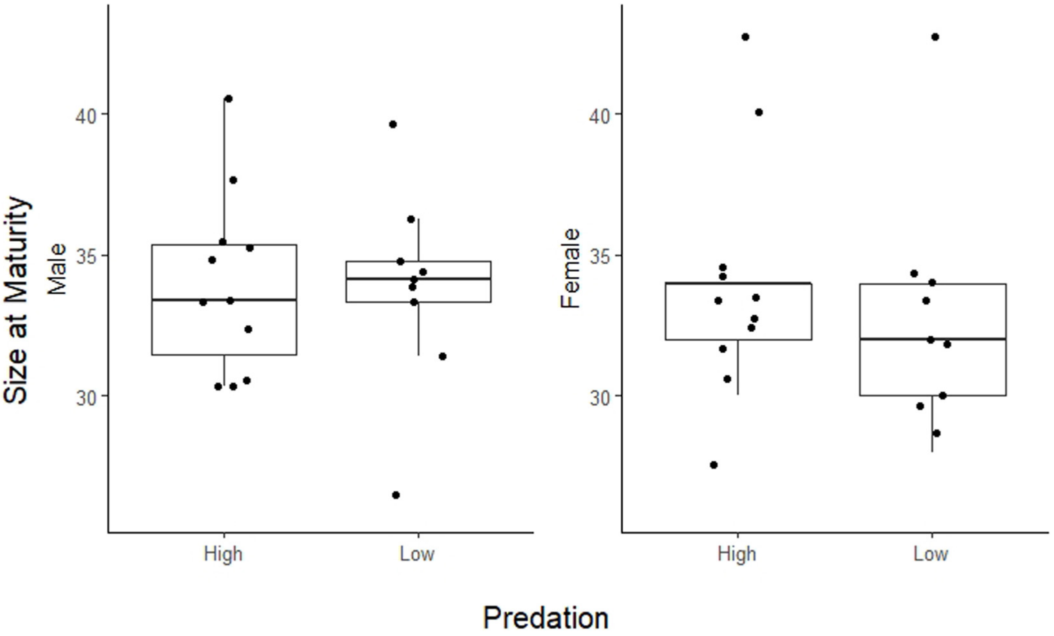

FIGURE 4 | Plot of size at maturity for males and females in high-predation and low-predation environments. Female size at maturity is the size where at least 1/2 females in that size category contain developing embryos. For males this is the mean size of mature males. Population points (dots) are jittered for better visualization.

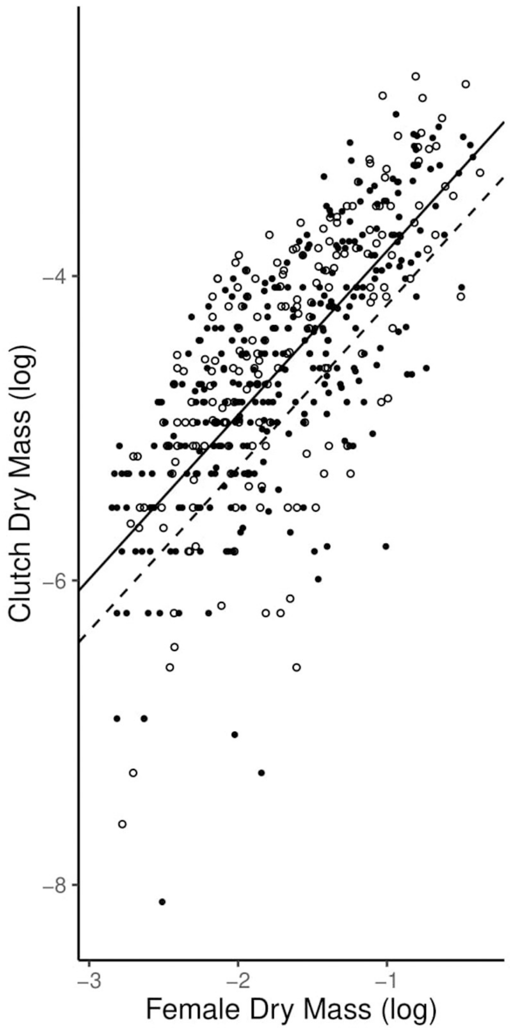

FIGURE 5 | Graph of allometric coefficients for high-predation and low-predation environments. Low-predation sites are open circles and high-predation sites are closed circles. The low-predation allometric coefficient (1.052) is the dashed line and the high-predation allometric coefficient (1.083) is the solid line.

DISCUSSION

There was no divergence in four life history traits or allometric coefficients for reproductive allotment in *A. cultratus* from different predation environments. All life history traits showed no significant difference between high-predation and low-predation environments, except for reproductive allotment, which did differ significantly. However, it differed in a direction opposite to what theory predicts (Reznick, 1990) – we found lower allotment in high-predation environments than in low-predation environments. This significant result and allotment pattern are driven by our collection from Quebrada Serena (site 3). With the removal of this site, the difference in reproductive allotment is no longer significant. This site appears to be unique in that all mature females had a large number of offspring (greater than 8). However, it does not appear to be unique in any other way. Thus, it is possible that the life history phenotype observed at this site is shaped by other selective pressures. One possible explanation is resource availability. High resource availability has been found to influence a high fecundity (Reznick and Yang, 1993) and with the high fecundity found at this site this may be a likely explanation. The allometric coefficient for reproductive allotment also did not differ among predation environments but instead showed an isometric pattern of allocation in both environments. This isometric pattern of investment is not consistent with the terminal investment hypothesis. Thus terminal investment is not evident in this species.

Lack of intraspecific life history variation is not unique to *Alfaro cultratus.* Absence of significant life history differences between populations is also seen in the species *Brachyrhaphis parismina* (Belk et al., 2011). However, differing predation pressures often evoke a divergent pattern of life history variation as is seen in *Brachyrhaphis rhabdophora*, *Brachyrhaphis episcopi*, and *Poecilia reticulata* (Reznick and Endler, 1982; Johnson and Belk, 2001; Jennions and Telford, 2002). The almost complete lack of intraspecific life history divergence across predation environments in *Alfaro cultratus* is unexpected and requires further exploration.

There are several possible explanations for the lack of divergence in life history in *A. cultratus*. It is possible that there may not be differences in environmental selective pressures among the sites. In environments where multiple factors are highly correlated, using one factor such as predator presence, is sufficient in representing a suite of putative selective agents at sites (Johnson, 2002). If environmental factors are not highly correlated, then using one factor such as predation may not adequately represent variation among selective environments. It is also possible that our predation environment as categorized here does not accurately predict mortality rates. Variation in actual mortality rates among localities could prove problematic to our simple placement of populations into either high or low mortality groups. This said, such categories have proved effective at predicting mortality rates in other systems (Johnson and Belk, 2001; Johnson and Zúñiga-Vega, 2009; Ingley et al., 2014; Belk et al., 2020). Lack of phenotypic divergence might also be attributed to gene flow between populations that can limit the ability of populations to adapt to selective pressures in their environment and therefore decrease differences between populations (Storfer, 1999). Unfortunately, we currently have no estimates of gene flow for this species – this said, the geographic distribution of high-predation and low-predation populations suggest that this explanation is not likely (**Figure 2**). Another alternative is that there is limited additive genetic variation. Again, we have no direct measure of additive genetic variation for *A. cultratus*. None of these explanations were examined in full in this study, but they may be a good direction for future research.

The most obvious explanation for lack of variation in this narrow-bodied species is that morphology acts as a constraint on reproductive traits. Body morphology influences swimming performance and predator avoidance (McPeek et al., 1996; Kolar and Wahl, 1998; Langerhans et al., 2004; Langerhans, 2009; Araújo et al., 2017). Pregnancy, in many species of poeciliids, can drastically change swimming performance causing predator avoidance to decline as pregnancy progresses (Ghalambor et al., 2004; Belk and Tuckfield, 2010). This may be caused by a morphological convergence across species during pregnancy which limits burst swimming near the end of pregnancy as the abdomen becomes distended and reproductive investment is favored over predator escape speed (Ghalambor et al., 2003; Wesner et al., 2011; Ingley et al., 2014). Some species are able to moderate the distension of the abdomen during pregnancy by superfetation, the simultaneous carrying of multiple broods, and thus maintain a more streamlined body morphology during pregnancy (Zúñiga-Vega et al., 2007; Fleuren et al., 2019). *Alfaro cultratus* does not exhibit superfetation, instead, their narrow body appears to limit abdominal distension during pregnancy. We suggest that the narrow body and distinctive ventral keel are important for swimming ability and that swimming ability may be favored in all environments in this species. Thus, limiting the space available at the end of pregnancy for a distended abdomen and contributing to the lack of difference seen among populations and individuals in life history characteristics.

For *Alfaro cultratus*, both the ventral keel and the narrow, streamlined body shape likely contribute to stabilized swimming ability. Morphological adaptations in fish are critical to increasing thrust and decreasing drag despite swimming style (e.g., stead or unsteady) (Webb, 1984). Small differences in morphology can have a large effect on locomotion (Webb, 1982), with body shape and fins both playing important roles in swimming performance (Blake, 2004; Langerhans and Reznick, 2010). First, a keeled ventral surface has been shown to be important in swimming performance (George and Westneat, 2019). In scombroid fishes, the presence of a keel on both sides of a caudal peduncle decreases drag and is more efficient than a cylinder or vertically elliptical peduncle (Walters, 1962). A keeled surface increases the surface area that is used for thrust (Graham and Lowell, 1987). Specifically, a ventral keel creates a negative pressure that increases stabilization and resistance to rolling (Van Wassenbergh et al., 2015). The ventral keeled surface of *Alfaro cultratus* may contribute to swimming performance by increasing stability for steady swimming. Second, a narrow-bodied morphology can likewise be beneficial for steady swimming. The body morphology of a fish influences energy demands by favoring either steady or unsteady swimming (Ohlberger et al., 2006). A streamlined body shape reduces turbulence and energetic costs (Araújo et al., 2017). Thus, the thin, streamlined body shape of *Alfaro cultratus* likely contributes to steady swimming, allowing for cruising at low energy costs (**Figure 1**). Both the ventral keel and the streamlined morphology of *A. cultratus* appear to be adaptations for steady swimming.

Typically, we would not expect to see stabilized swimming or the same morphology in all predation environments. Previous studies have found that in high-predation environments, unsteady swimming is favored but in low-predation environments steady swimming is favored (Langerhans, 2009; Langerhans and Reznick, 2010). Maintaining the same morphology in both high-predation and low-predation environments is thought to be costly as morphological divergence across predator regimes is commonly found in prey fish (Langerhans et al., 2004). The optimal morphology in a high-predation environment must be suboptimal in a low-predation environment or else we would expect to see the same morphology in both (Langerhans et al., 2004). No measure of morphological divergence in predation environments has been assessed in this species. Therefore, divergence may occur across predator regimes despite the persistence of a streamlined morphology. The narrow-bodied morphology may be influenced by other factors such as selective pressures like stream flow and resource acquisition as well as behaviors like habitat preference and foraging habits. For example, little is known about the habitat preference and foraging habits of *A. cultratus*. However, if foraging occurs in high flows, this may influence a steady swimming morphology despite suboptimal escape maneuvers that are limited in direction because of high flows (Anwar et al., 2016). It is important to acknowledge that body shape and life history may be unassociated. Fish with the same body shape may have varying patterns of life history if the gonads of the fish simply displace other internal organs (Zúñiga-Vega et al., 2011). Further work is needed to directly assess body shape, swimming performance, and selective pressures in this species.

In conclusion, divergent evolution in different predation environments was not seen in *Alfaro cultratus*. It appears that the ability for divergent evolution to occur in *A. cultratus* in response to predation pressures may be constrained by a narrow-bodied morphology adapted to stabilized swimming performance. Clearly, additional research focused on the cause of the lack of divergence in life history traits in this species will yield promising results.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this study was done on preserved specimens.

AUTHOR CONTRIBUTIONS

All authors formulated the idea for the study and conducted fieldwork. JJ and KG oversaw collection of life history data. KG collected 2019 female life history data, performed the analyses, and wrote the first draft of the manuscript. All authors reviewed and edited the manuscript, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.607802/full#supplementary-material

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Behavioral Variation in the Pygmy Halfbeak *Dermogenys collettei*: Comparing Shoals With Contrasting Ecologies

Alessandro Devigili[1][†], Erika Fernlund Isaksson[1][†], Nalini Puniamoorthy[2] and John L. Fitzpatrick[1]*

[1] Department of Zoology, Stockholm University, Stockholm, Sweden, [2] Department of Biological Sciences, National University of Singapore, Singapore, Singapore

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of Mexico, Mexico

***Correspondence:**
Alessandro Devigili
alessandro.devigili@zoologi.su.se

[†]These authors have contributed equally to this work

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Variation in biotic and abiotic factors among populations affects individual behaviors by transforming the social landscape and shaping mating systems. Consequently, describing behaviors in natural populations requires consideration of the biological and physical factors that different individuals face. Here, we examined variation in socio-sexual and locomotor behaviors in a small, livebearing, freshwater fish, the pygmy halfbeak *Dermogenys collettei*, across natural populations in Singapore. The pygmy halfbeak is a surface feeding fish that spends most of the time near the water surface, making it ideal for non-invasive behavioral observations. We compared behaviors between sexes among 26 shoals while simultaneously accounting for environmental variation. We demonstrated that sexual interactions and locomotor behaviors differed among shoals with varying levels of canopy cover and water flow. Specifically, in areas with greater canopy cover, sexual interactions decreased, whereas time spent in a stationary position increased. Sexual interactions were more numerous in still water, where fish spent less time swimming. Variation in the expression of socio-sexual and locomotor behaviors were not associated with differences in the amount of aquatic vegetation, water depth or halfbeak shoal size. Agonistic interactions were robust to environmental effects, showing little variation among environments. However, there were strong sex effects, with males performing more agonistic behaviors and spending less time in a stationary position compared to females, regardless of the environment. Moreover, sexual interactions, measured as actively performed by males and passively received by females, were on average more frequent in males than in females. Our findings help us explore the proximal causes of intraspecific behavioral variation and suggest that fundamental information on socio-sexual behaviors from wild populations can lead to a better understanding of how sexual selection operates when the strength of natural selection varies across environments.

Keywords: sexual selection, natural selection, predation, intrasexual competition, sexual activity, natural habitat, time budget

INTRODUCTION

Animals exhibit wide variation in behaviors when assessed within individuals, among individuals and among populations (Sih et al., 2004; Miller and Svensson, 2014). In natural populations, this ubiquitous behavioral variation is shaped by, and interacts with, varying biotic and abiotic characteristics of the environment (Emlen and Oring, 1977; Miller and Svensson, 2014). However, predicting how different behavioral responses vary with biotic and abiotic characteristics remains challenging. Individuals have limited time and energy budgets and may therefore face potential trade-offs among behaviors, which can themselves be mediated by environmental characteristics (Magnhagen and Magurran, 2008). Additionally, males and females often face varying costs associated with different behaviors, driven by a difference in reproductive investment and potentially mediated by the environment (Magurran and Seghers, 1994). Such behavioral trade-offs have important consequences for how animals respond to inter- versus intra-sexual selection (Ahnesjö et al., 2008) and defining such interactions can shed light on how selection acts in natural populations.

Socio-sexual (e.g., courtship and agonistic behaviors) and locomotor behaviors represent two categories of behaviors that may be especially subject to trade-offs and are often influenced by the environment (Endler, 1995; Penaluna et al., 2016; Suriyampola et al., 2017). Whether individuals invest more in courtship or agonistic behaviors may depend on the relative fitness benefits associated with performing either of these socio-sexual behaviors (Fitze et al., 2008; Weir et al., 2011). Environmental characteristics can mediate these gains, for example by influencing how information is transmitted (Sundin et al., 2016; Ehlman et al., 2018) and potentially modifying the type of interactions with conspecifics (e.g., Magellan and Magurran, 2006). Certain environmental characteristics may also result in differences in locomotor behaviors, particularly when enabling or interfering with individuals' movement through the environment (e.g., Marras et al., 2015). Trade-offs between socio-sexual and locomotor behaviors may also occur and be sensitive to the environment. For example, behaviors that increase survival (e.g., anti-predator behaviors, such as remaining stationary) potentially reduce the opportunity to perform behaviors that increase reproductive success (e.g., conspicuous mating behaviors), with this association being more relevant in environments with high predation risk (e.g., Rypstra et al., 2016; Edomwande and Barbosa, 2020). Explaining the variation in socio-sexual and locomotor behavior therefore requires these behaviors to be assessed across a range of environmental conditions.

Differences between the sexes also play a major role in determining among-population variation in socio-sexual and locomotor behaviors. Classically defined sex roles suggest that sexual selection should be stronger on males than females, as lower costs of gamete investment for males lead to greater benefits of multiple mating (Bateman, 1948; Lindsay et al., 2019). Consequently, males are generally expected to invest more of their time and energy engaged in courtship behaviors to attract partners (inter-sexual selection) and agonistic behaviors toward rivals to compete for reproductive opportunities (intra-sexual selection; Andersson, 1994). Males may also be more spatially active than females, particularly if locomotor activity increases the probability of finding a mate (e.g., Stark et al., 2005). In contrast, the strength of sexual selection is typically lower on females, who invest more resources in gamete and offspring production rather than mate acquisition (Kokko and Jennions, 2008), and generally devote time in choosing mates rather than courting them (e.g., Rosenthal, 2017). Moreover, carrying eggs or offspring increases cost of locomotion and risk of predation (Quicazan-Rubio et al., 2019), often accounting for a reduced mobility in females relative to males. Together, this suggest that across most taxa, selection acts differently on males and females and that sex-specific behavior may be influenced by biotic and abiotic conditions.

Measuring multiple behaviors across a range of environmental conditions is a powerful approach for identifying selection pressures and evolutionary constraints under ecologically relevant situations. Among viviparous fishes, the vast majority of research examining how behaviors are shaped by environmental characteristics in nature has been performed in Poeciliid fishes (order Cyprinodontiformes), a diverse group of fishes found primarily in the Neotropics, that represent a single evolutionary origin of viviparity (Blackburn, 2015). Here, we examine how behaviors change across different environments in a halfbeak fish from the family Zenarchopteridae (order Beloniformes). Halfbeaks are a relatively understudied group of fishes that represent an independent evolutionary origin of viviparity that occurred in a geographically distinct region (Southeast Asia) from the more commonly studied Poeciliids. Specifically, we focus on the pygmy halfbeak, *Dermogenys collettei*, a small, internally fertilizing, viviparous fish inhabiting freshwater rivers, streams and lakes in the Malay Peninsula (Downing Meisner, 2001; Nurul Farhana et al., 2018). Pygmy halfbeaks are characterized by an elongated lower jaw that facilitates prey capture at the surface of the water, where they spend the vast majority of their time. The halfbeaks live in mixed-sex shoals with frequent interactions among conspecifics and breed throughout the year (Greven, 2010; Ho et al., 2015). In this species, females give birth approximately every 30 days and males show a preference for females based on their breeding cycle (Ogden et al., 2020). Pygmy halfbeaks are sexually dimorphic in body size, with adult females being larger than males, and sexually dichromatic, with adult males expressing more red and yellow coloration than females, particularly on their modified anal fins (Greven, 2010). Females choose mates on the basis of the amount of red coloration displayed by a male (Reuland et al., 2020a), while male mate choice is based on the size of a sex-specific orange gravid spot on the female's abdomen that varies in size with the reproductive cycle (Ogden and Fitzpatrick, 2019). Sexual dimorphism, along with sex-specific behaviors, allows the sexes to be easily identified in natural populations. Male halfbeaks routinely perform a series of well-characterized courtship behaviors, whereas agonistic interactions are observed in both sexes (see the methods of this study and Greven, 2010; Baker and Lim, 2012; Ho et al., 2015). However, male halfbeaks are usually more aggressive than females (Greven, 2010) and form dominance hierarchies in laboratory conditions (Reuland et al., 2020b). These attributes make halfbeaks an ideal system for conducting non-invasive behavioral observations of natural populations across a range of environmental conditions.

In this study, we quantified socio-sexual (courtship and agonistic) and locomotor (swimming and stationary) behavior in 26 natural halfbeak shoals in Singapore. We tested the hypothesis that socio-sexual and locomotor behaviors differ between the sexes and are associated with differences in a range of environmental characteristics experienced among shoals (canopy cover, water depth, aquatic vegetation, and water flow). These environmental characteristics can influence fish behaviors in a variety of ways (**Table 1**). Previous research has focused on the association(s) between behaviors and environmental characteristics in species that swim throughout the water column [e.g., other viviparous species like Poeciliids (Head et al., 2010), other shoaling species like zebrafish, *Danio rerio* (Suriyampola et al., 2016, 2017) or stickleback, *Gasterosteus aculeatus* (Kim, 2016)]. In contrast, halfbeaks spend the vast majority of their time at the water surface and may therefore respond differently to environmental characteristics (e.g., water depth). This makes it challenging to derive specific *a priori* hypotheses and predictions about the associations between behaviors and environmental characteristics in this understudied group of viviparous fishes. Nevertheless, based on how environmental characteristics generally influence behaviors in other fish species (**Table 1**) and our knowledge on halfbeak biology, we predict that: (i) increases in canopy cover will be associated with reduced swimming behavior, fewer sexual and agonistic interactions and an increase in stationary behavior, (ii) increases in aquatic vegetation will be associated with a reduction in sexual and agonistic interactions and an increase in time spent stationary and/or swimming, (iii) increases in water depth will be associated with a reduction in time spent swimming, in sexual and agonistic interactions and an increase in time spent stationary, (iv) increases in water flow will be associated with a reduction in sexual interactions, an increase in agonistic interactions, a reduction in time spent swimming, and an increase in time spent stationary (keeping the same position against the flow). We also predict that, (v) males halfbeaks will perform more agonistic behaviors than females, while females will perform more stationary (anti-predator) behaviors than males.

MATERIALS AND METHODS

Field Sites

To quantify fish behavior, we performed non-invasive field observations on 26 pygmy halfbeak shoals in July–August 2019. All field observations were approved by the National Parks Board

TABLE 1 | Summary of how environmental characteristics can typically influence socio-sexual and locomotor behaviors in fishes.

Environmental characteristic	Effects of environment	Potential effects on behaviors
Canopy cover	Abundant canopy cover decreases food availability (Binckley and Resetarits, 2007; Zandonà et al., 2011) and/or quality (Wong and Fikri, 2016) for insect-eating fish, reduces light intensity and thereby the efficacy of visual cues used in courtship or the visual information available to predators (Vales and Bunnell, 1988; Binckley and Resetarits, 2007), and/or increases predation risk from piscivorous birds that use canopy as stakeout locations (Loegering and Anthony, 1999).	Increasing canopy cover may decrease sexual interactions due to reduced efficiency of sexual signaling and increased predation risk, reduce agonistic behaviors due to increased predation risk, reduce swimming due to reduced food availability, and increase stationary behavior due to increased predation risk.
Aquatic vegetation	Increasing aquatic vegetation can increase (Barreto et al., 2011; Bhat et al., 2015; Suriyampola and Eason, 2015) or reduce (Danley, 2011) aggression. In general it should reduce visual contact among conspecifics and thus social interactions (Basquill and Grant, 1998), unless it increases fish density (Kaspersson et al., 2010). Aquatic vegetation can also reduce predation risk (Orrock et al., 2013) by offering more refuges for prey against piscivorous fish (Camp et al., 2012) but not birds (Lantz et al., 2010).	Increasing aquatic vegetation may reduce agonistic behaviors unless aquatic plants are defensible resources. All social interactions should decrease due to fewer encounters with conspecifics. Aquatic vegetation may also reduce anti-predatory behaviors (e.g., remaining stationary) due to reduced predation risk and increase swimming as a search for conspecifics or sexual mates.
Water depth	Deeper water can increase predation risk, as piscivorous fish are more abundant in deeper water (Linehan et al., 2001; Rypel et al., 2007). Water depth may also alter shoal size and social dynamics, affecting hierarchy and social interactions (Sneddon et al., 2006; Flood and Wong, 2017).	Deeper water may reduce sexual and agonistic interactions if fish density decreases, while it may increase anti-predatory behaviors (e.g., remaining stationary and reducing swimming behavior) due to increased predation risk.
Water flow	Faster flowing water can restrict spatial movements of fish and thus reduce foraging areas (Salonen and Peuhkuri, 2007) and reduce or alter movement (Allouche and Gaudin, 2001). It can affect predators/prey interactions (Gemmell et al., 2014; Stewart et al., 2014), increase competition for food (Suriyampola et al., 2017) and increase energetic costs of swimming (Head et al., 2010). Water flow can alter fish communication (Mogdans and Bleckmann, 2012) and shoal size (Hockley et al., 2014) and shoal dynamics by either making shoals more (Suriyampola et al., 2016) or less cohesive (Suriyampola et al., 2017).	Faster flowing water may generally reduce all social interactions due to reduced communication possibilities. Sexual interactions may be reduced due to increased energetic costs, while agonistic behaviors may increase due to increased competition for resources. Fish may increase stationary behaviors and reduce swimming behaviors in response to increased flow (fish stay more in the same position).

We focus on four commonly studied environmental characteristics – canopy cover, aquatic vegetation, water depth and water flow – and describe how increases in each environment characteristics can potentially alter factors relevant for fish behaviors. We then suggest potential effect(s) of each environment characteristics on the socio-sexual (courtship and agonistic) and locomotor (swimming and stationary) behaviors we examined in this study.

of Singapore (permit number NP/RP19-077a). All shoals were located within three separate water systems (see **Supplementary Figure 1**), including eleven shoals at Jurong Lake Gardens (1°20′11.0″N 103°43′41.0″E), seven shoals in Seletar river in the Springleaf Nature Park (1°24′01.0″N 103°48′59.6″E), and eight shoals in an unnamed stream along Squirrel Trail located in Windsor Nature Park (1°21′36.3″N 103°49′36.6″E). Shoals were identified each day of observation within each water system as a semi-stable aggregation of halfbeaks along a stretch of water's edge. Shoals were not observed far from the water edge (within 2 m) and never covered an area larger than 2 m in diameter. Adjacent shoals were considered separated if at least 3 m distant. Occasionally, individuals or small subgroups (1–4 individuals) were observed to leave their shoal, possibly toward other shoals. In these cases, we still considered the shoals as independent units (see below). We performed real-time behavioral observations of adult male and female pygmy halfbeaks (described below) in each shoal.

Behavioral Observations

A total of 175 focal observations were performed on male ($n = 103$) and female ($n = 72$) halfbeaks across the 26 shoals. Focal observations were performed on 6.73 ± 0.50 (mean ± standard error) individual fish per shoal. For males, focal observations were performed on 3.96 ± 0.40 (mean ± standard error) males per shoal, with fish being observed for 136.2 ± 6.76 s (range = 7–325 s). For females, focal observations were performed on 2.77 ± 0.19 females per shoal, with fish being observed for 200.4 ± 7.10 s (range = 18–319 s). Observations ended when the focal individual swam out of view or after 330 s (i.e., ∼5 min) of recording. Behavioral observations were performed during the day between 9 AM and 5 PM (Singapore standard time; GMT + 08:00). None of the behaviors examined in this study were affected by the time of day during which observation occurred (LMM; $\chi^2 < 4.43$, $p > 0.11$, see **Supplementary Table 4** for all the details). Because our data is zero inflated, a common feature of behavioral data, we calculated average behavioral scores within each shoal and day of observation. Thus, we treated the shoal as the individual unit of replication in our analyses (note that we obtained qualitatively similar results when we treated individuals as the unit of replication in zero inflated models that accounted for shoal identity). In three cases, two shoals were sampled at the same location on different days. In these cases, we assumed that the observed fish were independent as it is unlikely we observed the same fish on different days.

Observation took place from the shore, bridges, or platforms, close to the edge of the water. After finding a shoal, the observers (AD and EFI) positioned themselves as close as possible to the water's edge and remained as still as possible. Focal observations started when the fish resumed normal behaviors (usually within few minutes). Briefly, a clearly visible focal individual was randomly chosen in the shoal, with no regard to its current behavior. After selecting a focal individual, the focal observation was immediately initiated. Sex was determined during the observation based on body size (adult females are larger than adult males) and occurrence or absence of the typical sex-specific behaviors (see section below for detailed descriptions). If sex of the fish was not clearly established, the observation was excluded from the analyses. Focal observations within the same shoal and day were performed consecutively. To minimize (not exclude) the risk of resampling the same individual, a new focal individual was selected from another area in the shoal from where the previous focal individual was last seen. Since a large number of observations in a small shoal increases the risk of observing the same individual twice, we limited the number of observations for all shoals and thereby additionally reducing the risk of pseudo-replication.

During observations, a verbal description of the behavior of a single focal individual was recorded using the audio-recording function of a smartphone. Socio-sexual and locomotor behaviors were categorized using a previously defined protocol, based on a consistent ethogram (modified from Greven, 2010), and are described below. Audio files where analyzed using BORIS software (Friard and Gamba, 2016) and behaviors were coded in an excel file for each individual observation. As observations varied in duration, we time-standardized all behavioral scores to obtain the number of behaviors performed per minute.

Sexual Behaviors

Halfbeaks spend considerable amounts of time performing sexual interactions, mainly in the form of courtship behaviors (Greven, 2010; Ogden et al., 2020. see also the **Supplementary video**). Courtship usually begins with an individual, generally the male, slowly *approaching* in the line of sight of an individual of the other sex. If courtship continues, the male either starts *following* the female, where the male swims within 2–3 body lengths of the female, or *circling* the female, where the male slowly swims in a circular path around the female (Greven, 2010). As courtship continues, males perform a *swimming under* behavior, where the male's head is positioned under the female's genital pore while she maintains a position that is more or less stationary (Greven, 2010). Swimming under is the most conspicuous and time-consuming sexual behavior performed by males (Ogden et al., 2020; see results). While swimming under a female, males can perform *checking* behaviors, where the male touches the female's body with his upper beak, and nipping behaviors, where the male rapidly opens and closes his beak (note that observations of checking behavior were rare in this study and nipping was not observed at all as it is impossible to observe when viewing fish from above, Greven, 2010). *Switching to new female* may occur at any point during these bouts of courtship, where a male changes the female that the courtship behaviors are directed toward. Following often prolonged bouts of courtship, males can perform a *mating attempt*, where the male rapidly (∼40–80 ms, Greven, 2010) moves from the position under the female, twists his body and attempts to transfer sperm to the female's genital pore using a modified anal fin (andropodium). Females can likely influence male mating success through her decision to maintain a still position, although this has yet to be empirically validated. These behaviors were scored differently between the sexes as only males perform these distinct courtship behaviors.

For males, we recorded the duration and number of courtship behaviors directed toward females, while for females we recorded the duration and number of courtship behaviors they experienced during the behavioral observation. This allows us to describe sex-specific sexual interactions. Thus, if males and females are equally likely to perform and receive courtship behaviors then we would not expect sex effects to emerge in our analyses.

We derived two metrics of total courtship behaviors for each shoal. Within sex, we first created an index of the *total duration of sexual interactions* by summing the shoal-mean values of all continuous measures of courtship (i.e., the total time spent following and swimming under). We then created an index of the *total number of sexual interactions* by summing the shoal-mean values of all discrete courtship events that were observed (i.e., the total number of approaching, circling, checking, switching to a new female and mating attempts).

Agonistic Behaviors

Halfbeak males (and to a lesser degree females, see the **Supplementary video**) perform frequent agonistic interactions both within and between sexes (Berten and Greven, 1991; Greven, 2006; 2010; Reuland et al., 2020b). We recorded agonistic interactions between individuals, which were clearly visible when observing fish from above (see **Supplementary video**). Agonistic behaviors include *chase* and *being chased* behaviors, which occurs when a focal individual either pursues or is pursued by a conspecific at higher than normal swimming speed (often in a sprint, Greven, 2010). We also recorded the number of *displace* and *being displaced* behaviors, where an individual approaches another causing one to move away (Greven, 2010). Furthermore, we recorded the number of *frontal threatening* behaviors, where an individual aggressively approached a conspecific, usually from the front and without increasing swimming speed, with the two individuals facing each other (Greven, 2010). The frontal threatening behavior is often the first step toward a sustained escalated contest, but the conflict is usually resolved by an individual moving away before escalation. We recorded *biting behavior*, when individuals rapidly approach a conspecific and bite or hit it on the body or fins (Greven, 2010). Generally, escalated agonistic behaviors involving body contact between two individuals are rare, but when they do occur they involve fish biting each other and locking their beaks together (known as *wrestling*). *Wrestling* was never recorded in our observations.

We generated an index of the *total number agonistic behaviors* for each shoal by summing the shoal-mean values of the total number of chase/being chased, displace/being displaced, frontal threatening and biting behaviors observed, separately for males and females. Under laboratory conditions, halfbeak males form dominance hierarchies, where dominant individuals are more likely to perform agonistic behaviors and subordinate individuals are more likely to receive agonistic behaviors (Reuland et al., 2020b). Therefore, by including agonistic behaviors that were both performed and received by focal fish during the observations, our metric of the total number agonistic behaviors captures overall levels of shoal agonistic behaviors, regardless of a focal individual's position in a potential dominance hierarchy.

Locomotor Behaviors

When not involved in agonistic or courtship activity, halfbeaks perform locomotor behaviors at the water surface. Locomotor behaviors include *swimming*, moving spatially without direct interactions with conspecifics, and *stationary* behaviors, where individuals exhibit no spatial translocation and do not interact with conspecifics. We focus on the *total duration of stationary behaviors* and the *total duration of swimming behaviors* by calculating the shoal-mean values of each behavioral category for each shoal.

Characterization of Environmental Variation

After behavioral observations, we characterized the ecological characteristics of the location were the shoal was observed (**Supplementary Figure 1**). Specifically, we described the amount of canopy cover above each shoal, the density of aquatic vegetation, the depth of the water, and water flow at each site. To quantify canopy cover, AD and EFI stood by the water edge where a shoal was found and noted whether and how much land-based vegetation hung over the shoal within 2 m upstream and downstream from the shoal location. Estimates of canopy cover were made regardless of how high the vegetation was above the water surface. We classified shoals as having canopy cover that was absent (n = 10, no canopy above the observation site), intermediate (n = 8, incomplete canopy cover above the site) or full (n = 8, complete canopy cover above the site). The amount of aquatic vegetation present in the water where each shoal was observed was scored as present (n = 15, at least part of the shoal was observed swimming near aquatic vegetation), or absent (n = 11, no aquatic vegetation was growing in the water where shoal was observed). Water depth was estimated for each shoal by placing a stick of known length inside the water to estimate whether it was deeper or shallower than 30 cm and classified as either shallow (n = 16, $<\sim$30 cm) or deep (n = 10, $>\sim$30 cm). Water flow was classified as either still (n = 13) or moving (n = 13), based on the movement of floating debris on the water surface. We expect halfbeaks to be affected in a similar way as floating debris since halfbeaks occupy the very top of the water column.

Where possible, we estimated the number of individuals present in each shoal. To do this, AD and EFI independently counted the number of halfbeaks present at the site three times each. The average of the six counts was then used. We were unable to estimate shoal size in four shoals, reducing the sample size to 22 in analyses assessing shoal size.

Statistical Analysis

Assessing Covariance Among Socio-Sexual and Locomotor Behaviors

We examined sex-specific patterns of covariance among socio-sexual behaviors (total duration of sexual interactions, number of sexual interactions, number of agonistic behaviors) and locomotor behaviors (duration of stationary behavior and duration of swimming behavior) by constructing a Spearman correlation matrix. Correlations were calculated using the *Hmsic* package[1] in R (version 4.0.2), with each sex treated separately.

Principal Component Analysis on Fish Behaviors

When examining how socio-sexual and locomotory behaviors are associated with ecological variables, we first used principal component analysis (PCA) to combine our five behavioral index variables (total duration of sexual interactions, number of sexual interactions, number of agonistic behaviors, duration of stationary behaviors and duration of swimming behaviors) into a reduced set of orthogonal principal components (PCs). This analytical step reduced the number of independent tests performed when assessing if behaviors differ according to ecological variables, accounts for potential collinearity among behavioral variables, and facilitates comparisons of duration and count data in a single analysis. The PCA returned three PCs with eigenvalues ≥ 1, which cumulatively accounted for 90.0% of the total variance in the data and were considered in further analyses (**Supplementary Table 2**). When interpreting each PC, we considered loading values that are ~70% of the variable with the largest loading to contribute strongly to that PC (Mardia et al., 1979).

Do Socio-Sexual and Locomotor Behaviors Differ Based on Environmental Characteristics and Sex?

We performed distinct linear mixed effects models (LMM) to assess how each of the three PCs vary accordingly to four environmental variables when considered separately (canopy cover, aquatic vegetation, water depth and water flow). In all models, we included sex as a fixed effect and the interaction between each environmental variable and sex. Non-significant interaction terms were removed and simplified models are presented. All models include shoal identity as a random effect to account for the fact that males and females were sampled from the same shoal within each of the 26 shoals assessed. We refrained from building more complex models that included multiple environmental variables as we were concerned about overfitting our models given the number of shoals assessed ($n = 26$) and total number of levels in our fixed effects ($n = 3$ levels for canopy cover, $n = 2$ levels for aquatic vegetation, $n = 2$ levels for water depth, $n = 2$ levels for water flow). The LMM models were performed using the *lmer* function in the *lme4* package[2] in R [example of model's syntax: $PC1 \sim Sex^{*}Canopy + (1| Shoal)$]. The interaction between fixed effects was removed if not significant. Significant main effects of canopy cover (the only predictor variable with more than two levels) were examined using pairwise Tukey *post hoc* comparisons in the *emmeans* package[3] in R. The direction of all other significant effects was determined by examining model parameters and visually with the aid of the *effects* package[4] in R.

Group Size, Environmental Characteristics and Behavior

When assessing how shoal size differed among different environments, we accounted for the left skewed distribution of shoal size using generalized linear models (GLM) fitted with a quasipoisson error distribution using the *glm* function in R. As above, we examined ecological variables (canopy cover, aquatic vegetation, and water depth) as fixed effects in separate models. To assess how shoal size was related to socio-sexual and locomotor behaviors we performed separate LMM using the *lmer* function in the *lme4* package, where PC1, PC2, and PC3 were entered as dependent variables (in separate models) whereas group size (log transformed) and sex were included as fixed effects and shoal identity was entered as random effect. Non-significant interaction terms were removed and simplified models are presented.

A Note on Water Systems

Although the shoals originated from three different water systems (i.e., Jurong Lake Gardens, Springleaf Nature Park, Windsor Nature Park), we were unable to incorporate this data structure into our analyses for several reasons. First, we could not model the different water systems as random effects, as three levels are too few to allow for reliable estimates of the among-sites variation (Harrison et al., 2018). Second, our dataset is not large enough to adequately incorporate many random factors (Harrison et al., 2018). Third, the ecological variables we investigated varied differently within each water system, which leads to overparameterized and unbalanced models when including water system as a fixed effect in our analyses. Given these limitations, we did not include water system in our analyses, as our primary aim was to assess how intraspecific behaviors vary across sex and ecological and social context.

RESULTS

Descriptive Summary of Halfbeak Behaviors and Shoal Size

Sexual, agonistic and locomotor behaviors were variable among shoals and between the sexes (summarized in **Supplementary Table 3**). Despite general similarities in the pattern of socio-sexual behaviors observed in male and female halfbeaks, the duration and number of sexual interactions, as well as number of agonistic behaviors and duration of swimming duration, was reduced in females. The mean shoal size was 34.59 (±7.68 S.E.) across 22 shoals but the number of individuals in each shoal varied widely, ranging from 6–128 individuals.

Within shoals, males *followed* females (16.18 ± 1.74 s/min, mean ± S.E.) nearly eight times longer than they *swam under* females (2.06 ± 0.54 s/min, mean ± S.E.). When considering the total duration of sexual interactions recorded, males spent nearly one third of their time performing either *following* or *swimming under* behaviors, although in one shoal males devoted as much as 74% of their time in performing courtship behaviors. Similarly, females were *followed* by males

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[1] https://cran.r-project.org/web/packages/Hmisc/index.html

[2] https://cran.r-project.org/web/packages/lme4/index.html

[3] https://cran.r-project.org/web/packages/emmeans/index.html

[4] https://cran.r-project.org/web/packages/effects/index.html

(10.06 ± 1.68 s/min, mean ± S.E.) eight times longer than they had males *swimming under* them (1.23 ± 0.33 s/min, mean ± S.E.). However, females had a total duration of sexual interactions (courtship behavior received from males) for around one fifth of their time. When considering discrete sexual interactions (see **Supplementary Table 3** for details), males performed on average 1 (0.97 ± 0.19 behaviors/min; mean ± S.E.) discrete sexual interactions (including *approaching, circling a female, checking, switching to a new female* or *attempting to mate*) every minute within the shoals, with *circling a female* being the most common (0.41 ± 0.13 behaviors/min, mean ± S.E.) discrete behavior observed. While females also received more *circling behaviors* (0.13 ± 0.04 behaviors/min, mean ± S.E.) than any other discrete courtship behavior, they experienced on average less than one sexual interaction every 2 min (0.43 ± 0.08 behaviors/min, mean ± S.E.).

Chasing behaviors made up the vast majority of agonistic behaviors observed in both males (0.85 ± 0.26 behaviors/min, mean ± S.E.) and females (0.15 ± 0.08 behaviors/min, mean ± S.E., see **Supplementary Table 3**). However, while within a shoal, males performed an average of 1.78 (±0.30 S.E.) agonistic behaviors each minute, females performed 0.23 (±0.10 S.E.) agonistic behaviors each minute. All other agonistic behaviors occurred less frequently, with females generally showing lower rates of agonistic behaviors than males (**Supplementary Table 3**).

Males spent slightly more time *swimming* than *stationary*, and together these locomotor behaviors accounted for an average of 57% of the males' time in the shoal (34.38 ± 2.14 s/min, mean ± S.E, see **Supplementary Table 3**). In contrast, females spent more than twice as much of their time remaining *stationary* than *swimming*, with these locomotor behaviors making up an average of 77% of a female's time in the shoal (46.22 ± 1.81 s/min, mean ± S.E., **Supplementary Table 3**). When only considering behaviors where durations were recorded, it was clear that males allocated roughly equivalent amounts of time to performing sexual (mean of 30.4%), stationary (mean of 24.4%) and swimming (mean of 32.9.5%) behaviors (**Supplementary Table 3**). Females spent about a fifth of their time on sexual interactions (receiving courtship behaviors from males, mean of 18.8%), a fifth on swimming (mean of 23.4%), and more than half of their time on remaining stationary (mean of 53.6%). The remaining time (12.3% for males and 4.2% for females) was not measured directly and was spent by individuals in performing discrete behaviors (e.g., *chasing, biting,* or *circling*) that were not considered when measuring continuous behavior.

Assessing Covariance Among Socio-Sexual and Locomotor Behaviors

There was evidence for covariance among socio-sexual and locomotor behaviors in both males and females (**Table 2**). In shoals where males performed longer durations of sexual interactions, males also performed greater numbers of sexual interactions. Males faced an apparent trade-off in their time budget, performing sexual behaviors for shorter durations and swimming for shorter durations when they stayed stationary for longer durations (**Table 2a**). Among females, there was apparently a similar trade-off, with stationary duration being negatively correlated with both the duration of sexual interactions (received courtship behaviors) and swimming behaviors. None of the other behaviors were correlated for either sex (**Table 2**). In particular, agonistic behaviors were not significantly associated with any other behavior for either males or females.

Principal Component Analysis of Fish Behaviors

The first principal component (PC1) explains 43% of total variability and is primarily loaded positively by the total duration of stationary behavior and negatively by the duration and number of sexual interactions (**Supplementary Table 1**). Therefore, increases in PC1 reflect individuals remaining stationary for long durations and performing (males) or receiving (females) less courtship behaviors. The second PC (PC2) explains 27% of total variability and is primarily positively loaded by the total duration of swimming behavior and negatively loaded by the duration of sexual interactions (**Supplementary Table 1**). Increases in PC2 are consistent with reductions in courtship behaviors and increases in swimming behavior. Finally, the third PC (PC3) explains 20% of total variability and is primarily loaded by the total number of agonistic behaviors (**Supplementary Table 1**), such that increases in PC3 indicate more frequent agonistic behaviors.

Do Socio-Sexual and Locomotor Behaviors Differ Based on Environmental Characteristics and Sex?

Variation in canopy cover was associated with a shoal-level difference in PC1, the composite measure of stationary behavior and sexual activity (LMM: $\chi^2 = 8.05$, $p = 0.02$, **Figure 1A** and **Table 3a**). *Post hoc* tests revealed that PC1 values were significantly lower when the canopy cover was absent than when canopy cover was full (Tukey *post hoc*: t-ratio = 1.13, $p = 0.03$), while PC1 values in sites with average canopy were intermediate and did not differ from sites where the canopy cover was either absent (Tukey *post hoc*; t-ratio = 0.24, $p = 0.83$) or full (Tukey *post hoc*: t-ratio = 0.89, $p = 0.12$). This indicates that longer and more numerous sexual interactions and shorter stationary behaviors were performed in sites where the canopy cover was absent, while the opposite occurred in sites where the canopy cover was full. PC2, the composite measure representing duration of swimming behavior and sexual activity, and PC3, the composite measure of agonistic behaviors counts, were not associated with variation in canopy cover (**Table 3a**).

Neither aquatic vegetation nor water depth were associated with any difference in PC1, PC2, or PC3, suggesting that halfbeak behavior did not differ among variants of these environmental characteristics (**Tables 3b,c**). The composite measure of duration of swimming behavior and sexual activity (PC2) significantly varied according to water flow (LMM: $\chi^2 = 5.53$, $p = 0.02$, **Table 3d**), with longer sexual interactions occurring for both males and females in sites with still water and longer durations

TABLE 2 | Spearman correlation coefficients (above the diagonal) and respective *p* values (below the diagonal) are shown for the total duration and number of sexual interactions, the total number of agonistic behaviors, and the total duration of stationary and swimming behaviors in each shoal in (a) males and (b) females.

	Sexual interactions (duration)	Sexual interactions (number)	Agonistic behaviors	Swimming behaviors	Stationary behaviors
a) Males					
Sexual interactions (duration)	NA	**0.64**	–0.13	–0.34	**–0.47**
Sexual interactions (number)	**<0.01**	NA	–0.33	–0.15	–0.31
Agonistic behaviors	0.53	0.10	NA	–0.23	0.01
Swimming behaviors	0.09	0.47	0.26	NA	**–0.44**
Stationary behaviors	**0.01**	0.12	0.97	**0.03**	NA
b) Females					
Sexual interactions (duration)	NA	0.08	0.06	–0.08	**–0.45**
Sexual interactions (number)	0.69	NA	–0.05	0.31	–0.31
Agonistic behaviors	0.76	0.81	NA	0.03	–0.22
Swimming behaviors	0.71	0.12	0.87	NA	**–0.78**
Stationary behaviors	**0.02**	0.12	0.27	**<0.01**	NA

The sexual interactions for males is based on the performed courtship behaviors while for females it is based on received courtship behaviors. Significant correlations are highlighted in bold.

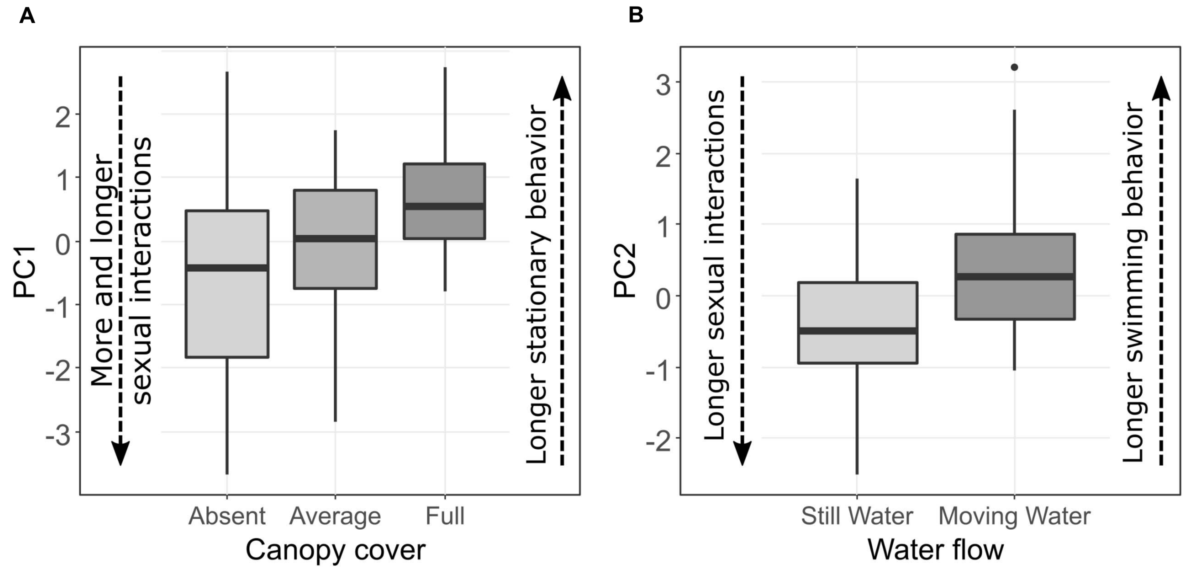

FIGURE 1 | The boxplots represent in **(A)** the association between PC1 (43% of variance explained) and canopy cover, where duration and number of sexual interactions decrease and stationary behavior increases with increasing canopy cover. In **(B)** the association between PC2 (20% of variance explained) and water flow, where duration of sexual interactions increases and time spent swimming decreases in still water compared to moving water.

of swimming behaviors in sites with moving water (**Figure 1B**). PC1 and PC3, on the contrary, were not affected by water flow.

PC1 values significantly differed between the sexes, with males performing more sexual interactions for longer durations, while females spent more of their time remaining stationary within each shoal (LMMs: $\chi^2 = 48.15$, $p < 0.001$; see **Tables 3a–d** and **Figure 2A**). PC2 did not differ between sexes (LMMs in **Tables 3a–d**, **Figure 2B**), suggesting that the contrast between the time spent in sexual interaction and swimming was not different across sexes. For PC3 there was a clear influence of sex, with males performing more agonistic behaviors than females (LMMs: $\chi^2 = 12.33$, $p < 0.001$; **Tables 3a–d**, **Figure 2C**). Significant differences between the sexes in PC1 and PC3 were consistent across all models (**Tables 3a–d**). In

TABLE 3 | Effect of sex and environmental characteristics on PC scores representing sexual, agonistic and locomotor behaviors.

Response (PC)	Predictor	df	χ^2	p
a) Canopy cover				
PC1	**Canopy cover**	**2**	**8.05**	**0.02**
	Sex	**1**	**48.15**	**<0.001**
PC2	Canopy cover	2	0.80	0.67
	Sex	1	0.36	0.54
PC3	Canopy cover	2	2.43	0.30
	Sex	**1**	**12.32**	**<0.001**
b) Aquatic vegetation				
PC1	Aquatic vegetation	1	0.52	0.47
	Sex	**1**	**48.15**	**<0.001**
PC2	Aquatic vegetation	1	0.49	0.48
	Sex	1	0.36	0.54
PC3	Aquatic vegetation	1	1.29	0.25
	Sex	**1**	**12.33**	**<0.001**
c) Water depth				
PC1	Water depth	1	0.01	0.90
	Sex	**1**	**48.15**	**<0.001**
PC2	Water depth	1	3.05	0.08
	Sex	1	0.37	0.54
PC3	Water depth	1	1.13	0.29
	Sex	**1**	**12.33**	**<0.001**
d) Water Flow				
PC1	Water Flow	1	0.51	0.44
	Sex	**1**	**48.15**	**<0.001**
PC2	**Water Flow**	**1**	**5.53**	**0.02**
	Sex	1	0.36	0.54
PC3	Water Flow	1	0.87	0.35
	Sex	**1**	**12.33**	**<0.001**

Each environment was tested separately for each PC. PC1 explains 43% of total variance and is the composite score representing duration and number of sexual interactions (negatively loaded) and duration of swimming (positively loaded). PC2 explains 27% of total variance and is the composite score representing duration of sexual interactions (negatively loaded) and duration stationary behavior (positively loaded). PC3 explains 20% of total variance and is the composite score representing agonistic interactions (positively loaded). Statistically significant results in bold.

general, these finding suggest that males perform less stationary and more agonistic behaviors as well as experienced more and longer sexual interactions than females across a range of environments.

Group Size, Environmental Characteristics and Behavior

Shoals were larger in the absence of aquatic vegetation (GLM; $\chi^2 = 7.61$, df = 1, $p < 0.01$) and in sites with still water (GLM; $\chi^2 = 6.10$, df = 1, $p = 0.01$). In contrast, canopy cover (GLM; $\chi^2 = 0.99$, df = 2, $p = 0.61$) and water depth (GLM; $\chi^2 = 0.99$, df = 1, $p = 0.32$) were not associated with shoal size. Furthermore, shoal size was not related to any of the composite measures of socio-sexual or locomotor behaviors (LMMs; PC1, $\chi^2 = 0.21$, df = 1, $p = 0.64$; PC2, $\chi^2 < 0.01$, df = 1, $p = 0.95$; PC3, $\chi^2 < 0.01$, df = 1, $p = 0.97$).

DISCUSSION

By comparing socio-sexual and locomotor behaviors among natural halfbeak shoals, we demonstrate that halfbeaks behave differently in different environments. However, contrary to our predictions, only two of the environmental characteristics considered influenced how halfbeak shoals invest time in different types of activities. Sexual interactions and locomotor behaviors varied according to both canopy cover and water flow. In contrast, neither the depth of the water nor the presence of aquatic vegetation showed any association with sexual or locomotor behaviors. Agonistic behaviors were frequent across all shoals and robust to changes in the external environment. Males were less stationary than females and spent more time swimming. Moreover, males were more aggressive and experienced on average more sexual interactions than females. We observed larger shoals in environments with still water as well as in sites characterized by the absence of aquatic vegetation. Shoal size did not affect any behavior considered, but large shoals may require more individuals to be sampled to get a better estimate of mean behaviors. Together, these results provide some support for the hypothesis that environmental characteristics covary with socio-sexual and locomotor behaviors among halfbeak shoals.

Canopy cover was associated with differences in socio-sexual and locomotor behaviors in wild halfbeak shoals, with halfbeaks performing more sexual interactions when canopy cover was absent and remaining stationary for longer durations when canopy cover was full. There are multiple non-mutually exclusive explanations for these results. First, dense canopy may reduce food (i.e., insect) availability (Binckley and Resetarits, 2007; Zandonà et al., 2011). As halfbeaks exclusively feed on small invertebrates on the surface of the water, changes in food availability associated with increased canopy cover may lead halfbeaks to prioritize energy conservation at the cost of socio-sexual interactions and movement or alter halfbeak feeding behaviors toward a 'sit and wait' strategy. Second, canopy cover limits the amount or intensity of light reaching the water surface with possible effects on mate choice based on visual stimuli (as seen for example in *P. reticulata* Endler, 1987; and in the poison frog *Dendrobates pumilio*, Summers et al., 1999). Halfbeaks may therefore perform fewer costly sexual displays when light conditions are poor. If this is the case, it would be interesting to test if shoals permanently inhabiting environments with reduced light intensity have evolved morphological differences compared to populations in open areas. Such variation in male ornamental coloration due to differences in light condition have been previously observed in sticklebacks (Rick et al., 2014) and guppies (Kemp et al., 2018). Third, canopy cover may alter predation risk. As pygmy halfbeaks are specialized surface feeders and spend most of its time at the water surface, avian predation is likely a main predation pressure in this species (Ho et al., 2015). The rich avian fauna of Singapore counts eight different kingfisher species, as well as twenty-two species of herons (Davison and Yeap, 2010; Nature Societey (Singapore), 2020), most of which potentially prey on halfbeaks. Canopy could increase shelter and stakeout locations for such piscivorous birds. Stationary behavior may be a successful anti-predator strategy against avian predators

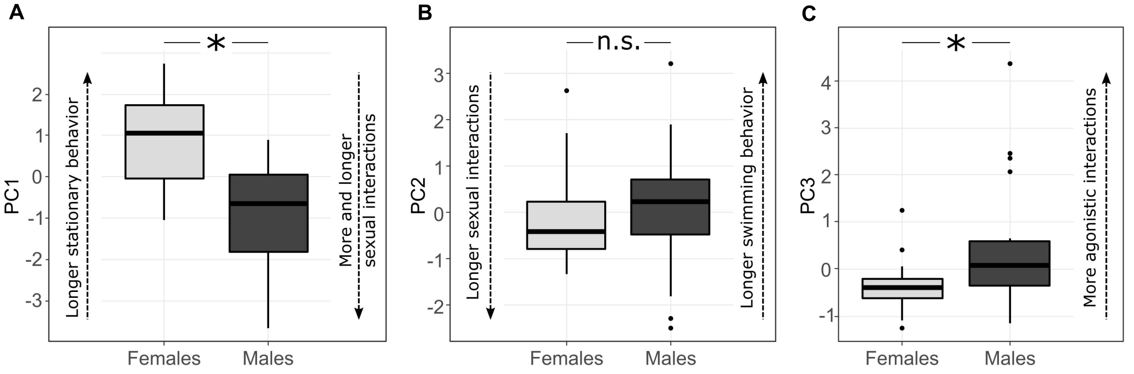

FIGURE 2 | The boxplots represent the differences between sexes in: **(A)** PC1 (43% of variance explained); females spent more time in stationary position and experienced less and shorter sexual interactions compared to males. **(B)** PC2 (27% of variance explained); males and females did not differ in how much time they were observed swimming or experiencing sexual interactions. **(C)** PC3 (20% of variance explained); Males performed more aggressive behaviors than females. *Significant effect of sex in all the LMMs, n.s.: non-significant effect of sex in all the LMMs (see **Table 3**).

in halfbeaks, and in our study we found a negative correlation between sexual interactions and remaining stationary, which supports a prediction from the predation hypothesis (Loegering and Anthony, 1999; Kelley, 2008).

Water flow rate was associated with an expected reduction in sexual interactions but also an unexpected increase in swimming behavior. Swimming across the water body may enhance fish success in finding floating food. Moreover, faster flowing water can reduce shoal size (Hockley et al., 2014) and shoal cohesion (Suriyampola et al., 2017). It seems plausible that in less cohesive shoals, males may spend more time moving in search of females and less time in courting them. Additionally, halfbeak mating success is strongly dependent on the ability of males and female to maintain synchronized positions. It is possible that faster water flow reduces fish ability to stay still and males therefore reduce mating effort as copulation success is low. Sexual interactions may be more common on days when the water flow is slower (e.g., in the dry season). Sampling the same locations on several different days and/or times of the year and directly quantify flow rates would help to further resolve how water flow shapes halfbeak behaviors.

Interestingly, our findings of how halfbeaks respond to differences in canopy cover and water flow differ from the patterns found in guppies, *P. reticulata*, a model system where the numerous interactions between environments and behaviors are well-studied. In most Trinidadian drainages, the original habitat of guppies, it is well established that guppy populations living upstream of waterfall barriers show different characteristics than populations living downstream of waterfalls (Endler, 1995). These differences include age and body size at maturity, male coloration, sexual behavior (reviewed in Houde, 1997) and ejaculate traits (Devigili et al., 2019). Specifically, male guppies in downstream populations are smaller and less colored, and perform fewer sexual displays. Downstream Trinidadian rivers are characterized by less canopy cover (Torres-Dowdall et al., 2012) and slower water flow (Reznick et al., 2001). Our finding of increased sexual behaviors when canopy cover is absent and water flow is low therefore contrast with the body of literature on guppies. Importantly, these among-population differences in guppies are related to predator abundance directly, rather than stream characteristics *per se*. Guppy predation is mainly due to other fishes, typically species of Characidae and Cichlidae families (Magurran, 2005). These guppy predators are absent in upstream sites, releasing upstream population from predation and resulting in evolutionary-derived differences between guppy populations. In contrast, the distribution of potential halfbeak predators in Singapore suggests piscivorous fish and birds are present throughout all of the local halfbeak habitats we evaluated (Davison and Yeap, 2010; Baker and Lim, 2012). Hence, we do not expect some water bodies to be deprived of potential predators. Instead, local variation in environmental conditions might influence the small-scale distribution of halfbeak predators within same sites or the antipredatory behavioral response of prey fishes. These differences may explain why our results suggest that guppies and halfbeaks respond differently to the environmental characteristics of canopy cover and water flow.

Water depth and aquatic vegetation were not associated with the behavioral traits measured in halfbeaks. Both of these environmental characteristics were hypothesized to influence the strength of predation from piscivorous fish. Our results therefore suggest that predation imposed by other fishes actually may be low or constant in the studied populations (Ho et al., 2015). However, additional surveys and experiments are necessary to validate this hypothesis. Contrary to our hypothesis, as well as a recent laboratory experiment demonstrating that adding a visual obstruction to the environment reduces aggression between male halfbeaks (Reuland et al., 2020b), in this study aquatic vegetation was not associated with differences in agonistic interactions in natural halfbeak populations. It is plausible that the specialized surface-dwelling lifestyle of halfbeaks may reduce the importance of both water depth and aquatic vegetation in shaping socio-sexual behaviors, particularly when compared with other fish species that swim throughout the water column.

We observed large variation in group sizes across shoals. Larger shoals were found in environments characterized by the absence of aquatic vegetation and slower flowing water. A simple explanation for this result is that when the space at (or just below) the water surface is restricted, individuals are unable to form large shoals. Alternatively, larger shoals may provide more benefits in obstacle-free environments. For example, in other systems, aquatic plants can work as refuge against predators (Lantz et al., 2010; Orrock et al., 2013; Penaluna et al., 2016) but in environments lacking such refuges, gathering in bigger groups can reduce predation through dilution, group guarding and improved collective decision making (e.g., Magurran et al., 1985; Godin, 1997; Hoare et al., 2004; Brown et al., 2006; Ward et al., 2011). Still or slowly flowing water has been linked with larger aggregations of fish compared to faster flowing water (e.g., Hockley et al., 2014; Eggertsen et al., 2016), but the opposite has been shown as well (e.g., Shelton et al., 2020). Many factors interact with flow to alter shoaling behavior, for example, parasite infection (Hockley et al., 2014) and the presence or absence of predators (Allouche and Gaudin, 2001). Furthermore, freshwater fishes like chub (*Leuciscus cephalus*) seek out more turbulent waters in areas with high flows since turbulence acts as a cover from avian predators (Allouche and Gaudin, 2001). Clarifying the interaction between shoal size, environmental characteristics and predation risk will be an important next step.

Socio-sexual and locomotor behaviors consistently differed when measured in males and females. Sexual interactions and agonistic behaviors were more pronounced in males than females and females stayed stationary for longer across all environments. Halfbeaks therefore seem to conform to classic sex roles, with males performing more agonistic behaviors, primarily directed at other males, and being the courting sex. The difference in the amount of sexual interactions measured in males and females support the recent finding of strong male mate choice in the halfbeak (Ogden et al., 2020). In the laboratory, male halfbeaks prefer females with large gravid spots which may indicate female sexual receptivity (Ogden et al., 2020). Similar male preferences for females with large gravid spots in natural populations could explain the sex-specific rate in sexual interactions we detected, although this idea requires further study. An alternative explanation is that natural shoals have female-biased sex ratios. This explanation seems unlikely however, as natural shoals appeared to have either equal or slightly male biased sex ratios (AD and EFI pers. obs.). Moreover, females likely spend time out of the mating pool when gravid, suggesting that the operational sex ratio is likely male biased. Therefore, we suggest that male mate choice represents the most parsimonious explanation for the sex-specific difference in sexual interactions.

CONCLUSION

In conclusion, we report that socio-sexual and locomotor behaviors vary in environments characterized by different canopy cover and water flow, but are not influenced by shoal size among natural populations of halfbeaks in Singapore. Key follow up studies should include, (i) assessing whether behavioral variation within and between sexes is influenced by more complex environmental interactions, (ii) testing these complex interactions experimentally, (iii) determining if separated populations evolved according to environmental differences, and (iv) whether there are genetic signatures of this difference across populations. This study represents the first examination of halfbeak behaviors in natural populations and highlights the suitability for studying behavioral ecology using this species. We also suggest that future research may focus on comparing the pygmy halfbeak, together with other halfbeaks species, with the well-studied Poeciliid fishes (sensu Reznick et al., 2007).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by National Parks Board of Singapore NP/RP19-077a.

AUTHOR CONTRIBUTIONS

AD, EFI, and JF designed the study and analyzed the data. AD, EFI, and NP organized the field work. AD and EFI collected the data. All authors contributed in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.607600/full#supplementary-material and includes the R code used to perform all statistical analyses.

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Con- and Heterospecific Shoaling Makes Invasive Guppies More Risk Taking

*Abigail Santiago-Arellano[1,2], Vianey Palomera-Hernandez[2] and Morelia Camacho-Cervantes[2]**

[1] Unidad Xochimilco, Universidad Autónoma Metropolitana, Mexico City, Mexico, [2] Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City, Mexico

Invasive species are one of the greatest threats to biodiversity. Behavioral traits are recognized as key to promote individual's survival in changing conditions. For social species being part of a group is key to carry out vital activities. Heterospecific social environments could provide exotic species with the opportunity to join groups and gain the advantages of being part of a larger population. Short latency to exit a refuge is a behavioral response that could be linked to invasion success as it increases the chances of individuals to locate food sources and other resources in novel environments. The guppy *(Poecilia reticulata*), a successful invader, has been found to take advantage of the presence of native species to reduce its refuge emergence latency and acquire information. The research was carried out in Mexico, we investigated the effect of heterospecific social contexts that include natives and other invasive viviparous fishes on guppies' refuge emergence latency. We found that guppies' emergence latency was shorter when accompanied by another guppy than when alone. Their latency was also shorter when with other invaders and when with native goodeids, but with one of the invaders (*Pseudoxiphophorus bimaculatus*) and with goodeids (*Skiffia bilineata*) latency reduction was not as high as when with conspecifics or with the invader *Poecilia gracilis*. Our experiment supports both the idea that already established invaders could provide benefits to new ones, and that native species also provide benefits but less than invaders. Increasing our knowledge about conspecific and heterospecific social interactions that could make an exotic species become invasive is key to assess the invasion risk of a community.

Keywords: social context, risk-taking behavior, heterospecific interactions, poeciliids, goodeids

Edited by:
Bart Pollux,
Wageningen University and Research,
Netherlands

Reviewed by:
David Bierbach,
Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Germany
Lysanne Snijders,
Wageningen University and Research, Netherlands
Andres Hagmayer,
Wageningen University and Research, Netherlands

Correspondence:
Morelia Camacho-Cervantes
mcc@cmarl.unam.mx

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INTRODUCTION

Accelerated changes in the world threaten biodiversity and favor the spread of invasive species that modify the structure of the communities they invade (Early et al., 2016). Behavior is important at each stage of the invasion process (i.e., movement, introduction, establishment, and spread) and understanding the mechanisms that promote or decrease individual's fitness at each stage is crucial when trying to predict the success or failure of unintentional introductions of species (Chapple et al., 2012). Invasive species are considered among the greatest threats to biodiversity, the main cause for extinction for birds and the second main cause for mammals and fish (Clavero and Garcia-Berthou, 2005; Simberloff et al., 2013). During the first stages of invasion, the low availability of conspecifics could decrease the chances for a species to establish due to the shortage of conspecifics to effectively perform vital tasks like foraging or avoiding predators (Liebhold and Bascompte, 2003). Even though animals can still forage and evade predators, they often do it better when in the company of others (Snijders et al., 2020).

Social animals tend to become part of groups when the advantages of doing so outweigh the disadvantages (Krause et al., 2002; Ward, 2012). When facing a low availability of conspecifics exotic individuals might choose to join heterospecific groups. Indeed, the "meltdown theory" states that previously established invaders promote invasion of new species as a result of interactions between them that enhance survival or population size, which could be through habitat modification, seed dispersal or any social interaction in which species benefit from each other (Simberloff and Von Holle, 1999; Simberloff, 2006; Rennó Braga et al., 2018). However, little research has been done on whether native species could act as facilitators as well. There are some examples in fish (Camacho-Cervantes et al., 2014a) and lizards (Damas-Moreira et al., 2018), but to the best of our knowledge not a comparison has been made on how an invasive species could act as a facilitator compared to a native one.

Risk-taking behavior has been recognized as valuable when establishing in a novel environment, as it could enhance individuals' response to changing conditions by enabling them to explore novel objects or areas, which might lead to the opportunity to find food, new refuges, partners, among others (Rehage and Sih, 2004; Reale et al., 2007; Sol et al., 2018). Risk-taking behavior is part of the shy-bold continuum and some of the ways in which variation of risk-taking behavior in fish is quantified are: novel object test (inspect-bold, do not inspect-shy), latency to feed under risk of predation (feed-bold, don't feed-shy) and the novel environment test (emerge quickly bold, emerge slowly shy) (Huntingford et al., 2013). Individuals must constantly assess costs and benefits of risk-taking behavior, and benefits (such as reproduction enhancement and food acquisition) should outweigh the risk of encountering predators or competition so that risk-taking behaviors actually results in benefits (Reale et al., 2007). An animal then must continually decide whether to remain sheltered or emerge into an open habitat depending on current factors such as its energy status and vulnerability to predation (Sih, 1992; Dowling and Godin, 2002).

The guppy (*Poecilia reticulata*) is an invasive species with a natural distribution range in Trinidad, Guyana, Venezuela and Suriname. They inhabit shallow rivers and ponds (Seghers et al., 1995; Magurran, 2005). As invaders, guppies are reported present in every continent except Antarctica (Deacon et al., 2011), including the Central Mexican Plateau where they are expanding its invasive range (Contreras-MacBeath et al., 1998; Gesundheit and Macías Garcia, 2018). They manage to survive and settle at temperatures (Chung, 2001; Reeve et al., 2014) and salinities (Chervinski, 1984) that are distant from those of their native environment. For example, guppies can be found in unusual locations such as the Moscow sewer (Zhuikov, 1993) and the River Lee in Essex, England (Wheeler, 1998); or in Germany where temperature can drop to 12°C (Lukas et al., 2017), in these places artificial heating effluents keep the water temperature high enough for them to survive. The guppy is also a very social species that performs most of its vital tasks in groups (Magurran, 1999, 2005) and one of the characteristics that is thought to have favored guppies' invasion in the Mexican Central Plateau (MCP) is their tendency to associate with native Goodeids, which translates into benefits such as locating food faster or acquiring information on food availability (Camacho-Cervantes et al., 2014a, 2015). The guppy is not the only invasive poeciliid present in the MCP, porthole livebearers (*Poeciliopsis gracilis*) and twospot livebearers (*Pseudoxiphophorus bimaculatus*) have also managed to establish populations in the area (Contreras-MacBeath et al., 1998; Gesundheit and Macías Garcia, 2018).

Given that guppies derive benefits from native species, and that this could be one of the tools they use during the first stages of invasion when conspecifics availability is low (Camacho-Cervantes et al., 2014a, 2015), the present investigation aims to research if the latency to exit a refuge when shoaling with conspecifics, other invasive or natives species changes. We hypothesize that guppies will show a lower latency to leave the refuge to an unknown area when they are accompanied by other invaders than when they are in the company of native Mexican goodeid twoline skiffia (*Skiffia bilineata*), given that poeciliid species are more associated with risk taking behaviors than goodeids (Valero et al., 2008; Brown et al., 2009). If so, then by being more prone to take risks invasive guppies could gain some of the benefits associated with it, such as locating food faster, however they might be under higher predation risk too, as this is also related to risk taking behavior. This information could contribute to the idea that poeciliids might be part of an invasive meltdown mechanism in the Mexican rivers.

MATERIALS AND METHODS

Subject Species

The guppy (*Poecilia reticulata*)—is a freshwater invasive species that belongs to the Poeciliidae family. It is one of the most popular aquarium fish with many standardized varieties in the world for its easy maintenance. Guppies are able to establish populations in a wide range of conditions (Gibson and Hirst, 1955; Chung, 2001); in the wild, guppies can occupy aquatic habitats that are highly turbid as well as pristine ponds, canals or lakes (Rodriguez, 1997). They belong to a group of ovoviviparous fish whose females are able to store sperm for up to 6 months and their offspring can be from different males (Meffe and Snelson, 1989; Hain and Neff, 2007). Thus the release of even one sexually mature female can result in the establishment of viable invasive populations in the wild (Deacon et al., 2011). Guppies are omnivorous and have a strong sexual dimorphism. Females are light grayish in color and continue to grow throughout their lives, reaching a body length of around 4 cm but up to 6 cm. Males grow to sexual maturity and do not usually measure more than 4 cm, they present a color pattern composed mainly of yellow, orange and black spots (Magurran, 2005). Guppies are carriers of exotic parasites, and are considered a threat to native fishes, as the decline of several endangered species have been linked to its invasion (Magellan and Magurran, 2009; Global Invasive Species Database, 2020).

The Porthole livebearer (*Poeciliopsis gracilis*)—is a freshwater species from the Poeciliidae family. Native to central America found from Southern Mexico to Honduras. The species has been introduced in the MCP in the Balsas river basin, in the states of Guerrero and Michoacán (Miller et al., 2009). This species inhabits quiet water bodies of streams, flood-water ponds, lagoons, micro reservoirs and lakes, and is normally found within waters that can range from clear to muddy (Meffe and Snelson, 1989; Miller et al., 2009). Males and females are only differentiated by the modified anal fin of males (gonopodium). Males grow up to 5 cm while females are usually larger reaching up to 6.6 cm (MCC Pers. Obs.). However, little is known about the biology and particular mechanisms of invasion of this species, particularly what is related to their behavior because its study is a relatively new approach to elaborate managing plans and to identify potential invaders (Carere and Gherardi, 2013). To the best of our knowledge, there are no published records of the particular way in which porthole livebearers pose a threat to native species, but Gesundheit and Macías Garcia (2018) recognized invasive poeciliids in general as a threat to native species in the MCP.

The Twospot livebearer (*Pseudoxyphophorus bimaculatus*)—is a freshwater species belonging to de Poeciliidae family. Is native to the Atlantic slope drainages of Central America, occurring from the Misantla River in central Veracruz, Mexico, southwards to the Prinzapolka River in Nicaragua (Miller et al., 2009). This species has also been translocated into a number of drainages in Mexico, including the Teuchitlan drainage in Jalisco and the upper Balsas drainage (Ayotac River) in Guerrero (Ramírez-García et al., 2018). Is an habitat generalist that can be found in lotic and lentic systems, lakes and seasonal wetlands (Pérez Alvarado et al., 2004; Escalera-Vázquez et al., 2017). The twospot livebearer has the ability to change its diet according to the alterations in the habitat structure, water quality, and biotic integrity of places it inhabits, it is recognized as omnivore (Carbajal-Becerra et al., 2020). As the porthole livebearer, the twospot livebearer has not been as thoroughly studied as the guppy in its invasion contexts. However, this species is more aggressive than the guppy and grows up to 6.7 cm (MCC Pers. Obs.). They are also carriers of fish parasites that are exotic to the MCP (Salgado-Maldonado et al., 2014).

The Twoline skiffia (*Skiffia bilineata*)—the native fish in this study, is a freshwater species, belonging to the Goodeidae family. This species is endemic to the MCP, where it is found in the Rio Lerma Grande de Santiago basin and the Rio Grande de Morelia basin within the States of Michoacán and Guanajuato. This species can be found in freshwater river systems, in quiet, shallow, muddy, and typically slow waters (depths of less than 1 m over mud), with occasional dense vegetation (Koeck, 2019). The species is threatened by a continuing decline in the quality of its habitat, as a result of water pollution from human activities, predation and competition from introduced invasive alien species (De La Vega-Salazar et al., 2003a.). Because of this it is considered an endangered species in the IUCN Red List (Koeck, 2019). Twoline skiffias are also omnivores and are amongst the smallest goodeid species. They are sexually dimorphic, females reach up to 5 cm while males reach up to 6 cm. Its reported optimum temperature range is 19–22°C (Ornelas-Garcia et al., 2012).

Experimental Design

Experiments were carried out in the Institute of Marine Sciences and Limnology of the Universidad Nacional Autónoma de México in Mexico City from the 4th to the 26th of June 2019. Twoline skiffias used in this experiment were collected from outdoor ponds in the Institute of Ecology (19°18′44′′N 99°11′46′′O); guppies, porthole livebearers and twospot livebearers were collected from Mixquiahuala in the Tula River (20° 30′ 25′′ N, 99° 14′ 44′′ W), each species was collected at different ponds created by trees' roots on the river side. The ponds were apart from each other by 10–15 m. The fish were collected with scoop nets and traps on the riverbank, in periods of 15 min. After collection, all fishes were carefully transported to the aquarium at the Institute of Marine Sciences and Limnology in breathable bags, with Pentabiocare®, salt and zeolite, which is a filter substrate. Fish were kept in the aquarium for 3 weeks prior to the trials in separate stock tanks (40 L) for each species that contained between 25 and 30 fish per tank. Tanks were set up with aged tap water—which is tap water left in a container with an aerator for at least 8 days to evaporate any trace of chlorine. Water was also treated with a solution called Pentabiocare®, which is a high-performance colloidal conditioner with a high concentration of Thiamine (Vitamin B1, also known as antineurotic vitamin). This solution has the property of helping fish to better withstand stress conditions in the different stages of collection, such as transport, capture and during fish adaptation period to the conditions of a new aquarium. Each tank contained a filter, gravel at the bottom, a water pump and plastic plants. Photoperiod was 12L:12D. Water daily temperature ranged between 19 and 22°C. Tanks were visually isolated from one another and individuals used in each trial were kept separated for at least 2 weeks prior to observations to avoid familiarity effects (Griffiths and Magurran, 1997). Fish were fed with commercial flake food daily. To avoid sexually motivated behavior we only used female fish of all species in our experiment, at least in the case of guppies females have been found to allocate more time to shoaling and foraging behavior than males (Sievers et al., 2012).

Our experiment had an independent measurement approach, as all observations were made independently from each other; each fish (focal guppy or companion) were observed only once and discarded to a tank in a different section of the aquarium to avoid confusions. We observed focal guppies that were randomly selected prior to each observation in five different treatments: (1) alone, (2) focal guppy accompanied by a conspecific, (3) by a porthole livebearer, (4) by a twospot livebearer and (5) by a twoline skiffia. Observations for each treatment were made randomly but ensuring that at least one of each was made each day. The observation tank was 50 × 30 × 30 cm and was filled with water up to 26 cm. In addition, it contained gravel on the bottom and plastic plants that formed a 15 cm long refuge on one side of the tank (**Figure 1**).

Observations started when the focal guppy or couple were gently released in the refuge section of the tank by submerging the edge of the transporting container (100 ml) and letting the fish switch to the observation tank, which reduced stress (i.e., shaking, freezing, nervous swimming patterns). Both fish left the transporting container almost immediately to swim into the plants refuge. Observations lasted until both fish left the refuge. We recorded fish (both focal and companion) had exited the refuge when they were at least one body length away from the edge (this distance was calculated after averaging the standard length of 10 randomly selected fish from the stock). To ensure exiting the refuge was considered equally in each observation, we drew an indicating mark at the end of the refuge followed by another mark separated by the average standard length of fish, both marks were invisible to the fish. In none of the observations made fish displayed antagonistic behaviors. A total of 17 replicates for each treatment were made, we thus used 120 guppies, 20 porthole livebearers, 20 twospot livebearers and 20 twoline skiffias. Observations were made between 9:00 and 13:00 h. Focal guppy fish and companions were paired in size but still photographed after the end of each observation to measure their standard length. Average sizes of individuals used for each species were: guppies 49.4 mm (*SD* = 10.3 mm), porthole livebearers 41.8 mm (*SD* = 12.7 mm), twospot livebearers 50.5 mm (*SD* = 5.6 mm), and twoline skiffias 51.1 mm (*SD* = 9.7 mm). The average size difference between focals and companions was 2.5 mm (*SD* = 1.7 mm). Size difference between focals and companions was not normally distributed thus we ln-transformed it to achieve normality (Shapiro-Wilk: W = 0.96, p = 0.053). We tested for differences between treatments and since we did not find any [lm: $t_{(3,68)} < -0.38$, $p > 0.71$] we removed the difference in size between focals and companions from the analysis to improve clarity. Still, we incorporated size of the focal fish to explore its effect on emergence latency. Variables recorded and analyzed were: frequency of focal guppies exiting the refuge first, latency of the focal guppy to exit the refuge (s) and latency of the companion to exit the refuge (s). In addition, using exit latencies of focals and companions we calculated the difference between the time one and the other fish left the refuge by subtracting the time when the focal left from the time when the companion left, thus positive numbers mean focal exited first and negative companions left first.

Statistical Methods

We performed a *Z*-test to compare the proportion of times focal guppies were the first to exit the refuge in each treatment against the 0.5 null hypothesis proportion—focal and companions exiting first an equal number of times. Latency to exit the refuge of focal guppies, of the companion and difference between their emergence latencies were not normally distributed. Given that time is a continuous variable, we performed generalized linear models (glm) specifying Inverse Gaussian and Gamma distributions to later select the best model through the distribution of their residuals and their Akaike information criterion (AIC), models with the lowest AIC value score indicates the most parsimonious model to explain variation in data (Burnham and Anderson, 2002; Johnson and Omland, 2004). We included treatment, fish size, as well as the interaction between treatment and fish size as explanatory variables in all our models. However, in all models, the interaction between treatment and fish size was not significant, and thus, it was removed from the analysis to increase clarity. Since neither Gamma nor Inverse Gaussian distributions work with negative values we added a fixed number (200) to differences between emergence latencies of focals and companions in order to shift all values to positive. Finally, for variables that had a significant

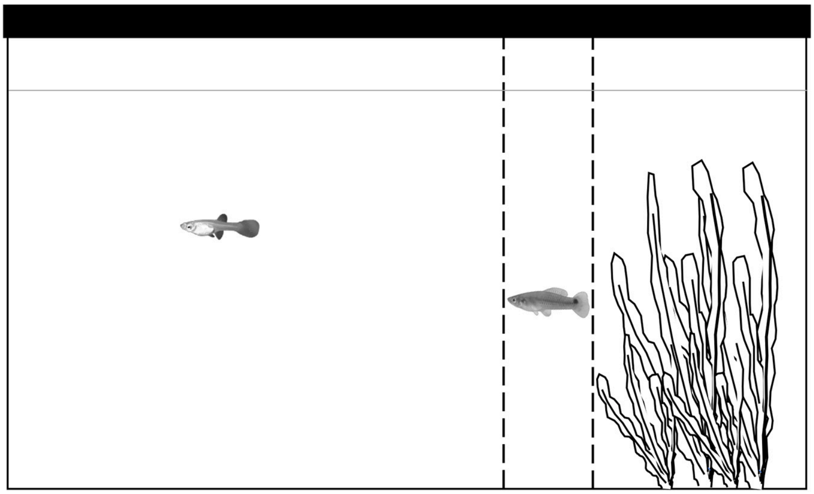

FIGURE 1 | Diagram of the experimental tank set up. Plastic plants were used to simulate a refuge, the refuge was identical for all observations. Observations started when fish were gently released in the upper right corner and lasted until both fish left the refuge, we considered fish had left the refuge when they were one body length (distance between dashed lines) away from the end of the refuge (dashed line closest to plants). We averaged the standard length of 10 fish randomly selected from the stock to calculate the distance between dashed lines, these lines were indicated for the observer and invisible to the fish.

p-value we carried out a Tukey *post hoc* (glht) analysis from the "multcomp" R package to identify the treatments that were significantly different (Bretz et al., 2016). The more parsimonious model to test focal's emergence latency was the one that specified Gamma distribution, for partner's emergence latency was the one that specified Inverse Gaussian and for the difference between focal's and partner's emergence latencies was Gamma; please refer to our **Supplementary Material 1** file for the complete output of our models plus their Residuals vs. Fitted, Normal Q-Q, Scale-Location and Residuals vs. Leverage plots. All our analysis were carried out in the R Studio statistical software (R Core Team., 2020).

RESULTS

All fish from all treatments left the refuge before reaching 6 min after release. In the four treatments that included a companion, guppies and the companion exited the refuge a similar number of times as the proportion of times guppies exited the refuge first was not significantly different from the 0.5 null hypothesis proportion ($Z_{17} < 1.2$, $p = 0.2$). Focal guppies' emergence latencies were different between treatments (glm: $|t|_{4,85} > 2.4$, $p < 0.02$, **Figure 2A**). Focals took longer when alone than when accompanied by conspecifics or porthole livebearers (glht: $|Z| > 2.7$, $p < 0.05$), and as long when with twospot livebearers or twoline skiffias (glht: $|Z| < 2.4$, $p > 0.1$). Size had no effect in the emergency latency of focal guppies (glm: $|t|_{4,85} < 0.1$, $p > 0.91$). Emergence latency of companions was similar between treatments and size did not had an effect on it [glm: $t_{(3,68)} < 1.27$, $p > 0.21$]. Regarding the difference between the emergence latencies of focals and companions, we found no difference between treatments and no effect of focals' size [glm: $t_{(3,68)} < 2$, $p > 0.054$, **Figure 2B**].

DISCUSSION

Behavior of an individual is influenced by the changing presence or absence of conspecifics and/or heterospecifics in the

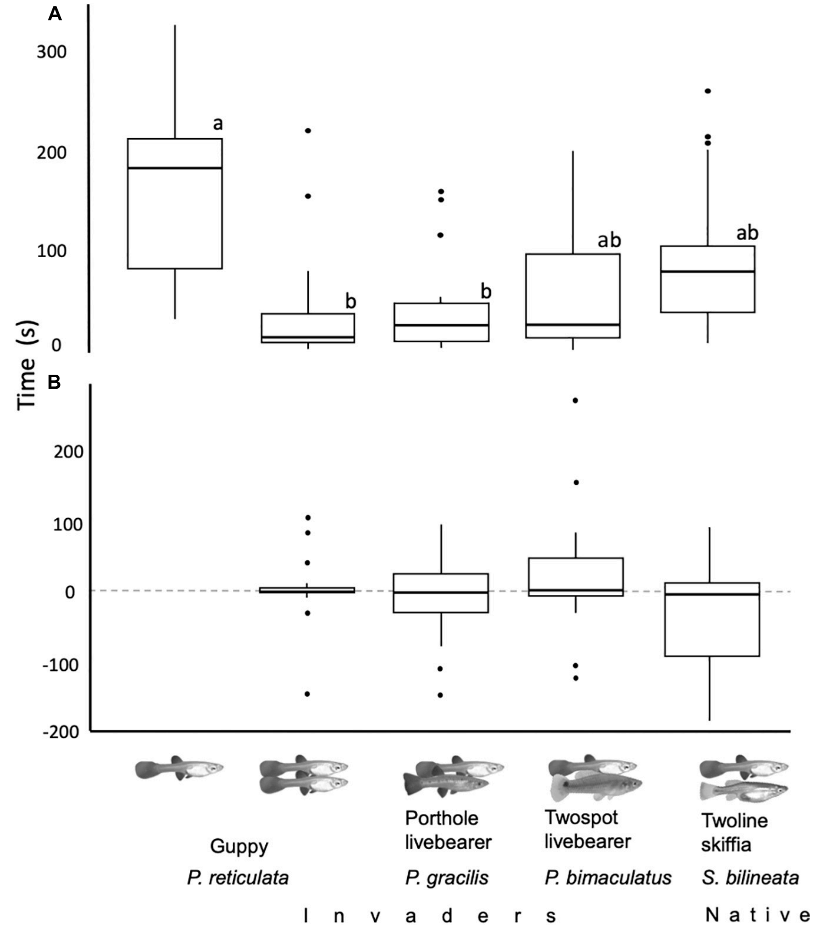

FIGURE 2 | (A) Time it took focal guppies to exit the refuge between treatments was different. They took longer to exit the refuge when alone than when accompanied, lower case letters represent significant differences between treatments. **(B)** Differences between the emergence latency of the first and the second fish were similar between treatments; positive numbers mean focal exited first and negative companions exited first. For both panels centerlines of boxplots represent the median, top and bottom of boxes represent the 25th and 75th percentile, while dots outliers.

environment (Ward, 2012). Traits of temperament, such as the tendency to take risks, affects how an individual interacts with conspecifics and heterospecifics they socialize with, as well as their reactions to predators, food sources, and habitat (Reale et al., 2007). In our study, guppies emerged quicker from a refuge when with a conspecific or an invasive porthole livebearer than when alone. When accompanied by a twospot livebearer or a goodeid, focal guppies emergence latencies were intermediate, similar as when accompanied by conspecifics and other species but not significantly different from when alone. We hypothesize that when with twospot livebearers guppies might not feel as secure as when with porthole livebearers being that twospot livebearers are generally more aggressive. However, we did not record any agonistic behavior in any of our observations, thus more research on the topic should be made before reaching any conclusion. Regarding focals emergence latency when with goodeids, the fact that the native twoline skiffias used in our experiment are less related and not sympatric to guppies might contribute to the decreased facilitation effect compared to the other (invasive) species.

Previous studies have found that size has an effect in fish risk taking behavior, Bierbach et al. (2020) found a pattern of "the bigger the better" in the leadership in schools of guppies, they proved that fish more easily followed a larger leader regardless of the size of the follower, while Lukas et al. (2021) found smaller individuals tend to be bolder. Nevertheless, in our experiment, we did not find evidence that body size influenced the emergence latency of guppies. Our results are in accordance with those of Harris et al. (2010). They found the variables that influence emergence latency of guppies the most are sex (males showed a higher tendency to risk taking behaviors) and predation risk in their origin locations (guppies from high predation risk locations were more prompt to risk taking behaviors) while size had no effect. This is also the case for *Brachyrhaphis roseni* and *Brachyrhaphis terrabensis*, Ingley et al. (2014) found these species' tendency to risk related behaviors are related to their sex (males are more eager to engage in risk taking behaviors than females) and the presence of predators in their origin locations (fish from high predation risk locations were more prompt to risk taking behaviors) while size had no effect. However this is not the case for all poeciliid species, Brown and Braithwaite (2004) examined the relationship between size and risk taking in eight populations of the Poeciliid *Brachyraphis episcopi* and found size showed influence in this species since larger fish took longer to exit the refuge.

Previous research has shown that when accompanied by others, fish spend longer in open (higher risk) areas and resume faster their foraging activities after a simulated predation attack (Webster et al., 2007; Magnhagen and Bunnefeld, 2009). Similarly, in our experiment, focal guppies showed a shorter emergence latency from a refuge when accompanied, by conspecifics or heterospecifics, than when alone. We hypothesize this could be due to the fact that social individuals, such as guppies, form groups to increase their safety from predators via collective detection and dilution effect (Krause et al., 2002). We acknowledge that our results are valid only for females as we only tested them, thus the behavior of males is still to be investigated. Generally speaking, guppy males have been found to be bolder than females (Godin and Dugatkin, 1996; Harris et al., 2010), and that females tend to be more at risk when accompanied by males (Piyapong et al., 2010). However, females could be more important than males in the dispersion of the species and colonization of further areas as a single sexually mature female could found a viable population (Deacon et al., 2011). Still, in the case of both sexes being present, males could even enhance females' tendency to take risks, but this idea would also need further testing before making any assumption.

When established invaders and exotic individuals have mutualistic associations or simply benefit from being close, there could be a facilitation mechanism for invasion of exotics, known as invasional meltdown (Simberloff, 2006; Green et al., 2011). This phenomenon remains controversial as some studies demonstrate empirical evidence for positive interactions that confirm the invasional meltdown hypothesis (Green et al., 2011). While other studies propose that non-native species have additive effects at ecosystem, community and population level, which does not support the invasional meltdown hypothesis (Rennó Braga et al., 2018).

Risk taking behaviors could aid fish populations to better cope with novel conditions as it might contribute to their spread into novel areas; individuals with a higher tendency to take risks could be at a better chance to find food sources, mating partners or shelter, which in turn could influence their invasion success (Rehage and Sih, 2004; Chapple et al., 2012). Our results suggest that, when conspecific availability is low, guppies could be as quick to exit a refuge to an unknown environment when associating with other invasive poeciliids as when they associate with conspecifics. This could represent a tool to take advantage from in their invasion context if conspecific individuals to associate with are scarce.

Although we only researched one behavioral response, emergence latency from a refuge, our results could be evidence to build on the idea of an invasion meltdown mechanism when certain other invaders are present, while still a facilitation mechanism when only natives are present. Previous research has found that guppies do prefer to associate with conspecifics than with native heterospecifics (Camacho-Cervantes et al., 2014b, 2018) and they derive more benefits from conspecifics than from native individuals (Camacho-Cervantes et al., 2014a). Which has been discussed as a native facilitation mechanism at least for food finding, transmission of information on food availability and boldness (Camacho-Cervantes et al., 2014a, 2015). We found a similar trend in our experiment and this could suggest that deriving benefits from either other invaders or natives is a tool that would be useful only during the initial stages of their invasion process, while conspecifics availability is low. Furthermore, given that species from which they derive benefits have similar ecological requirements, they could become competitors for space and food creating an invasional interference after settling down (Rauschert and Shea, 2017). This possibly suggests that the invasional meltdown facilitation is only a short stage until offsetting the disadvantages of being part of small populations in the early invasional stages (Drake, 2004; Tobin et al., 2011). However, in the case of our experiment, this would only prove true if indeed a higher tendency to take risks is advantageous (e.g., through finding food sources or novel areas to disperse and establish). More research is needed to test for different behavioral traits (e.g., predator avoidance, food finding) and population dynamics in time to be able to fully support this.

Our results build on the idea that sites that are already invaded may be at increased risk for new invasions, as natives and established invaders could aid exotic individuals to engage in risk taking behaviors (such as abandoning a refuge to explore an unknown area), which have been linked with invasion success (Sol and Maspons, 2016). Since we only researched one behavioral response, we acknowledge that more research on the positive interactions between invaders, other invaders and natives is needed to fully understand how social exotic species become successful invaders. Specially because species with similar ecological requirements, as the ones tested here, would be expected to be competitors and not allies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the experiments for the purposes of this article were conducted at the Universidad Nacional Autónoma de México (UNAM) in Mexico City using fish of four different species, three of which are invasive and considered pests in the site of collection and thus the Mexican Ministry of Environmental and Natural Resources (SEMARNAT) resolved we did not need a permit from them to collect these species. The native species we used has been kept for over 10 years in an outdoors pond in the Institute of Ecology (UNAM) for research purposes, we were granted a verbal permit to use these fish to perform our experiment by the researcher responsible of the population, Prof Constantino Macías-Garcia. Fish were transported to the laboratory following the Official Mexican Norm NOM-051-ZOO-1995 for humanitarian treatment in the mobilization of animals. Field and laboratory protocols followed all guidelines provided by the Mexican Official Norm NOM-062-ZOO-1999 for the use and maintenance of vertebrates for research purposes. Given that the experimental design involved behavioral observations in glass aquarium tanks, which did not include any surgery, anesthesia, or other invasive procedure that could have produced distress in the fish we were not required to get a permit from the Ethics Committee of the Institute of Marine Sciences and Limnology.

AUTHOR CONTRIBUTIONS

AS-A and MC-C designed the experiment. VP-H and AS-A collected the data. MC-C obtained the funding to carry out this research. All authors analyzed, discussed, and prepared this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.624245/full#supplementary-material

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Diurnal Changes in Hypoxia Shape Predator-Prey Interaction in a Bird-Fish System

Juliane Lukas[1,2]*, Felix Auer[3], Tobias Goldhammer[3], Jens Krause[1,2,4], Pawel Romanczuk[4,5,6], Pascal Klamser[5,6], Lenin Arias-Rodriguez[7] and David Bierbach[1,2,4]

[1] Department of Biology and Ecology of Fishes, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany, [2] Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt University of Berlin, Berlin, Germany, [3] Department of Chemical Analytics and Biogeochemistry, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany, [4] Cluster of Excellence "Science of Intelligence", Technical University of Berlin, Berlin, Germany, [5] Institute for Theoretical Biology, Humboldt University of Berlin, Berlin, Germany, [6] Bernstein Center for Computational Neuroscience Berlin, Berlin, Germany, [7] División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco, Villahermosa, Mexico

Animals often face changing environments, and behavioral flexibility allows them to rapidly and adaptively respond to abiotic factors that vary more or less regularly. However, abiotic factors that affect prey species do not necessarily affect their predators. Still, the prey's response might affect the predator indirectly, yet evidence from the wild for such a classical bottom-up effect of abiotic factors shaping several trophic levels remains sparse. In many aquatic environments, daily changes in oxygen concentrations occur frequently. When oxygen levels drop to hypoxic levels, many fishes respond with aquatic surface respiration (ASR), during which they obtain oxygen by skimming the upper, oxygenated surface layer. By increasing time at the surface, fish become more vulnerable to fish-eating birds. We explored these cascading effects in a sulfidic spring system that harbors the endemic sulphur molly (*Poecilia sulphuraria*) as prey species and several fish-eating bird species. Sulfide-rich springs pose harsh conditions as hydrogen sulfide (H_2S) is lethal to most metazoans and reduces dissolved oxygen (DO). Field sampling during three daytimes indicated that water temperatures rose from morning to (after)noon, resulting in the already low DO levels to decrease further, while H_2S levels showed no diurnal changes. The drop in DO levels was associated with a decrease in time spent diving in sulphur mollies, which corresponded with an increase in ASR. Interestingly, the laboratory-estimated threshold at which the majority of sulphur mollies initiate ASR (ASR_{50}: <1.7 mg/L DO) was independent of temperature and this value was exceeded daily when hypoxic stress became more severe toward noon. As fish performed ASR, large aggregations built up at the water surface over the course of the day. As a possible consequence of fish spending more time at the surface, we found high activity levels of fish-eating birds at noon and in the afternoon. Our study reveals that daily fluctuations in water's oxygen levels have the potential to alter predator-prey interactions profoundly and thus highlights the joined actions of abiotic and biotic factors shaping the evolution of a prey species.

Keywords: predator-prey interactions, bird predation, poeciliidae, hypoxia, hydrogen-sulfide

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Andrea S. Asphury,
Texas State University, United States

Reviewed by:
Michael Tobler,
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Jennifer Kelley,
University of Western Australia,
Australia

****Correspondence:***
Juliane Lukas
contact@julianelukas.com

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INTRODUCTION

Almost all organisms have to cope with changing environments during their lifetime (Bernhardt et al., 2020). The rate of change can occur at different temporal scales including diel and seasonal (e.g., light, temperature, oxygen, precipitation, wind, and water regime), as well as multiannual cycles (e.g., El Niño events), or can be completely unpredictable (aperiodic such as some human disturbances). Many environmental variables are correlated with selective conditions (e.g., resource availability or predation) and thus animals have evolved a suite of traits and strategies that allow them to detect and exploit environmental fluctuations to maximize their fitness (Bernhardt et al., 2020). For many animals, the first response to altered conditions is often behavioral (as opposed to physiological; Wong and Candolin, 2015), allowing for a rapid and (often) reversible response, which can be especially crucial in highly fluctuating environments.

Environmental fluctuations have the potential to affect not only individuals and populations, but also higher (community) levels through species interactions such as competition and predator-prey relationships (Nilsen et al., 2009; Cherry and Barton, 2017; Turner et al., 2017). For one, abiotic changes alter species distribution and consequently species overlap due to habitats becoming more or less accessible or favorable for survival. As a consequence, existing biotic interactions may cease or new ones may arise. For example, during periods of unfavorable water conditions, mobile species will seek out habitat refuges, but therein experience increased pressure of competition and predation (e.g., demersal species such as blue crab, croaker and spot in a river estuary with spatially and temporally dynamic hypoxia; Eby and Crowder, 2002). For another, the nature of the species interaction may change. Two species may respond differently to the same environmental condition, which can drastically shift interaction outcomes in favor of the one with greater tolerance or exploitation capacity [e.g., water turbidity affecting damselfly–fish interactions (Van de Meutter et al., 2005), temperature and oxygen affecting prey selection of an predatory insect (Cockrell, 1984), hypoxia increasing susceptibility of benthic prey to fish predation (Long and Seitz, 2008) or decreasing a fish predator's foraging efficiency (Hedges and Abrahams, 2015)].

In aquatic ecosystems, oxygen is often a limiting factor and the lack of oxygen can cause detrimental stress to some animals (Wannamaker and Rice, 2000; Pollock et al., 2007; Galic et al., 2019). Many species leave oxygen-depleted (hypoxic) areas and move to normoxic regions when necessary (Pihl et al., 1991; Wannamaker and Rice, 2000; Eby and Crowder, 2002; Brady and Targett, 2013). Others are able to remain in the hypoxic areas due to specialized physiological, morphological, and behavioral adaptations that help minimize the effects of severe hypoxia (Pihl et al., 1991; Timmerman and Chapman, 2004). In fish, a common response to severe hypoxia is a compensatory behavior termed "aquatic surface respiration" (ASR), during which they utilize dissolved oxygen diffusing through the air-water interface (Kramer and Mehegan, 1981; Kramer and McClure, 1982; Chapman et al., 1995; Timmerman and Chapman, 2004; Tobler et al., 2009). Time allocated at the surface increases as oxygen decreases and while there are high costs associated with this behavior (both opportunity and predation; Kramer, 1983; Poulin et al., 1987), it is highly efficient against hypoxia-induced mortality (Kramer and Mehegan, 1981).

As a consequence of ASR, fish undergo a habitat compression, which often leads to dense aggregations at the surface and thus greater overlap with competitors and potential predators. Predators in the same medium (i.e., aquatic piscivores) will be exposed to the same hypoxic conditions. Depending on physiological tolerances of the specific interaction pair, this can hinder (Poulin et al., 1987) or favor the predator (Wolf and Kramer, 1987), yet, when predators cross ecosystem boundaries (e.g., aerial or terrestrial to aquatic), conditions experienced by one species may not be experienced by or even affect the other. Piscivorous birds, for example, will have advantages during periods of aquatic hypoxia. As prey becomes more clustered within surface waters (vertically, horizontally or both; Kramer, 1987; Eby and Crowder, 2002), it is easily accessible for avian predators. Also, hypoxia affects swimming activity and numerous anti-predator behaviors in fish (Domenici et al., 2000, 2007), thus shifting predation outcomes in favor of the birds. Empirical evidence for a link between hypoxia-induced ASR in fishes and aerial predation by birds was established in a laboratory setting, where an increase of hypoxia-induced ASR significantly reduced the survival of six species preyed on by a heron (Kramer et al., 1983). However, to date, evidence for such a classical bottom-up control of abiotic factors shaping predator-prey interactions in the wild remains sparse (but see Kersten et al., 1991). This is not least owing to the fact that predator-prey interactions are often complex (e.g., include multiple aquatic and aerial predators) and it is not always feasible to capture them at relevant temporal and spatial scales.

Here we report on a freshwater system that circumvents many of the above-mentioned observational shortcomings. In southern Mexico, poeciliids colonized multiple hydrogen-sulfide rich springs (Tobler et al., 2006; Palacios et al., 2013; Culumber et al., 2016). Hydrogen sulfide (H_2S) is toxic to most metazoans and often contributes to hypoxic conditions (Bagarinao, 1992). Consequently, respiratory adaptations are essential for survival in this environment. Evidence suggests that sulfide-adapted ecotypes can evolve in as little as 250 years (Brown et al., 2018). In the case of our study species, the sulphur molly *Poecilia sulphuraria*, sulfidic and non-sulfidic lineages genetically diverged between 15 and 30 ky ago (Greenway, 2019). While several morphological and physiological adaptations allow this species to persist in these conditions (e.g., mouth and gill area enlargement for enhanced oxygen uptake, modified toxicity targets and detoxification pathways; Tobler and Hastings, 2011; Tobler et al., 2011; Greenway et al., 2020), they are still dependent on aquatic surface respiration (ASR) for survival. A previous study estimated that sulphur mollies spend up to 84% of their time performing ASR (Tobler et al., 2009), which was three times higher than a closely related species (*Poecilia mexicana*) from a nearby (slightly less) sulfidic spring system. As a result of the species' high ASR rates, these fish frequently form large aggregations, likely to offset some of the risks associated with surfacing (equivalent to synchronous air-breathing;

Kramer and Graham, 1976; Chapman and Chapman, 1994). These aggregations likely attract non-aquatic predators, and bird predation rates are estimated to be 20-fold increased compared to surrounding clearwater habitats (Riesch et al., 2010a). As such, this system provides a unique natural laboratory to investigate how changes in oxygen can affect ASR in fish and upscale to influence the interaction with fish-eating aerial predators.

With hypoxia and high avian predation being ubiquitous features of this sulfide spring habitat (albeit some spatial variation in DO and H_2S along the 2.5 km long stream; Culumber et al., 2016), sulphur mollies need to respond behaviorally to mitigate the risks of hypoxia and predation, which will likely vary also temporally. Our objective was thus to examine the role of diurnal environmental variation in altering the bird-fish predator-prey interactions at the Baños del Azufre sulfidic springs. Our first aim was to observe the extent to which water conditions (especially temperature, DO and H_2S) varied during daytime. On the basis of the inverse relationship between temperature and DO, we predicted that daytime warming of the water would likely reduce DO levels and possibly affect H_2S throughout the day. We measured physico-chemical water parameters using standard methods for a period of 6 days at three times each day to capture diurnal variation of the system. Our second aim was to determine the role of hypoxia in influencing fish's ASR tendency and the concomitant surface use. Fish perform ASR to compensate for oxygen shortages in the water. However, H_2S also requires high amounts of oxygen for detoxification, and thus both may affect fish's ASR rates (and accompanied times at the surface). We predicted the highest ASR rates during the most unfavorable water conditions (low DO, high H_2S). Using a similar sampling regime (6×3), we quantified fish's ASR tendency by observing the duration of fish's voluntarily dives into the water column (with long dives equating to low ASR tendency). To further pinpoint how hypoxia levels affect ASR rates in this system, we established the threshold DO level at which fish initiate ASR in H_2S-rich water in a laboratory experiment. Our third and last aim was to examine how hypoxia-related variation in fish's ASR (surface) behavior affects bird predators. We hypothesized that high ASR rates would be associated with high bird activity, because birds are attracted by the easily accessible prey. We predicted piscivorous birds would exploit times when fish only dove for short periods (and thus spent most of their time at the surface) by increasing foraging efforts through staying longer and attacking more. To test this prediction, we quantified piscivorous bird activity whenever we measured the fish's ASR tendencies. To our knowledge, the present study is the first to investigate temporal variation in abiotic and biotic stressors and their implications for the surface behavior of a sulfide-adapted freshwater fish.

MATERIALS AND METHODS

Study System

Several springs in southern Mexico (states of Tabasco and Chiapas) are fed by sulfidic groundwater aquifers with high concentrations of hydrogen-sulfide (H_2S) generated from volcanic deposits and bacterial sulfate reduction (e.g., drainages Pichucalco, Tacotalpa and Puyacatengo; Rosales Lagarde et al., 2014). The Baños del Azufre (17°33′ N, 93°00′ W) describes an approximately 2.5 km section of the Rio El Azufre, which is associated with multiple sulfidic springs. A previous study characterized it as a freshwater habitat with high temperatures and sulfide content, low oxygen and pH, and high conductivity, which showed little temporal variation across years (T: 31.9 ± 0.7°C, H_2S: 190.4 ± 119.7 μmol/L, DO: 1.06 ± 0.92 mg/L, pH: 6.9 ± 0.1, EC: 2.7 ± 0.2 mS/cm; mean ± SD of 4 years; Tobler et al., 2011), but some spatial variation due to differences in habitat structure and spring discharge (Culumber et al., 2016).

The Baños del Azufre spring complex is inhabited by the endemic sulphur molly (*P. sulphuraria*) that shows distinct adaptations to these severe hypoxic and sulfidic conditions (for details see Plath et al., 2007; Riesch et al., 2010b; Tobler and Hastings, 2011; Tobler et al., 2011, 2018; Pfenninger et al., 2014; Kelley et al., 2016; Brown et al., 2017; Barts et al., 2018; Camarillo et al., 2020; Greenway et al., 2020). Most notably, fish spend a majority of their time performing aquatic surface respiration (ASR), but frequently dive to engage in benthic foraging as well as aggressive or reproductive activities under water (Tobler et al., 2009). Fish also dive in response to predation (Lukas et al., in review). Main predators in this system consist of fish-eating birds such as kingfishers, herons and egrets (**Supplementary Table 1**, Riesch et al., 2010a), while aquatic predators are mostly absent (see Riesch et al., 2009 for rare exceptions in less-sulfidic up- or downstream parts).

General Study Design

Preliminary observations revealed considerable temporal variation in fish's tendency to perform ASR during daytime hours, with large aggregations of fish building at the surface throughout the day (**Supplementary Video**). This pattern was reliably observable at various sites of the Baños del Azufre (pers. observations of the authors, 2016–2019). To allow for higher temporal resolution we based our investigations at a single, representative site exposed to moderate to high levels of sulfidic and hypoxic stress (refer to site 1 in Culumber et al., 2016). Additionally, this site allowed for access during all sampling times and fish and birds were already habituated to a degree of human presence.

To explore whether the observed diurnal differences in fish behavior are driven by physicochemical water conditions and how they link to predator activity, we conducted two field surveys and one laboratory experiment. During one field season (subsequently termed field survey I), we first quantified how variable physicochemical water conditions were throughout the day. In another field season (subsequently termed field survey II), we investigated the link between fish's behavior and predatory bird activity.

To link observations from both surveys, they were carried out at the same location and followed the same regimen by sampling each morning (07:00 – 09:30), midday (12:00 – 14:30) and afternoon (16:00 – 18:30) for six subsequent days. Surveys were matched for season (i.e., end of dry season; I: 12–17 April

2019, II: 05–10 May 2016) and we subsequently verified that no major deviations from the temperature trend occurred between years (compare **Figure 1A** and **Supplementary Figure 1**). Due to the spatial dynamics of predator-prey interactions, observations on water chemistry, fish and bird behavior were performed on slightly different spatial scales. While fish were clearly clustered in shoals with very little movement between, avian predators were much more mobile. Fish observations were based on a focal shoal, which reliably built up at the same location every day (crossing the Survey I transect; **Supplementary Video**). At its largest dimension, the focal shoal spanned completely across the stream's width and was estimated to reach about 15 m in length. For bird observations, we considered a slightly longer stretch of the stream (50 m with focal shoal in the upper half) and included predators along the stream's bench (∼2 m on each side), which were walking or perching close by (∼400 m^2 total study area). Ultimately, this approach did not allow for abiotic and biotic observations to be directly linked, nor for the behavior of individual prey to be correlated with a single predator. Still, it does provide first useful insights into the overall trend of the relationship between water chemistry, prey's surface aggregations and predator activity (see Statistical analyses on how we approached this).

Extremely high fish densities at times prevented us from reliably scoring fish's ASR (surface time) under field conditions as one would lose focus of individual fish at the surface. However, fish would interrupt ASR and drop below the surface for voluntary dives [i.e., without external disturbance; see description of the behavioral repertoire of *P. sulphuraria* above and similar observation on *Poecilia reticulata* (Kramer and Mehegan, 1981)]. To the human eye, these individuals were more conspicuous and their trajectory through the water column could be easily followed until they resurfaced again. While dive duration can merely present an inverse proxy of ASR tendency, we argue that it is a conservative assessment, considering that many individuals did not leave the surface at all when DO levels were low (see also **Supplementary Video**).

Lastly, to corroborate some of the above observations in a controlled laboratory experiment, we explored the proportion of time sulphur mollies spent performing ASR as well as the number and duration of dives at different dissolved oxygen levels without the confounding effects of variation in water chemistry or predator activity. It also allowed us to validate our ASR proxy by verifying that fish did not compensate shorter lasting dives by diving more often.

In their natural habitats sulphur mollies perform ASR in large groups. Such high social tendency has also been reported for sulfidic surface ecotypes of the closely related *P. mexicana* from a high predation site similar to ours (Bierbach et al., 2018, 2020). We therefore chose to test fish in groups. In nature, some individuals likely have to make compromises by surfacing earlier or later than they need to conform with group behavior. It is conceivable that a similar effect occurred in our experiment, so that some individuals (possibly due to differences in personality or metabolic demand) may have triggered the response of the entire group (Kramer and Graham, 1976; Chapman and Chapman, 1994; Borowiec et al., 2018; Killen et al., 2018). Nonetheless, when naturally shoaling species are tested in isolation, they are stressed and consume significantly more oxygen (see evidence for group's "calming effect": Queiroz and Magurran, 2005; Schleuter et al., 2007; Nadler et al., 2016). We are thus confident that our setup enabled us to gain biologically most relevant data on the DO thresholds for ASR behavior.

Field Survey I: Physicochemical Water Parameters

At each sampling, we took multiple measurements of water temperature, dissolved oxygen (DO), pH and electrical conductivity (EC), and – due to logistical constraints – one sulfide sample along a cross section of the sulfidic stream. Temperature, DO, pH and EC were measured using a multiparameter probe (WTW Multi 3630 IDS) fixed to a height-adjustable pole facing upstream. Calibration and measurements were carried out according to the manufacturer's recommendations. To capture variation introduced through flow regime and/or stratification within the water column, measurements were taken according to a transect grid (i.e., one sample each meter starting at 0.5 m away from the stream bench and at depths of 0.05, 0.25, and 0.5 m as water levels would allow). With a width of 5.6 m and a depth of less than 0.8 m at the site, this resulted in 10–11 subsamples per sampling.

For the quantification of total free sulfides (i.e., sum of concentrations of H_2S, HS^- and acid-soluble metallic sulfides), we collected a water sample from the stream center (width: 2.5 m, depth: 0.25 m) with a syringe. The first 1 ml sample was discarded to clear the tubing and the subsequent 0.2 ml sample was diluted with 3.8 ml distilled water to obtain a concentration within the desired measurement range. The total sulfide concentration was immediately analyzed by cuvette test (Hach Lange LCK 653) using a spectrophotometer (Hach Lange DR 2800) with automated recognition of the measurement program and internal calibration. We calculated the pH-dependent speciation of measured total free sulfide into H_2S and HS^- using a pKa1 of 6.9 and pKa2 of 17 (Stumm and Morgan, 1996).

Field Survey II: Diving Behavior of Sulphur Mollies

We assessed fish behavior using a focal animal sampling approach (see Tobler et al., 2009 for a similar approach to quantify fish's time budgets on site). An observer (i.e., field assistant with no prior knowledge of the hypothesis or its predictions) sat quiescent on the stream bench, and within a few seconds of arrival fish resumed normal swimming activity.

Twenty diving fish of similar size were chosen randomly from a focal shoal and observed from the moment they initiated diving until they resurfaced to calculate the mean *dive duration*. In rare cases, observations had to be terminated and repeated because a disturbance occurred during the diving period (e.g., shadow of an overflying bird) or because a fish swam out of sight.

Field Survey II: Activity of Avian Predators

We assessed bird activity prior to any fish observations to minimize disturbances caused by human activity. We made observations from a natural hide alongside the stream using binoculars. Three observers recorded all sightings of piscivorous birds in the predefined study area within a 30-min period. Observers were versed in identifying the bird species previously described to predate on *P. sulphuraria* (refer to Riesch et al., 2010a).

For each bird, we determined species (to lowest feasible taxonomical level), entry and exit times, as well as the number of attacks launched. Given the highly stochastic nature of predation in time and space, we selected two measures of bird activity that we deemed robust against differences in predator abundance and species differences in foraging styles. We calculated *presence time* as the mean time piscivorous birds spent in the study area (excluding mere fly-throughs, i.e., flying through the transect without landing or attacking) and *attacks* as the total number of bird attacks launched in a sampling period.

Experiment: ASR Tendency in Response to Hypoxia

We exposed fish to dissolved oxygen concentrations ranging from near-anoxic to normoxic conditions (0.6 – 5.1 mg/L DO). Due to logistical restraints (field lab in tropical climate), water temperature could not be controlled completely. As a consequence, we assigned fish *post hoc* to one of two temperature regimes (26.5°C or 28°C). Both temperatures are ecologically relevant as they represent the daily variations commonly experienced by the species between morning and afternoon.

We collected fish and water for the experiment from the same site previously used in Survey I and II. Fish were left to habituate in insulated coolers for at least 1 h without external disturbance. During habituation, fish were held in aerated water from the nearest freshwater source (5.5 mg/L DO). Treatment water was aerated to the desired DO level under constant mixing. DO and temperature were monitored with a multiprobe (OxyGuard Polaris 2) directly prior and after each trial to calculate a mean treatment value. Water was exchanged after each trial and testing was done under natural light.

We tested fish in groups of five adults visually matched for size. Under consideration of the sulphur molly's ecology, we deemed group testing both ecologically relevant and necessary to avoid isolation stress. We tested a total of 27 groups ($n = 135$) with 12 groups experiencing the morning (26.5°C; mean ± SD: 26.6 ± 0.3°C) and 15 groups the afternoon temperature regime (28°C; 27.9 ± 0.1°C). Fish were netted haphazardly and introduced into the front third of a test tank (30 × 20 × 30 cm, water depth of 20 cm). A trial lasted 10 min, in which experimenters left the area and fish's position in the water column was recorded with a camera (GoPro Hero6) facing the tanks' front. As part of the acclimation protocol, we did not analyze the first 5 min of each trial to ensue fish had recovered from handling and resumed swimming. We quantified the cumulative time spent at the surface by all five fish (see Video analysis) and calculated a percentage *surface time*. To link laboratory and field observations of fish's behavior (Survey II), we also assessed the *total number of dives* and *mean dive duration* performed by each group.

Experiment: Video Analysis

We analyzed videos obtained from the lab experiment using EthoVision 12 (Noldus Information Technology). The front view of the water column allowed for good monitoring of fish's depth position, but the view of individual fish was sometimes occluded as they moved slowly along the surface and thus did not allow for trajectories with individual identity. As a prerequisite for ASR, fish need to have direct surface contact, but experiments on another poeciliid (*P. reticulata*) showed that these fish rarely spent time near the surface without being in surface contact (Kramer and Mehegan, 1981). Based on fish' body depth, we defined the area less than 1 cm below the water surface as zone of interest, which was tracked for presence/absence of fish and corrected manually. We note that this approach may slightly overestimate ASR duration (e.g., due to brief transitions during up- and downward diving), nonetheless, we deem this negligible considering the overall observation period of 5 min.

Statistical Analyses

In order to make both field surveys comparable, it was necessary to reduce some information of the obtained variables for statistical analyses. Multiple measurements taken during a single sampling were pooled into one mean score (sample pooling). Data from the morning of day 1 was excluded in both surveys due to increased human activity affecting the surveys (Sunday morning church), resulting in $n = 17$ observations for each parameter. We tested for temporal variation in all parameters of interest by performing separate (generalized) linear regressions (package *lme4*; Bates et al., 2015) with time (morning/midday/afternoon) as a fixed effect. Regressions of dive duration and predator presence time were performed with Gaussian error structure. Both variables were $\log(y)/\log(y + 1)$-transformed to avoid negative predictions and normalize model residuals. We assumed predator attacks to be (approximately) Poisson distributed. Models were validated by visual inspection of the residuals. Significance of coefficients was evaluated via Likelihood-Ratio tests.

We further explored the association between parameters (cor.test in package *stats*; R Core Team., 2020). Observations of temperature, oxygen and H_2S were directly paired and fulfilled the assumption of a Pearson's correlation. For prey and predator behavior, we used Mann-Kendall rank correlations, which are robust against outliers and appropriate for small sample sizes.

To explore the relationship between oxygen and aquatic surface respiration of *P. sulphuraria*, we used a model selection approach. Eight hypothesis-driven candidate models were considered, allowing for linear and non-linear effects of DO on fish's surface time (**Table 2**). We did not include higher degree effects (>2) as we did not consider them biologically plausible. To account for an effect of test temperature, half of the models included the parameter but did no interactions due to the sample size. We fitted all models with binomial logit-normal

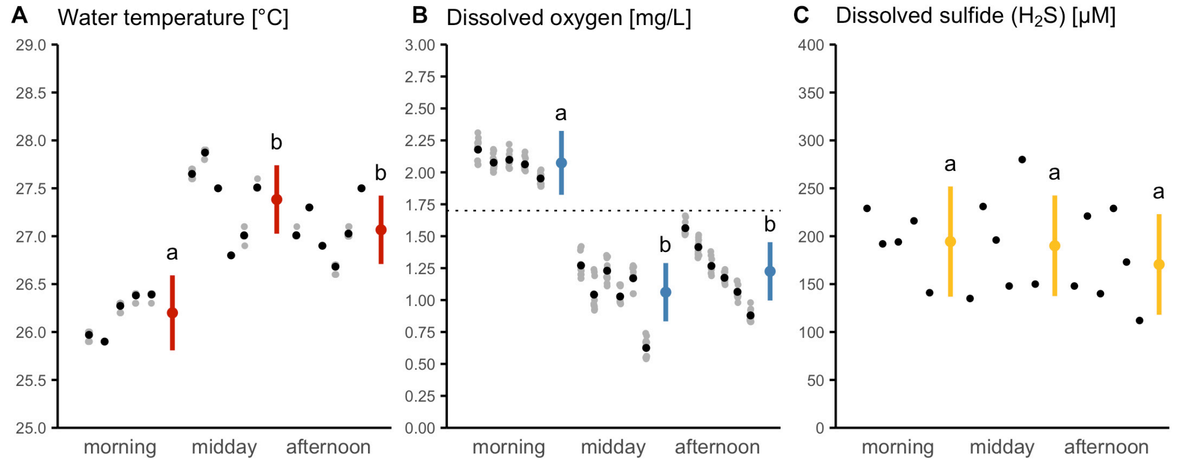

FIGURE 1 | Diurnal variation in water parameters of a sulfidic stream at Baños del Azufre, Mexico. A cross section of the stream was repeatedly sampled during three daytime periods on 6 consecutive days ($n = 17$ sampling occasions; morning Day 1 excluded). Shown are multiple measurements (gray), which were sample-pooled for analysis (black). Time of day affected **(A)** water temperature and **(B)** dissolved oxygen concentration, but not **(C)** dissolved sulfide concentration (see model-estimated marginal means with 95% CI in red, blue, yellow). Letters indicate results from *post hoc* pairwise comparisons. The dashed line indicates a laboratory-established threshold at which *Poecilia sulphuraria* predominantly performed aquatic surface respiration (ASR_{50}; see results below).

TABLE 1 | Descriptive statistics of diurnal variation in abiotic and biotic parameters at the Baños del Azufre sulfur spring complex.

Time of day	N	Water temperature (°C)	pH	Specific conductivity (mS/cm)	Dissolved oxygen (mg/L)	Dissolved free sulfides (μmol/L)		Prey dive duration (s)	Predator presence time (min)	Predator attacks (N)
						Total sulfide	H_2S			
Morning (07:00 – 09:30)	5	26.2 ± 0.2	6.7 ± 0.0	1.6 ± 0.0	2.1 ± 0.1	302.8 ± 50.9	194.4 ± 33.6	134.4 ± 6.6	0.3 ± 0.2	2.4 ± 2.5
Midday (12:00 – 14:30)	6	27.4 ± 0.4	6.6 ± 0.0	1.6 ± 0.1	1.1 ± 0.2	290.2 ± 85.5	190.0 ± 56.9	34.2 ± 8.4	3.4 ± 2.3	38.8 ± 28.7
Afternoon (16:00 – 18:30)	6	27.1 ± 0.3	6.6 ± 0.0	1.6 ± 0.0	1.2 ± 0.2	260.7 ± 70.5	170.5 ± 46.6	34.7 ± 9.0	3.5 ± 1.4	36.7 ± 18.4

Values expressed as mean ± SD derived from pooled-sample measurements from field survey I (shaded) and II (unshaded).

error structure (glmer function in package *lme4*; Bates et al., 2015) and added an observation-level random effect to account for overdispersion (Harrison, 2015). Models were evaluated by using Akaike's information criterion for finite sample sizes (AICc; package *MuMIn*; Bartoň, 2020). With this approach, the model with the lowest AICc as well as all models within 7 ΔAICc units are considered equally supported (Burnham et al., 2011). In addition, we used multimodel inference to compare the relative importance of main effects (i.e., sum of the relative evidence weights for all models in which the parameter appeared). Estimations of half-maximal DO concentration (ASR_{50}) for both temperature regimes were done on the basis of the global model.

Lastly, the more controlled laboratory experiment allowed us to validate our proxy of dive duration (Survey II) using direct measurements of ASR surface time. We first tested for differences in both number and duration of dives tested either above or below the established ASR_{50} threshold using Wilcoxon Signed-Ranks tests. We then estimated a Spearman correlation between surface time and dive duration (cor.test in package *stats*; R Core Team., 2020). All analyses were performed in R (see R script; R Core Team., 2020, version 4.0.2).

RESULTS

Dynamics of Physicochemical Water Conditions

Overall, the study site exhibited high temperatures, high concentrations of sulfides (especially H_2S), high specific conductivity, as well as low dissolved oxygen concentrations and low pH (**Table 1**). Temperature and DO varied significantly throughout the day (effect of *time:* temperature $F_{2,14} = 19.2$, $p < 0.0001$, DO $F_{2,14} = 36.8$, $p < 0.0001$), but were inversely related (Pearson's *rho* = −0.82, $t = -5$, $p < 0.0001$, $n = 17$). While mornings were associated with temperatures below 26.5°C and DO levels around 2 mg/L, temperatures rose and DO decreased to severely hypoxic levels toward later parts of the day (i.e., <1.7 mg/L DO; **Figures 1A,B**). Dissolved sulfide concentrations showed considerable variation between samplings (**Figure 1C**). We found no evidence for a diurnal cycle ($F_{2,14} = 0.4$, $p = 0.67$) or an association with temperature (Pearson's *rho* = −0.18, $t = -0.7$, $p = 0.5$, $n = 17$) or DO (Pearson's *rho* = 0.23, $t = 0.9$, $p = 0.4$, $n = 17$).

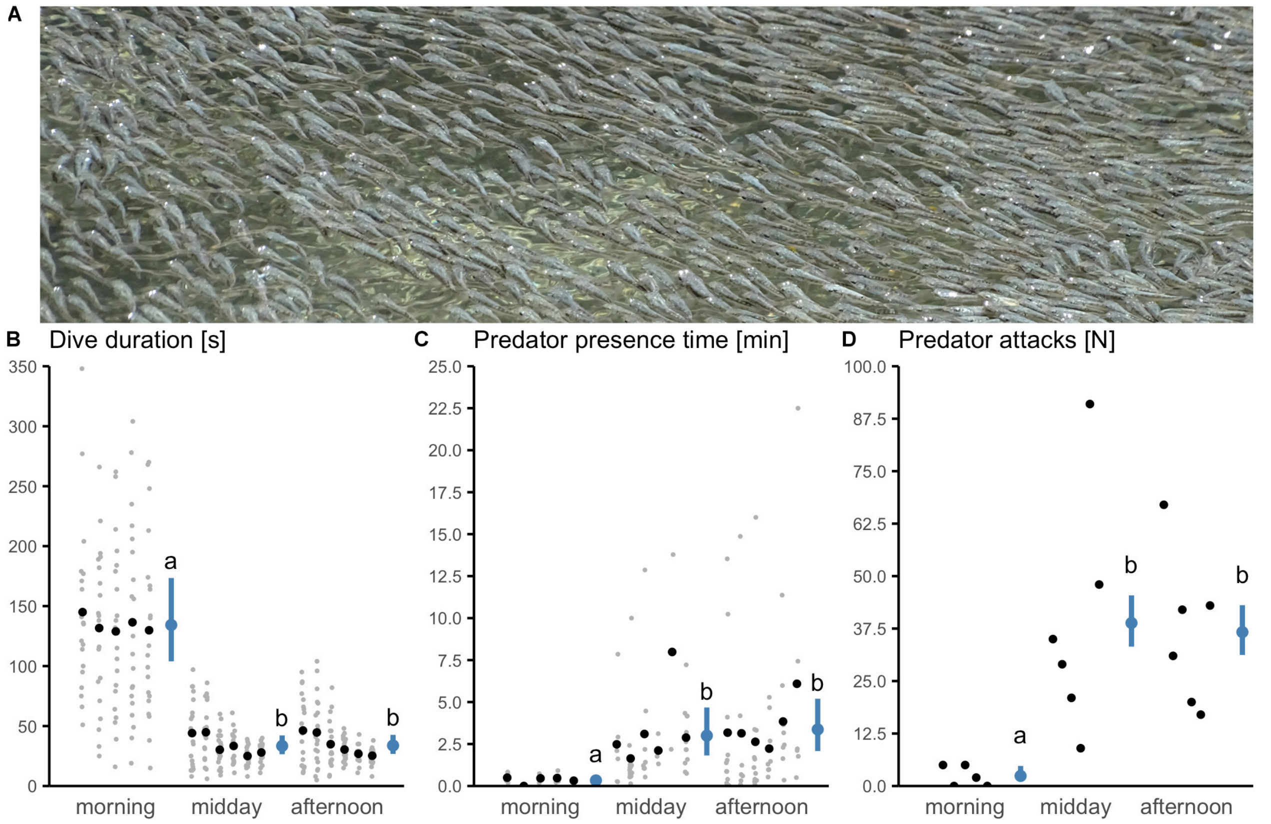

FIGURE 2 | Diurnal variation in sulphur mollies' diving behavior and predatory birds' activity. **(A)** During daytime hours, sulphur mollies spent large amounts of time at the surface performing aquatic surface respiration, often resulting in densely-packed shoals. A stream section was repeatedly sampled during three daytime periods on six consecutive days (n = 17 sampling occasions; morning Day 1 excluded). Shown are measurements (gray), which were sample-pooled for analysis (black). Time of day affected **(B)** prey's behavior (mean dive duration) as well as predator's activity [**(C)**: mean presence time and **(D)**: total sum of attack; see model-estimated marginal means with 95% CI in blue]. Letters indicate results from *post hoc* pairwise comparisons.

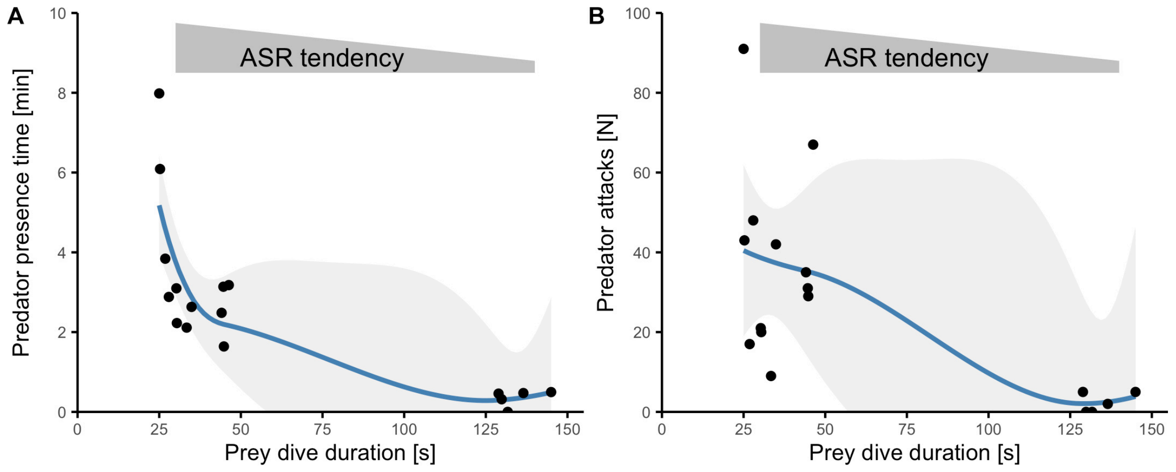

FIGURE 3 | Interplay among prey behavior and predator activity. Relationship between dive duration and either **(A)** predator presence time (mean) or **(B)** predator attacks (total sum) at different daytime periods. Each point represents data for one sampling occasion (sample-pooled measurements). Blue lines represent loess-smoothed curve fits with 95% CI for significant Kendall rank correlations.

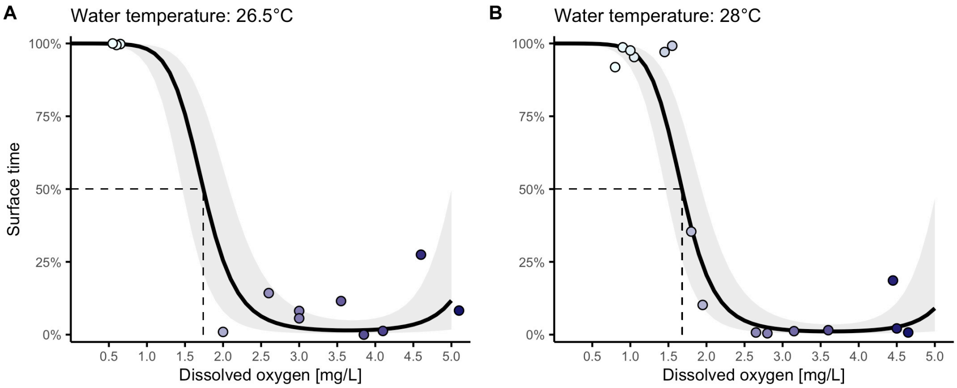

FIGURE 4 | Hypoxia-driven ASR tendency of *Poecilia sulphuraria* for a morning **(A)** and midday temperature scenario **(B)**. Time spent at the water surface performing ASR increased with decreasing oxygen concentrations at both test temperatures in a laboratory setting. Each data point equals the cumulative time spent at the surface for one group of 5 adult fish (*n* = 27 groups). Darker points represent data collected at higher levels of aquatic oxygen. The half-maximal concentration at which fish spend 50% of their time at the surface (ASR_{50}) was estimated at 1.68 mg/L DO for 28°C and 1.74 mg/L DO for 26.5°C (M2-estimated trend lines in black).

TABLE 2 | Overview of GLMMs tested to predict surface time of *Poecilia sulphuraria*.

(A) Model selection	df	AICc	ΔAICc	w_i
M1: (Intercept) + DO + DO^2	**4**	**302**	**0**	**0.78**
M2: (Intercept) + DO + DO^2 + Temp	**5**	**304.9**	**2.9**	**0.18**
M3: (Intercept) + log(DO)	**3**	**308.4**	**6.5**	**0.03**
M4: (Intercept) + log(DO) + Temp	4	311.1	9.1	0.01
M5: (Intercept) + DO	3	320	18	0.00
M6: (Intercept) + DO + Temp	4	322.3	20.3	0.00
M7: (Intercept)	2	340.6	38.6	0.00
M8: (Intercept) + Temp	3	343.1	41.2	0.00

(B) Model averaging	Coefficient	Variance	Number of models	Importance
(Intercept)	10.9	1.6	8	1
DO – Linear trend	−8.2	1.5	4	0.96
– Quadratic trend (DO^2)	1.1	0.3	2	0.96
– Logarithmic trend (log[DO])	−0.2	0.4	2	0.04
Temp	−0.06	0.2	4	0.19

*Results of model selection **(A)** and model averaging **(B)** based on GLMMs. Variables tested include a linear, logarithmic, or quadratic effect of dissolved oxygen concentration (DO), as well as temperature regime (Temp). Degrees of freedom (df), Akaike's Information Criterion for finite sample sizes (AICc), Delta AIC (ΔAICc) and model weights (w_i) are listed. Models with substantial support (ΔAICC < 7) are in bold.*

Dynamics of Diving Behavior of Sulphur Mollies and Predatory Bird Activity

Fish frequently surfaced during mornings, middays and afternoons, but continuously aggregated at the surface toward the later parts of the day (**Figure 2A** and **Supplementary Video**). Fish performed occasional voluntary dives (i.e., without predatory or human disturbance) into the water column during all three periods. The duration of these dives varied throughout the day (effect of *time:* $F_{2,14} = 75.8$, $p < 0.0001$). Dives lasted significantly longer during mornings than later in the day (**Figure 2B**).

Activity of avian predators was generally high. Within 8.5 h over 6 days, we observed nine different piscivorous bird species (**Supplementary Table 1**) and witnessed 465 attacks (**Figure 2D**). The time birds spent in the transect as well as the number of attacks peaked during middays and afternoons (effect of *time:* presence time $F_{2,14} = 23.1$, $p < 0.0001$; attacks $\chi^2(2) = 234$, $p < 0.0001$; **Figures 2C,D**). Periods of short dives (and an associated extension of surface time) were associated with high activity of avian predators. When fish were able to dive for longer periods, predators spent less time near the prey (Kendall's *tau* = −0.62, $T = 26$, $p = 0.0003$, $n = 17$; **Figure 3A**) and launched fewer attacks (*tau* = −0.47, $z = -3$, $p = 0.008$, $n = 17$; **Figure 3B**).

ASR Tendency of the Sulphur Molly in Response to Hypoxia in a Laboratory Setting

Time spent at the surface performing ASR varied as fish were exposed to dissolved oxygen concentrations ranging from near anoxia to normoxia (0.6 – 5.1 mg/L). We observed a non-linear (sigmoidal) relationship between surface-time and DO (**Figure 4**). This was supported by model selection (all top models included quadratic or logarithmic effects of DO; **Table 2A**) and multimodel interference, as a linear and quadratic effect of DO showed the highest relative importance (**Table 2B**). Under severe hypoxia, fish spent more than 90% of their time at the surface performing ASR, while fish in more normoxic conditions surfaced only rarely. The point at which fish switched from utilizing the water column to mainly perform ASR (50% of the time spent at the surface; ASR_{50}) was at 1.7 mg/L DO. This behavioral switch was observable for fish tested at 26.5°C and 28°C (**Figures 4A,B**). Temperature exhibited low relative importance on surface time (**Table 2B**), although a more fine-scaled investigation of intermediate DO levels will be necessary to eliminate a possible shift of the behavioral threshold with temperature.

In accordance with field observations, dive durations were shorter under severe hypoxia (i.e., <1.7 mg/L DO; mean ± SD: 7.1 ± 6.7s, n = 9) than under more favorable oxygen conditions (105.1 ± 83.1 s, n = 18; W = 0, $p < 0.0001$; **Supplementary Figure 2A**). This reduction in dive time was inversely proportional to surface time (Spearman's *rho* = −0.86, S = 6089, $p < 0.0001$, n = 27), as fish also dove less with hypoxic stress (<1.7 mg/L DO: 10.4 ± 10.5, >1.7 mg/L DO: 23.7 ± 17.1; W = 38, p = 0.01; **Supplementary Figure 2B**).

DISCUSSION

This study revealed that predator-prey activity patterns in a bird-fish system from a sulfidic habitat are subject to a diurnal cycle, matching that of water temperature and dissolved oxygen. In a laboratory experiment, we disentangled that variation in DO drove changes in sulphur mollies' surfacing behavior in a way that when oxygen levels dropped below 1.7 mg/L (i.e., daytime hypoxia) fish significantly increased the time proportion for aquatic surface respiration (ASR) and consequently reduced time in the water column (i.e., shorter-lasting but not more dives). Coinciding with predictions, activity of piscivorous birds was highest during times of high ASR tendency, suggesting that birds spent more time at the springs and launched more attacks when fish were concentrated at the surface. Despite some constraints with such correlative studies, our results provide first empirical evidence for a potential link between physicochemical water conditions, aquatic prey's behavior and the activity of aerial predators.

The Baños del Azufre spring system is characterized by oscillations of temperature and dissolved oxygen (i.e., magnitude of ∼1°C and ∼1 mg/L DO). Many shallow waterbodies commonly experience periodic fluctuations which are predominately driven by biological processes, where submerged vegetation or algae blooms typically produce oxygen through photosynthesis during daytime and consume oxygen at night (i.e., daytime supersaturation and nighttime hypoxia; see French Camargue marshlands: Kersten et al., 1991; tidal creeks of Chesapeake Bay, United States: Shen et al., 2008). In the Baños system, where aquatic vegetation is absent, oxygen supply seems to be strongly driven by temperature-dependent solubility. Daytime hypoxia may be further aggravated by the presence of dense mats of sulfide-oxidizing bacteria, which increase their metabolic rate with temperature (Hotaling et al., 2019), yet the presence of dissolved hydrogen sulfide did not follow a diurnal rhythm suggesting that, at least during the day, the influx of H_2S likely exceeded its consumption by bacterial activity.

Hypoxia and H_2S elicit similar physiological and behavioral responses in fishes (Bagarinao, 1992) and through interactions with temperature induce even greater stress than reported for either stressor separately (Gee et al., 1978; Kramer and Mehegan, 1981; Skandalis et al., 2020). Our observations suggest diurnal fluctuations in DO concentration are a dominant factor driving the observed changes in fish's diving behavior and associated ASR tendency. However, the relative importance of H_2S, temperature or other factors such as food availability (which was not considered here), is still uncertain and will require further study. Our laboratory test indicates that the intensity with which sulphur mollies perform ASR is oxygen-driven with temperature having little effect. We attribute this to the relatively small temperature range tested here (26.5 versus 28°C), compared to the studies that found diurnal temperature effects on aquatic surface respiration in minnows (6–31°C; Gee et al., 1978) and guppies (25–32°C; Kramer and Mehegan, 1981). Nonetheless, for temperatures commonly experienced by sulphur mollies in the morning (26.5°C) or later parts of the day (28°C), responses were very similar and fish of either treatment spent the majority of their time at the surface below 1.7 mg/L DO (ASR_{50}). Interestingly, similar thresholds have been established for the poeciliid *Gambusia affinis* both in the lab and in the field (i.e., ASR became obligatory below 1 and 2 mg/L DO, respectively; Cech et al., 1985; Kersten et al., 1991), but this species at least partially relied on ASR until values exceeded 3 mg/L (lab at 20°C) or even 6 mg/L DO (field during June/July in temperate climate). If temperature conditions were as high as in the present study, due to an increase in oxygen demand we would expect this species to rely on ASR at even higher DO concentrations. Hence, it is intriguing that, despite warm water temperatures, sulphur mollies do not seem to rely much on ASR at values above 2 mg/L and may in fact convey that many adaptations to tolerate hydrogen sulfide also improve hypoxic tolerance [especially morphological adaptations facilitating oxygen acquisition (Tobler and Hastings, 2011; Tobler et al., 2011), upregulation of oxygen transport genes (Barts et al., 2018), shifts toward anaerobic metabolism (Kelley et al., 2016) and reduced energy demand (Passow et al., 2017; Camarillo et al., 2020) as well as modulations of pathways maintaining mitochondrial function and aerobic ATP production (Pfenninger et al., 2014; Tobler et al., 2018; Greenway et al., 2020)].

Piscivorous bird activity peaked at times when fish showed reduced diving and hence were mostly aggregated at the surface. Many birds are easily disturbed by human-related presence and may leave an area or trade off foraging for increased vigilance (Burger and Gochfeld, 1998). Similarly, during periods of intense heat, bird's foraging activity is often reduced or can cease completely (Edwards et al., 2015; Funghi et al., 2019). Nonetheless, we observed peak predator activity during the parts of the day with highest temperatures and increased human activity (noon and afternoon), rendering both unlikely explanations for the observed bird behavior in our system. While our study was not suitable to disentangle whether increased bird activity at the Baños del Azufre is due to a circadian rhythm or an exploitation of vulnerable prey, we argue in favor of the latter explanation. Herons, egrets, and kingfishers are the main predators in this system (**Supplementary Table 1**), all of which are visual hunters. As such, these species will maximize their foraging efforts during periods of good visibility and light conditions (mainly around midday). For avian predators, large surface aggregations are not only more easily accessible but also more conspicuous compared to times when these fish are less clustered (morning). On top, fish's escape performance is likely limited during later parts of the day. Within an optimum range, swimming performance usually increases with temperature (Colchen et al., 2017), but hypoxia and high temperatures often act synergistically in hampering escape performance (Domenici et al., 2000, 2007; Lefrançois et al., 2005). This effect might be especially pronounced in sulphide-adapted fish, which are selected for more energy efficient swimming, resulting in a lower burst start performance compared to non-sulfidic populations (Camarillo et al., 2020). This line of evidence clearly suggests an interaction shift in favor of the avian predators with more severe hypoxia and as such is similar to observations made on little egrets (*Egretta garzetta*) in the Camargue that showed highest capture rates when mosquitofish performed ASR during morning hypoxia (Kersten et al., 1991). Through individual identification, future studies could examine whether birds frequently assess the quality of different foraging sites and only initiate foraging when profitability is high or if they have learned to predict when fish aggregate.

With an average of about 1 attack per minute, especially when DO levels dropped during the later parts of the day, our study confirmed the extreme predation pressure that sulphur mollies experience (see also Riesch et al., 2010a; Lukas et al., in review). This selection pressure likely promotes the formation of the dense, synchronized fish aggregations (**Supplementary Video**; Riesch et al., 2010a), which may help to reduce individual sulphur mollies' predation risk through risk dilution, improved predator detection and predator confusion (Kramer and Graham, 1976; Krause and Ruxton, 2002). However, during times of reduced predation pressure (morning), costs of grouping may outweigh its benefits (e.g., foraging costs; Sogard and Olla, 1997) and thus favor solitary strategies. In fact, this balance might be further modulated by severe hypoxia rendering some solitary strategies (e.g., predator avoidance, escape and crypsis) less effective. As a result, sulphur mollies should flexibly adjust shoal density and anti-predator behavior during the day to match local conditions of predation intensity and hypoxia, which is an aspect of our research that will deserve further attention.

We note that all the information gathered here comes from the dry season (April–May), and it is essential to also understand the ecological pressures of *P. sulphuraria* during the rainy season. For example, sustained rain could majorly influence water chemistry and likely reduce hypoxic events, thus profoundly changing the system dynamics. On the other hand, rain also increases spring discharge so that the system would likely (with some lag) return to its former state. Anecdotal observations by one of the authors (*DB*) suggest sulphur mollies perform similar ASR behavior within dense shoals also in the rainy seasons (July to September), leading us to concur that this pattern is observable year-round in this species.

In summary, this study showcases a predator-prey system that is influenced by diurnal fluctuations in abiotic factors. Sulphur mollies face different selection pressures, namely hypoxia and predation, and adaptations to both have been found, highlighting their complex evolutionary trajectories. In this context, the sulfide spring systems of the Mexican states of Tabasco and Chiapas with their endemic fish fauna represent interesting natural laboratories to study periodicity, which is often not feasible in other ecosystems as they involve complex interactions and occur on much harder to capture temporal and spatial scales (e.g., population density cycles of wolf, elk and aspen (Fortin et al., 2005; Hebblewhite et al., 2005; Vucetich et al., 2005) or lynx and roe deer or hare (Nilsen et al., 2009; Lavergne et al., 2019)).

DATA AVAILABILITY STATEMENT

The raw data and analysis script supporting the conclusions of this article are available at: https://doi.org/10.6084/m9.figshare.14135045.

ETHICS STATEMENT

The animal study was reviewed and approved by the Mexican "Comisión Nacional de Acuacultura y Pesca" (CONAPESCA; DGOPA.09004.041111.3088, PRMN/DGOPA-003/2014, PRMN /DGOPA-009/2015, and PRMN/DGOPA-012/2017).

AUTHOR CONTRIBUTIONS

JL, DB, JK, PR, PK, and LA-R conducted the predator-prey survey. JL and DB performed the hypoxia experiment. JL and FA collected the water samples. FA and TG performed the physicochemical analyses of water samples. JL performed the statistical analysis and wrote the manuscript with input from all authors. All authors approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.619193/full#supplementary-material

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Consuming Costly Prey: Optimal Foraging and the Role of Compensatory Growth

Randall Brian Langerhans[1]*, Taylor R. Goins[1], Kenzi M. Stemp[2], Rüdiger Riesch[3], Márcio S. Araújo[4] and Craig A. Layman[5†]

[1] Department of Biological Sciences, W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, United States, [2] Department of Biology, Appalachian State University, Boone, NC, United States, [3] Department of Biological Sciences, Centre for Ecology, Evolution and Behaviour, Royal Holloway, University of London, Egham, United Kingdom, [4] Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, Brazil, [5] Department of Applied Ecology, North Carolina State University, Raleigh, NC, United States

Some prey are exceptionally difficult to digest, and yet even non-specialized animals may consume them—why? Durophagy, the consumption of hard-shelled prey, is thought to require special adaptations for crushing or digesting the hard shells to avoid the many potential costs of this prey type. But many animals lacking specializations nevertheless include hard-bodied prey in their diets. We describe several non-mutually exclusive adaptive mechanisms that could explain such a pattern, and point to optimal foraging and compensatory growth as potentially having widespread importance in explaining costly-prey consumption. We first conducted a literature survey to quantify the regularity with which non-specialized teleost fishes consume hard-shelled prey: stomach-content data from 325 teleost fish species spanning 82 families (57,233 stomach samples) demonstrated that non-specialized species comprise ∼75% of the total species exhibiting durophagy, commonly consuming hard-shelled prey at low to moderate levels (∼10–40% as much as specialists). We then performed a diet survey to assess the frequency of molluscivory across the native latitudinal range of a small livebearing fish, *Gambusia holbrooki*, lacking durophagy specializations. Molluscivory was regionally widespread, spanning their entire native latitudinal range (>14° latitude). Third, we tested for a higher frequency of molluscivory under conditions of higher intraspecific resource competition in Bahamian mosquitofish (*Gambusia* spp.). Examining over 5,300 individuals, we found that molluscivory was more common in populations with higher population density, suggesting that food limitation is important in eliciting molluscivory. Finally, we experimentally tested in *G. holbrooki* whether molluscivory reduces growth rate and whether compensatory growth follows a period of molluscivory. We found that consumption of hard-shelled gastropods results in significantly reduced growth rate, but compensatory growth following prior snail consumption can quickly mitigate growth costs. Our results suggest that the widespread phenomenon of costly-prey consumption may be partially explained by its relative benefits when few alternative prey options exist, combined with compensatory growth that alleviates temporary costs.

Keywords: durophagy, food web, growth compensation, niche partitioning, predator-prey interactions, prey choice, resource competition, trophic ecology

Edited by:
J. Jaime Zúñiga-Vega,
National Autonomous University of Mexico, Mexico

Reviewed by:
Regina Vega Trejo,
Stockholm University, Sweden
Geoff Smith,
Denison University, United States

***Correspondence:**
Randall Brian Langerhans
langerhans@ncsu.edu

†Present address:
Craig A. Layman,
Department of Biology, Center for Energy, Environment, and Sustainability, Wake Forest University, Winston-Salem, NC, United States

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INTRODUCTION

Competition for food resources serves as one of the most important factors driving major ecological and evolutionary patterns (e.g., Tilman, 1982; Schluter, 2000; Chase and Leibold, 2003; Morin, 2011; Pfennig and Pfennig, 2012). This fact has led to an array of adaptations, where particular species have evolved different resource specializations for acquiring and consuming certain types of foods. For instance, animals have evolved specialized means of feeding on potentially costly prey, such as those characterized by toxins, thorns, spines, and hard shells (e.g., Savitzky, 1983; Benkman, 1993; Coley and Barone, 1996; Brodie, 1999; Wainwright, 2006). Nevertheless, many animals exhibit a broad diet, opportunistically feeding on a variety of organisms, and appear to regularly include the consumption of seemingly costly prey (Westoby, 1978; Rex et al., 2010).

Throughout, we refer to "costly prey" as those prey whose acquisition and consumption can cause reduced fitness relative to feeding on most other prey regularly available and consumed by a given species (e.g., decreased survival, fecundity, mating success, parental care). Reduction in fitness can result from impacts such as reduced growth, condition/health, egg/embryo development, and age/size at maturity. Natural selection should generally favor diets comprising more easily acquired and consumed prey having higher net energy intake, i.e., more beneficial cost-to-benefit ratios (MacArthur and Pianka, 1966; Schoener, 1971; Pyke et al., 1977; Pyke, 1984; Stephens and Krebs, 1986). But then what might explain the widespread consumption of costly prey?

We suggest four adaptive, non-mutually exclusive mechanisms might explain the phenomenon of eating costly prey items (**Box 1**). The four mechanisms represent two categories of explanation: (1) consumption of costly prey could reflect adaptive feeding strategies (mechanisms I-III), and (2) costs can potentially be offset through evolved mechanisms that provide a delayed compensation for the costs (mechanism IV). In this study, we seek to explain why so many animals consume prey items that not only seem relatively less profitable than other options, but seem particularly costly. That is, we are specifically concerned with the consumption of especially costly prey in non-specialized taxa, and not merely the inclusion of poor-quality or low-cost prey in diets, or the very rare (perhaps inadvertent) consumption of moderate- to high-cost prey. We suggest that two particular mechanisms, optimal foraging, and compensatory growth, seem especially likely to prove important in explaining this phenomenon in nature, as the conditions conducive for their operation are common: decades of literature have revealed strong support for optimal foraging theory, and compensatory growth is widespread throughout the animal kingdom (see **Box 1**). In essence, costly-prey consumption may be largely explained by animals making the best of a bad situation (optimal foraging) and having the ability to offset its negative impacts by rapidly achieving a body size or condition associated with high fitness after the resumption of a high-quality diet (compensatory growth; **Figure 1**).

Here we test the predictions of optimal foraging and compensatory growth to evaluate their role in explaining durophagy in fishes. Durophagy describes the consumption of hard-shelled prey, including snails, bivalves, crabs, and urchins. This type of diet is typically thought to demand distinctive adaptations for crushing the hard shells, such as blunt teeth and strong jaws (Liem, 1986; Wainwright, 1988; Grubich, 2003; Hulsey et al., 2008). So why would animals lacking such specializations consume hard-bodied prey that will likely result in fitness costs? Durophagy can induce costs such as reduced growth of somatic or reproductive tissue owing to the large proportion of indigestible material consumed, spatial constraints within the gut as hard prey slowly evacuate (restricting entry, digestion, and evacuation of other prey, and potentially constraining available space for eggs or developing embryos), and possible injuries during consumption or excretion. Yet previous work on fish diets suggests that many fish lacking specializations might commonly include hard-shelled prey in their diet (see literature survey below). In this study, we (1) conduct a literature survey to quantify the regularity with which non-specialized teleost fishes consume hard-shelled prey, (2) perform a non-invasive diet survey to assess the frequency of molluscivory across the native latitudinal range of a wide-ranging, small, livebearing fish, *Gambusia holbrooki*, (3) test optimal foraging predictions of higher frequency of molluscivory under conditions of higher intraspecific resource competition in Bahamian mosquitofish (*Gambusia* spp.), and (4) conduct an experimental test in *G. holbrooki* for a cost of molluscivory (i.e., reduced growth rate) and the occurrence of compensatory growth following a period of molluscivory.

MATERIALS AND METHODS

Literature Survey of Durophagy in Teleost Fishes

To estimate the occurrence of hard-shelled prey in the diet of wild teleost fishes, we conducted a literature search of diet studies that employed stomach-content analysis. Rather than attempt to provide an exhaustive review of fish diets, we limited our search to the first 100 studies we encountered that matched the criteria described below. In this way, our search should provide a representative sample of fish diets, approximating what an exhaustive literature review might find. Using Google Scholar, we searched for studies of fish diets that directly examined stomach contents of wild-caught teleost fish, and provided quantitative information relevant to determining the inclusion of hard-shelled prey in the diets. Quantitative diet information was gathered for five different estimates of hard-shelled prey consumption: (1) percent occurrence (number of stomachs with hard-shelled prey / total number of stomachs that contained prey items), (2) percent by number (number of hard-shelled prey items / total number of prey items), (3) percent by weight (weight of hard-shelled prey / weight of all prey), (4) percent by volume (volume of hard-shelled prey / volume of all prey), and (5) percent index of relative importance. This latter metric is a commonly employed compound index of fish diets, and incorporates occurrence, bulk, and amount: percent occurrence × (percent volume + percent by number), expressed as a percentage of the sum of all index of relative importance values for all prey items [for details, see Pinkas et al. (1971), Cortes (1997)]. This large set of studies

BOX 1 | Why animals consume costly prey

We describe and evaluate four adaptive explanations for the consumption of costly diet items in non-specialized animals (**Table I**).

TABLE I | Four adaptive, non-mutually exclusive mechanisms to explain the consumption of costly prey in non-specialized taxa.

Mechanism	Description
I. Direct benefits	Costly prey provide direct advantages that result in net fitness benefit, such as essential limiting nutrients or buffer effects of toxic compounds.
II. Individual specialization	Some individuals possess specialized traits that reduce the costs of costly prey.
III. Optimal foraging	Costly prey are relatively less costly than alternative prey or no prey at all.
IV. Compensatory growth	Mitigate negative effects of costly-prey consumption by later exhibiting accelerated growth of somatic or reproductive tissue following consumption of higher-quality resources.

Mechanism I–Direct benefits: Animals could attain a net fitness gain from consuming particularly difficult-to-eat prey because they confer important benefits. Despite substantial costs, certain prey might provide important limiting nutrients, aid in detoxifying compounds derived from other foods, support mechanical digestion, or facilitate the removal of obstructions, parasites, or harmful microbiota (Bernays et al., 1994; Provenza et al., 2003; Simpson et al., 2004; Voigt et al., 2008). While consumption of some inedible items, such as clay or soil, might offer some important benefits at low to moderate cost, this "direct benefits" mechanism seems unlikely to commonly explain the consumption of prey that induce considerable costs, as the gains would need to be quite large to overcome the costs—unless the costly prey was only infrequently consumed in small amounts. Thus, although theoretically possible, and potentially important in combination with other mechanisms, we doubt that this mechanism alone can explain frequent or moderate levels of consumption of particularly costly prey.

Mechanism II–Individual specialization: Competition for food can drive intraspecific resource partitioning to reduce the intensity of competition, where individuals within a population differ in their ranked preferences of prey items. That is, some individuals rank costly prey items higher than others due to frequency-dependent competition (Bolnick, 2001) and specialized phenotypes that reduce costs of acquiring and consuming those prey resources (Bolnick et al., 2003). For instance, individuals might possess certain behaviors, morphologies, or physiologies that enhance foraging or feeding performance for costly prey, and thus more readily consume those prey than other individuals less equipped to contend with these resources (Olsson et al., 2007). This scenario results in individual diet specialization (e.g., Bolnick et al., 2003; Svanbäck and Persson, 2004; Svanbäck and Bolnick, 2007; Araujo et al., 2011), with certain individuals consistently feeding on costly prey more frequently than other individuals. Certain cases of intraspecific resource polymorphisms provide one source of known examples of this scenario, where phenotypically-specialized subsets of populations consume costly prey items, such as snail-feeding within several cichlid fishes and hard-seed consumption within some finches (e.g., Smith and Skúlason, 1996; Swanson et al., 2003). However, for individual specialization to account for the widespread consumption of costly prey in "non-specialized" species, there must be considerable cryptic phenotypic variation within populations that has so far gone undetected. While the occurrence of individual diet specialization appears pervasive across many taxa (e.g., Bolnick et al., 2003; Araujo et al., 2011), whether or not such specialization often comprises specialized phenotypes that reduce the impact of costly-prey consumption is currently unknown.

Mechanism III–Optimal foraging: Animals might adaptively consume costly prey when the net energetic gain associated with that particular prey type is greater (or when the net energetic loss is smaller) than alternative prey options. This comprises a fundamental prediction of optimal foraging theory (e.g., Stephens and Krebs, 1986), where animals should increase consumption of suboptimal prey as higher-quality resources become more difficult to acquire. Specifically, if individuals rank prey resources largely based on net energy intake, and include different resources in their diet according to this criterion, then situations can arise where consumption of costly prey represents an adaptive foraging behavior because these prey become relatively less costly in comparison with other available prey. For instance, this might occur in cases of reduced abundance or absence of higher-quality resources, reduced access to higher-quality resources (e.g., interference competition, predation), increased abundance of costly prey, or increased encounter rates with costly prey within less risky foraging areas. This might be most prevalent during particular seasons, times of drought, in the face of strong resource competition, when particular individuals or species guard high-quality resources, or when elevated predation risk confines individuals to regions without higher-quality resources. Put simply, this mechanism provides a means by which animals can make the best of a bad foraging situation. This mechanism could prove common for suboptimal prey resources of moderate costliness, while particularly severe conditions might be required for this mechanism to wholly explain moderate levels of consumption of highly costly prey. Thus, the importance of this mechanism depends on the balance between the costliness of the prey and the severity of the alternative option of not eating the costly prey (e.g., starving, eating lower-quality resources, suffering a high risk of injury or death in attempting to acquire higher-quality resource).

Mechanism IV–Compensatory growth: Animals might endure temporary costs of consuming difficult-to-eat items, but exhibit compensatory growth that offsets these costs once they subsequently obtain higher-quality food. Compensatory growth describes a phase of accelerated growth when high-quality nutrition is restored after a period of growth depression (Osborne and Mendel, 1916; Bohman, 1955; Hornick et al., 2000), and is widespread in animals after diet/nutrient restriction and in plants following herbivory (Wilson and Osbourn, 1960; Tanner, 1963; McNaughton, 1983; Ryan, 1990; Ali et al., 2003; Hector and Nakagawa, 2012; Won and Borski, 2013). Because body size often has a strong link to fitness (Roff, 2002), compensatory growth has been thought to represent an adaptation to avoid negative consequences of reduced body size caused by episodes of reduced growth. Thus, animals might more readily accept costs of suboptimal prey consumption partially because elevated growth later will compensate for these costs, resulting in little to no net reduction in fitness. Natural selection might often favor such a strategy in systems that meet two criteria: (1) temporally or spatially patchy distributions of high-quality food resources, occasionally requiring consumption of costly prey (e.g., via mechanism III), and (2) high likelihood of acquiring higher-quality food within a relatively short period after costly-prey consumption. The latter criterion implies a low mortality risk during the compensatory growth phase, as an individual cannot offset fitness costs at a later date if it cannot survive until that time. These two criteria are often met in natural populations, and combined with both the pervasiveness of compensatory growth and the ability of compensatory growth to minimize fitness costs, this mechanism might play an important, previously unrecognized role in explaining the common phenomenon of costly-prey consumption in natural animal populations. Whether animals initially consume costly prey because of direct benefits, to reduce intraspecific competition via individual specialization, or through optimal foraging strategies, compensatory growth might provide a common solution for offsetting costs. That is, complete elimination of costs is unlikely through any of the three mechanisms described above, and thus selection should often favor an additional mechanism that can largely offset those costs. Based on our current knowledge, compensatory growth seems to hold considerable promise as a widespread, influential factor in permitting costly-prey consumption.

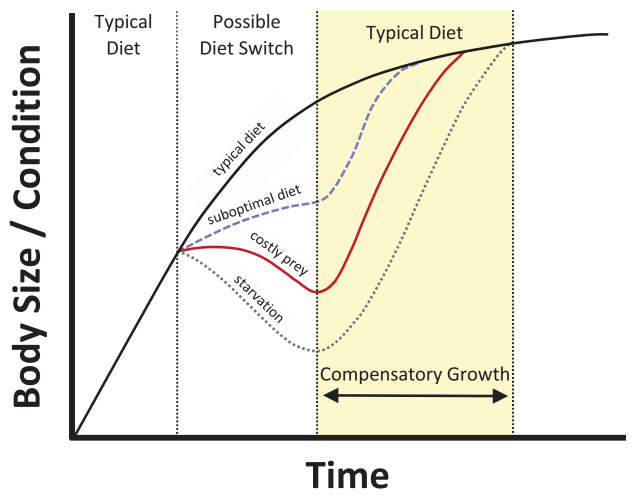

FIGURE 1 | Illustration of possible negative consequences of costly-prey consumption, and the rapid offsetting of costs via compensatory growth. Costly-prey consumption might often entail strong fitness costs relative to other prey items (especially in the absence of mechanisms I or II, **Box 1**), but still offer more energetic return than starvation, with compensatory growth providing a generalized response to counteract periods of growth depression.

should provide reasonable estimates of our parameters of interest (see Results for species diversity and sample size). All studies included in this review, and their associated data, are presented in **Appendix 1** in the **Supplementary Material**.

To avoid ambiguities and maintain consistency across studies, we only included molluscs and crabs in our category of "hard-shelled prey" even though some other prey may have shells or other dense parts (e.g., ostracods). For mollusc prey, unequivocal quantitative data for hard-shelled molluscs must have been provided in the study for inclusion in our dataset. For some studies, we could not obtain estimates of hard-shelled prey consumption even though quantitative data were provided; we excluded those studies. For instance, we excluded studies that only provided percent occurrence data separately for different mollusc species because this did not allow us to determine the overall percent occurrence of all molluscs (i.e., stomachs could have contained multiple mollusc species). Inclusion of crab prey data followed the same criteria, with the exception that if a study only provided values for the entire group of "crustaceans" or "decapods," we ignored those values and assumed the groups did not contain crabs. This conservative approach likely biased our estimates downward only slightly, as this only occurred in three studies, and in all these cases it was unlikely that crabs comprised a substantial part of the diet owing to the habitat use and diet of these species.

We classified each fish species included in the dataset as either a "specialist" or "non-specialist" based on the presence or absence of specialized morphological features for crushing and consuming hard-shelled prey. This classification was straightforward and unambiguous in all cases, as species with durophagous specializations are well-known. Typically, durophagous specialists possess specialized pharyngeal jaw morphologies accompanied by large, strong epaxial muscles (e.g., Liem, 1986; Wainwright, 1988; Meyer, 1990; Grubich, 2003; Hulsey et al., 2008). We further classified each species as either exhibiting durophagy or not, based on the presence or absence of hard-shelled prey in their diet. Because durophagy can be rare in non-specialists, low sample sizes might fail to detect durophagy within populations where it is present at low frequency. To test whether the detection of durophagy depended on sample size in our dataset, we conducted logistic regression for non-specialists with the presence of durophagy as the dependent variable (0 vs. 1) and $\log_{10}$-transformed sample size (number of stomachs examined) as the independent variable. We did not include specialists in this test because durophagy was detected in all specialists, regardless of sample size (see below). If sample size affected the detection of durophagy, then we would exclude species with low sample sizes (either <5 or 10 individuals, using separate analyses) where durophagy was not detected, and re-perform the logistic regression to determine whether the sample-size dependence of durophagy detection could be eliminated by excluding species with particularly small sample sizes. If so, then we would exclude those species with small sample sizes for all analyses. All analyses in this study were performed in the programs SAS v.7.15 and JMP v. 14.2 (SAS Institute Inc., Cary, NC, USA).

Field Survey of Molluscivory in *Gambusia holbrooki*

Gambusia fishes are small, livebearing fish (family Poeciliidae) that exhibit wide-ranging diets (Meffe and Snelson, 1989; Pyke, 2005), occasionally consuming hard-shelled molluscs by swallowing them whole (Bay and Anderson, 1966; Hubbs et al., 1978; Walters and Legner, 1980; Hubbs, 1990; Pen and Potter, 1991; Araujo et al., 2014). We selected *G. holbrooki* for detailed investigation because of its broad diet, extensive geographic range, lack of any adaptive specializations for durophagy, potential for substantial costs of molluscivory in terms of both growth and reproduction, and ability to exhibit compensatory growth (Kahn et al., 2012; Livingston et al., 2014).

We collected *G. holbrooki* in August 2011 from 10 natural populations along the eastern coast of the United States, spanning their entire native latitudinal range (**Figure 2A**, **Supplementary Table 1**). Specimens were immediately euthanized and preserved in 95% ethanol upon collection. We used digital x-ray imaging to examine the presence/absence of molluscs in gut contents of preserved adult *G. holbrooki* (261 females, 170 males). We captured a digital x-ray of each fish in the lateral perspective using a custom-built digital x-ray unit comprising a micro-focus x-ray source (Hamamatsu L6731-01) and a digital x-ray detector (PaxScan 2520E) housed in a lead-shielded cabinet. We inspected each image for the occurrence (presence/absence) and number of molluscs within the stomach. Previous work demonstrated the feasibility of this technique for detection of hard-shelled prey, such as molluscs (Beckmann et al., 2015). Because direct stomach-content analysis of a subset of fish revealed that snails smaller than 1 mm shell

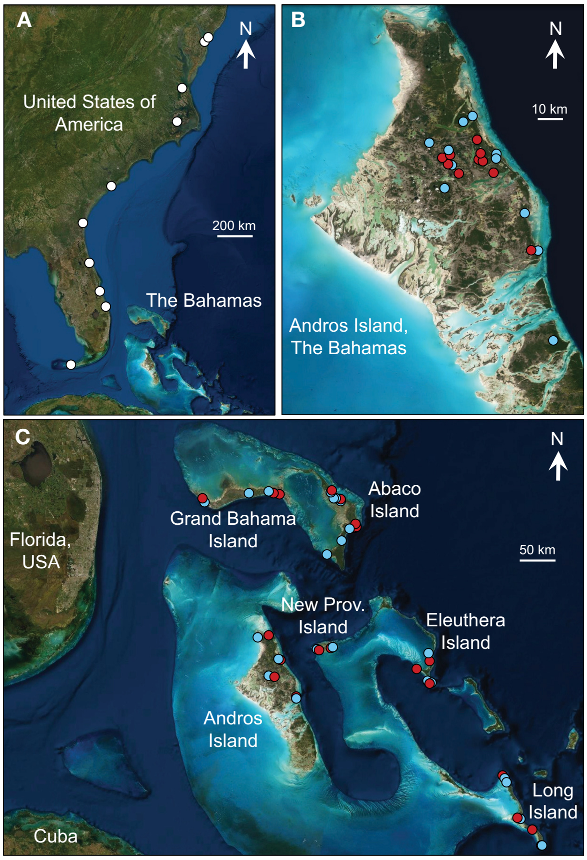

FIGURE 2 | Map of collection localities for the field studies of molluscivory in **(A)** *Gambusia holbrooki*, **(B)** *G. hubbsi* in blue holes, and **(C)** three species of Bahamian mosquitofish in tidal creeks. Blue symbols: high density/low predation; red symbols: low density/high predation.

length were rarely detected by x-ray imaging, our estimates provide lower bounds for the frequency of molluscivory in *G. holbrooki*, capturing cases of particularly dense mollusc shells (see **Figure 3**). Moreover, because x-ray imaging could not unequivocally determine which stomachs were empty and which contained prey items (Beckmann et al., 2015), our estimate of percent occurrence of molluscivory was calculated as the number of fish with snails in their stomachs divided by the total number of fish examined (rather than the total number of fish with prey items in their stomach).

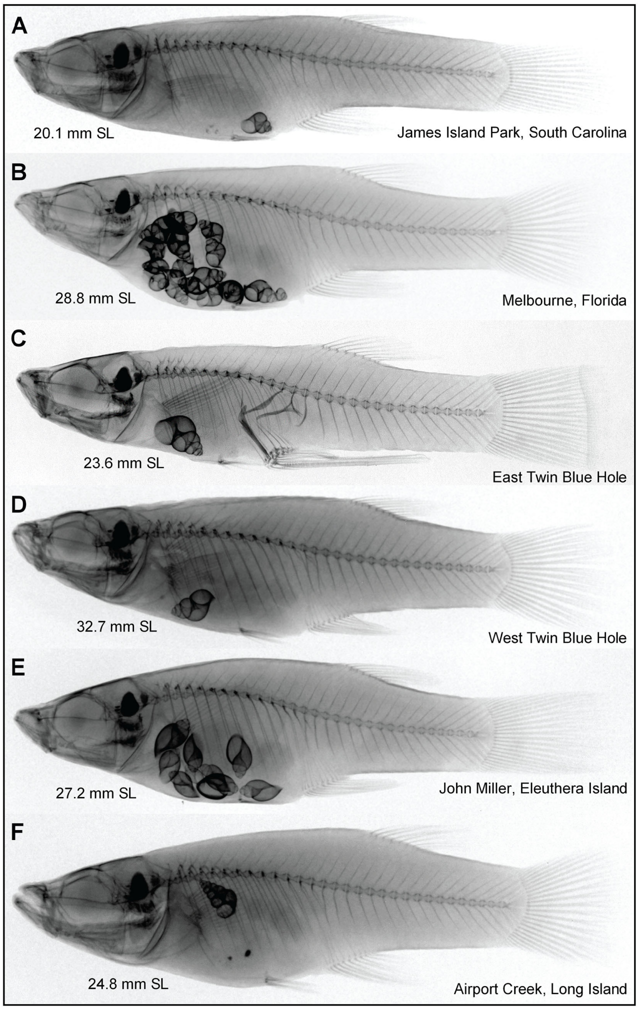

FIGURE 3 | Representative x-ray images of wild-caught **(A,B)** *Gambusia holbrooki*, **(C,D)** *Gambusia hubbsi*, and **(E,F)** *Gambusia manni* that had consumed snails (standard length of each fish provided).

Molluscivory and Resource Competition in Bahamian Mosquitofish

To test whether increased levels of resource competition are associated with molluscivory, as predicted by optimal foraging theory, we examined endemic livebearing fish in The Bahama Archipelago. Bahamian *Gambusia* were selected for study for reasons similar to *G. holbrooki*, with the added feature that they inhabit environments known to vary substantially in population density, with prior work finding multiple lines of evidence indicating stronger resource competition in populations with higher densities (Heinen et al., 2013; Riesch et al., 2013, 2015; Araujo et al., 2014; Heinen-Kay et al., 2016; Langerhans, 2018). We conducted our tests in two separate systems: (1) *Gambusia hubbsi* in inland blue holes on Andros Island and (2) three *Gambusia* species in tidal creeks across six islands. In all cases, we immediately euthanized and preserved individuals in 95% ethanol upon collection.

During the past ∼15,000 years (Fairbanks, 1989), *G. hubbsi* colonized many inland blue holes (water-filled, vertical caves) and have subsequently undergone adaptive diversification in a large number of traits [reviewed in Langerhans (2018)] and evolved varying levels of reproductive isolation among populations (e.g., Langerhans et al., 2007; Langerhans and Makowicz, 2013). A primary driver of evolutionary divergence in this system stems from the fact that in some blue holes *G. hubbsi* experience a relatively predator-free environment devoid of any piscivorous fish, and consequently exhibit high population densities with elevated competition for food resources. In other blue holes, *G. hubbsi* are heavily preyed upon by the much larger bigmouth sleeper (*Gobiomorus dormitor*) and have much lower population densities (e.g., Langerhans et al., 2007; Heinen et al., 2013; Martin et al., 2015). Because these two categories of blue holes do not systematically differ in abiotic environmental variables (Langerhans et al., 2007; Heinen et al., 2013; Riesch et al., 2013; Björnerås et al., 2020), this system provides a remarkable opportunity to test for the role of altered predatory and competitive environment on diet.

For *G. hubbsi* inhabiting blue holes on Andros Island, we tested for increased frequency of molluscivory under scenarios of higher resource competition by examining x-ray radiographs of 2,248 adult fish collected from 21 populations (11 with high density and no predators, 10 with low density and predatory fish present; **Figure 2B**, **Supplementary Table 2**). For each site, we calculated the overall proportion of fish with molluscs in their guts. For statistical analysis, we conducted a one-way analysis of variance (ANOVA) using arc-sin square-root transformed proportional molluscivory as the dependent variable and the dichotomous factor "predation regime" (high-density/low-predation vs. low-density/high-predation) as the independent variable.

Bahamian tidal creeks are shallow, tidally influenced estuaries typically having a relatively narrow creek mouth that broadens landward. Water flux largely arises from tidal exchange (freshwater input only provided via rainfall and aquifer percolation), so salinities in unfragmented systems are typically around 35 ppt and the biotic communities comprise marine taxa (Layman et al., 2004; Valentine-Rose et al., 2007a,b; Araujo et al., 2014; Riesch et al., 2015). Three species of Bahamian mosquitofish (*G. hubbsi*, *G. manni*, and *G.* sp.) inhabit tidal creeks across the archipelago, with each species within these systems found on different islands (Heinen-Kay et al., 2014). Fragmentation of Bahamian tidal creeks—the process by which connectivity with the ocean is restricted or cut off entirely—is principally caused by road construction, and results in strong and persistent ecological change. Most road construction that fragmented tidal creeks occurred during the 1960s and 1970s. Fragmentation dramatically reduced tidal exchange (tidal amplitude ∼0–10 vs. ∼40–80 cm in unfragmented creeks), leading to increased sedimentation rates, reduced animal biomass, reduced species diversity, and changes in the community composition of fishes, macroinvertebrates, plants, and macroalgae (Layman et al., 2004; Valentine-Rose et al., 2007a,b, 2011; Valentine-Rose and Layman, 2011; Araujo et al., 2014; Riesch et al., 2015). Key among these changes are markedly reduced densities of piscivorous fishes (e.g., great barracuda, *Sphyraena barracuda*; needlefish, *Strongylura* spp.) and increased densities of Bahamian mosquitofish. Previous work suggests that these drastic changes in intraspecific resource competition and predation represent the drivers of rapid phenotypic change in Bahamian mosquitofish in these systems (Araujo et al., 2014; Heinen-Kay et al., 2014; Giery et al., 2015; Riesch et al., 2015).

We employed two methodological approaches to test for consistent differences in molluscivory of Bahamian mosquitofish between the high-competition conditions of fragmented tidal creeks and the low-competition scenarios of unfragmented tidal creeks. First, we used x-ray radiographs of 2,463 adults from 44 populations across six Bahamian islands (two islands for each of three species; 1,466 females, 997 males; **Figure 2C**, **Supplementary Table 3**) to measure the proportion of fish with molluscs in their guts at each site. Again, the limitations of this method mean that our estimates provide a lower bound, likely underestimating the frequency of molluscivory because small molluscs (<1 mm shell length) can go undetected and because all fish, not only those with prey items in their stomachs, were used as the denominator in the percent occurrence calculation. We conducted a general linear model with arc-sin square-root-transformed proportional molluscivory as the dependent variable to test for effects of fragmentation regime, species, the interaction between species and fragmentation regime, island nested within species, and the interaction between fragmentation and island nested within species. Second, we complemented this non-invasive method with direct examination of stomachs because this could reveal smaller, less dense snail, and bivalve shells than detectable with x-rays. For this reason, and to additionally examine individuals particularly vulnerable to costly growth reductions caused by molluscivory, we included juveniles in our stomach-content analyses. We examined stomachs of 625 *G.* sp. on Abaco Island (373 females, 122 males, 130 juveniles) from 13 tidal creeks that span wide, continuous variation in population density (**Supplementary Table 4**). Some of these fish (156 females) were previously examined in Araujo et al. (2014), which can be consulted for details, but overall mollusc consumption has never previously been examined for these fish. Briefly, we removed the stomach of each individual in the laboratory and analyzed the gut contents under a stereo microscope, counting and identifying all prey items to the lowest feasible taxonomic level. To estimate the frequency of molluscivory, we calculated the average proportion of molluscs in the stomachs of each sex-age class for each population (number of molluscs divided by total number of prey items present in each stomach). We tested for greater molluscivory in populations with higher densities using an analysis of covariance (ANCOVA) with arc-sin square-root transformed proportion of molluscs as the dependent variable, and sex-age class and $\log_{10}$-transformed density as independent variables (interaction between sex-age class and $\log_{10}$-transformed density was excluded due to non-significance, $P = 0.93$).

Experimental Test of Cost of Molluscivory and Compensatory Growth in *G. holbrooki*

We experimentally tested whether molluscivory induces a cost in terms of growth in *G. holbrooki* and whether these fish can exhibit compensatory growth after snail consumption to reduce costs. Because our field survey found that females consumed snails more frequently than males (see Results), and because males exhibit minimal growth after sexual maturity in *G. holbrooki* (reducing our ability to detect growth-rate changes), we only examined adult females in this experiment. We collected adult fish from a single population in Cary, North Carolina. We first confirmed the presence of molluscivory in this population through fecal examination of live fish (i.e., expelled whole shells), and verified willingness to consume molluscs for all fish before experimentation by feeding live *Physa acuta* snails to the fish. All fish were maintained in the lab several months prior to experimentation. Although pregnancy status varied among fish used in the experiment (15 of 24 fish were determined to be pregnant by examination after the experiment), none of the fish gave birth during the experiment, and pregnancy status was highly non-significant when included as a covariate in analyses described below (main effect and all interaction terms: $P > 0.65$). Thus, we did not include pregnancy status in our final analyses.

We conducted a 17-day feeding experiment examining 24 female *G. holbrooki* [32.5–47.0 mm standard length (SL)]. The experiment was conducted in two temporal blocks, where we applied all the same experimental procedures twice, separated by 6 months (194 days), with 12 females examined within each block. For each block, each fish was individually placed in a 4.5-L container with an aerator on day 1, starved for 48 h, and then received a single feeding of a prescribed treatment per day until day 17. We housed tanks side-by-side on two shelves within a single room, and assigned a feeding treatment, hard-bodied prey vs. soft-bodied prey, alternately to each tank to avoid any potential confounding of treatment with spatial location or shelf effects. Fish in the hard-bodied prey treatment were fed two *P. acuta* snails with shells intact per day for 8 days (days 3–10), and then received Tetra-min Pro flakes for 6 days (days 11–16). Fish in the soft-bodied prey treatment were fed two *P. acuta* snail bodies removed from their shells per day for 8 days (days 3–10), and then received a similar 6 days of Tetra-min Pro flakes (days 11–16). Thus, the only difference between prey treatments involved the inclusion/exclusion of the shell along with the snail body during the snail-feeding period of the experiment—i.e., nutritional value remained constant across treatments, assuming no nutritive content of the hard shell itself for *Gambusia* fishes. We weighed (g) and measured standard length (mm) of each fish on four occasions: days 3, 7, 11, and 17. Fish of relatively similar size were selected for experimentation, and body size did not differ between treatments (*t*-test, initial mass: $P = 0.58$; initial SL: $P = 0.79$), nor did the average amount of snail mass fed to fish ($P = 0.82$). Fish mass changed considerably during the experiment, but length did not. Thus, we used initial SL as a potential covariate when examining variation in growth rate (g/day) during the experiment. To confirm low measurement error in our estimates of body size, we weighed and measured six similarly sized adult female *G. holbrooki* three times each and calculated repeatability as the intraclass correlation coefficient. Even though overall variation in body size was not high in this test (coefficient of variation = 0.14 and 0.07 for mass and SL, respectively), we found that repeatability was extremely high in both cases (mass: $r > 0.999$, SL: $r > 0.998$), supporting our use of these estimates of body size.

We collected all *P. acuta* snails from the same locality as the *G. holbrooki* used in the experiment, and prepared snails for feeding in advance of the experiment by weighing and freezing each individual (thawed immediately prior to feeding). We assigned each snail to be fed to a particular fish in a manner that maintained consistency throughout the experiment in the average mass of snails fed per day to a given fish. We prepared the Tetra-min Pro flakes in advance by weighing the flakes to ensure that each fish received flakes weighing twice the average mass of snails fed to each particular fish. During the experiment, tanks were vacuumed of debris and fecal waste every other day. Tanks were checked periodically throughout each day to note any feces or passed snail shells, which were removed once sighted.

We calculated the average daily growth rate (g/day) of each fish during both feeding periods (snail-feeding and flake-feeding periods) using the mass data collected during the experiment and conducted a repeated-measures general linear mixed model to test for effects on growth rate for the following terms: feeding period (snails vs. flakes), prey treatment (hard vs. soft), the interaction between feeding period and prey treatment, fish SL, and snail mass (average daily snail mass fed to each fish). The latter two variables were included as covariates to control for potential effects of body size or the amount of prey consumed on growth rate. We also initially included all two-way interactions between main effects and covariates and excluded all highly non-significant interaction terms ($P > 0.4$) from our final model. We included individual and block as random effects. We were especially interested in the interaction between feeding period and prey treatment, as we hypothesized that fish in the hard-bodied prey treatment would suffer reduced growth rate during the snail-feeding period, but subsequently exhibit elevated (compensatory) growth during the flake-feeding period.

To visualize changes in mass throughout the experiment, we plotted the relative mass of *G. holbrooki* over time (the 17 days of the experiment, with four measurement periods), and used cubic regression to summarize growth trajectories within each treatment. We estimated relative mass as back-transformed residuals of a linear regression of mass on SL (residuals + mean), which resulted in values of mass for each fish for each measurement period, controlling for body length, in g units. To provide an intuitive metric of recovery in the hard-prey treatment, we calculated the "compensatory index" following Wilson and Osbourn (1960). This index expresses the magnitude of compensatory mass gain as a percentage of the maximal mass differential between treatments, with a value of 100% indicating full recovery. We calculated the compensatory index as the difference in mass between treatments at the end of the snail-feeding period minus the difference in mass between treatments at the end of the flake-feeding period, divided by the mass difference at the end of the snail-feeding period.

RESULTS

Literature Survey of Durophagy in Teleost Fishes

We accumulated a dataset of 366 teleost fish species from 23 orders and 84 families, comprising stomach-content data from a total of 57,511 individual fish (**Supplementary Appendix 1**). Fifty one species from 18 families within this dataset were "durophage specialists," possessing adaptive morphological modifications for crushing and consuming hard-shelled prey—all of these species exhibited durophagy in our dataset. Within non-specialists, we detected a strong, positive effect of sample size on the detection of durophagy ($\chi^2 = 19.32$, $P < 0.0001$). Thus, the detectability of durophagy within non-specialists depended on the sample size of stomachs examined. Excluding species with <5 stomach samples did not eliminate this sample-size dependency ($\chi^2 = 7.08$, $P = 0.0078$), but excluding species with <10 stomach samples did ($\chi^2 = 1.20$, $P = 0.2731$). This resulted in a reduced dataset of 325 species from 22 orders and 82 families, comprising 57,233 stomach samples (48,579 for non-specialists, 8,654 for specialists). The reduced dataset did not remove any study completely from the analysis, as all excluded species derived from three studies that reported diets for multiple species, including some with large sample sizes (Randall, 1967; Winemiller and Ponwith, 1998; Lopez-Peralta and Arcila, 2002). We used this reduced dataset for all results presented here.

We found that over 63% of the species in our dataset exhibited durophagy (206 of 325 species). These 206 species spanned 14 orders (63.6%) and 60 families (73.2%). The majority of fish species that included hard-shelled prey in their diet did not possess specialized features for processing these prey: non-specialists represented ∼75% of the species (155 of 206 species). Despite potential costs for consuming hard-shelled prey, non-specialists typically consumed 10–40% as much hard-shelled prey as specialists, depending on the estimate of durophagy (**Table 1**). Even for specialized fishes, hard-shelled prey tended to comprise less than half of the diet on average (**Table 1**), consistent with broadly opportunistic foraging in most fishes, with partial reliance on specialized prey.

Field Survey of Molluscivory in *G. holbrooki*

We observed molluscivory in 8 of the 10 *G. holbrooki* populations examined, spanning their entire latitudinal range (**Supplementary Table 1**). While molluscivory appears widespread in *G. holbrooki* at the regional scale (especially in females), it was always locally uncommon within populations (3.3–13.3% occurrence; based on all fish examined, not only those with prey items present), at least based on x-ray images, which likely failed to detect consumption of small molluscs (<1 mm). Overall, we detected molluscs in the stomachs of 13 of 261 females (5.0%) and 2 of 170 males (1.2%). For fish with mollusc shells apparent within their stomachs in x-ray images, we found 1–20 shells present within a single stomach (**Figures 3A,B**), with a total of 56 shells observed. Individuals with molluscs in their stomach spanned a range of body size (18.2–33.8 mm SL, mean = 26.5 mm SL), suggesting that adults of any body size might consume molluscs in the wild. All shells appeared to be gastropods (primarily *Physa* spp.), except for two bivalves.

Molluscivory and Resource Competition in Bahamian Mosquitofish

In blue holes on Andros Island, we detected molluscivory in *G. hubbsi* within all 11 blue holes having high population density and no predatory fishes, but detected molluscivory in only 4 of 10 blue holes with low population density and predatory fish present (**Supplementary Table 2**). Overall, we found a much higher frequency of molluscivory in high-density/low-predation blue holes compared to low-density/high-predation blue holes ($F_{1,19} = 14.48$, $P = 0.0012$; **Figure 4A**), consistent with the prediction from optimal foraging theory for a higher frequency of costly-prey consumption in populations experiencing stronger resource competition. Our lower-bound estimate of molluscivory indicated that it was generally rare, with an average of 3% of fish examined having molluscs in their guts for populations where molluscivory was detected. When molluscs were observed, between 1 and 18 shells were present within stomachs (**Figures 3C,D**). Again, individuals with molluscs in their stomach spanned a large range of body size (17.0–49.2 mm SL, mean = 28.4 mm SL).

In Bahamian tidal creeks across six islands, we detected molluscivory using x-ray radiographs in 18 of 44 populations (41%), 47 of 1,466 females (3.2%), and 7 of 997 males (0.7%) (**Supplementary Table 3**). Consistent with the prediction from optimal foraging theory, we observed a higher frequency of molluscivory in the high-density scenarios of fragmented tidal creeks compared to unfragmented tidal creeks ($F_{1,32} = 6.80$, $P = 0.0137$; **Figure 4B**). We found no differences between the three species in molluscivory ($F_{2,32} = 0.42$, $P = 0.66$), nor any effects of the interaction between species and fragmentation status ($F_{2,32} = 1.41$, $P = 0.26$). Similarly, we found no significant variation among islands within species ($F_{3,32} = 0.96$, $P = 0.42$) or for the interaction between fragmentation status and island ($F_{3,32} = 1.28$, $P = 0.30$). Fish with mollusc shells in their guts again spanned the full range of adult body size (14.1–36.9 mm SL, mean = 22.4 mm SL), and had between 1 and 10 shells within their stomachs (**Figures 3E,F**).

Using direct examination of stomach contents for Bahamian mosquitofish in 13 tidal creeks on Abaco Island, we found a much higher frequency of molluscivory than when using x-ray methodology. Our observations indicated that this derived from smaller molluscs being detected using direct stomach-content analysis. In all, we detected molluscivory in 12 of the 13 populations, with an average percent by number of

TABLE 1 | Summary of average quantitative diet information for the 206 fish species exhibiting durophagy within our dataset derived from our literature survey.

Durophage specialization	*N*	% O	% N	% W	% V	% IRI	Stomachs
Non-specialized	155	7.68	8.64	12.61	19.99	4.53	34,273
Specialized	51	39.83	28.07	42.14	43.67	39.96	8,654

% O, percent occurrence; % N, percent by number; % W, percent by weight; % V, percent by volume; % IRI, percent index of relative importance.

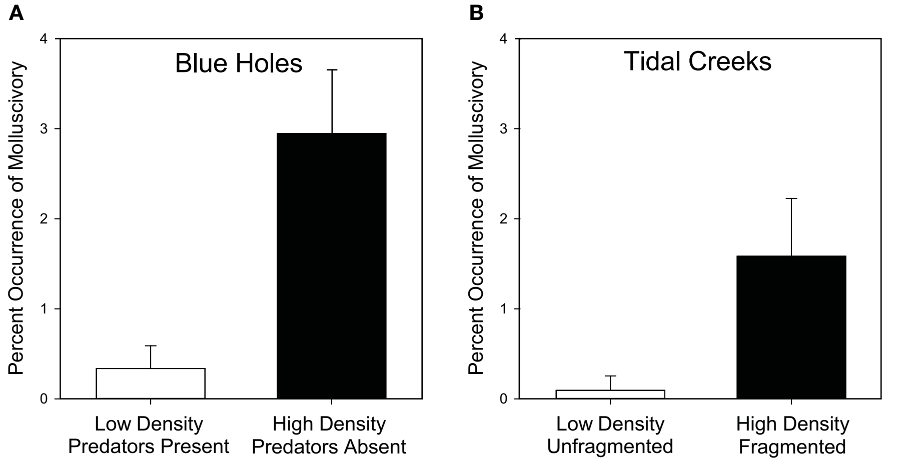

FIGURE 4 | Higher frequency of molluscivory associated with higher densities in Bahamian mosquitofish inhabiting **(A)** inland blue holes on Andros Island and **(B)** tidal creeks across six Bahamian islands. Back-transformed means ± SE depicted.

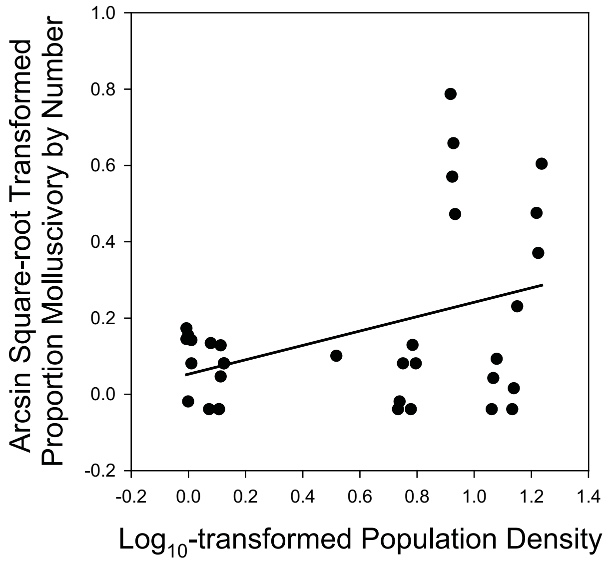

FIGURE 5 | Increased proportion of molluscs in the diet in sites with higher conspecific densities based on stomach-content analyses of male, female, and juvenile Bahamian mosquitofish inhabiting tidal creeks on Abaco Island, The Bahamas (see **Supplementary Table 4**) based on our general linear model (see text).

16.9% (molluscs as a percent of total prey items in the stomach). Consumption of molluscs was positively associated with population density ($F_{1,\,30} = 5.58$, $P = 0.0248$; **Figure 5**), while variation among sex/age classes was not significant ($F_{1,\,30} = 0.82$, $P = 0.45$).

Experimental Test of Cost of Molluscivory and Compensatory Growth in *G. holbrooki*

During the experiment, fish consumed the majority of prey given to them (184 of 192 snails in hard-prey treatment, 178 of 192 snail bodies in soft-prey treatment). For the hard-prey treatment, all snail shells were likely swallowed whole, as intact and empty shells of 94% of the snails consumed by *G. holbrooki* were found on the bottom of the aquarium (remaining shells were not found), and no case of shell crushing has ever been documented for any *Gambusia* fish.

TABLE 2 | Results of repeated-measures general linear mixed model examining variation in *Gambusia holbrooki* growth rate in the feeding experiment.

Source	*F*	*df*	*P*
Feeding period	16.37	1.48	0.0002
Prey treatment	0.07	1.48	0.7964
Standard length	2.26	1.48	0.1393
Snail mass	2.94	1.48	0.0929
Prey treatment × Feeding period	8.70	1.48	0.0049
Snail mass × Feeding period	5.20	1.48	0.0271

In our repeated-measures general linear mixed model, we found significant effects of two key terms on *G. holbrooki* growth rate: the prey treatment-by-feeding period interaction term and the snail mass-by-feeding period interaction term (**Table 2**). The clearest result from the experiment indicated that fish in the hard-prey treatment exhibited a reduced growth rate during the snail-feeding period relative to the soft-prey treatment, but elevated growth rate during the flake-feeding period (**Figure 6A**). This reflects a growth-rate cost of consuming hard-shelled prey, but also the presence of compensatory growth in fish receiving a high-quality diet after a period of consuming costly prey items. The second effect revealed by this experiment indicated that snail mass only influenced *G. holbrooki* growth rate during the snail-feeding period (not the flake-feeding period), with lower growth rates in fish fed larger snails on average—a trend primarily evident in the hard-prey treatment (**Figures 6B,C**).

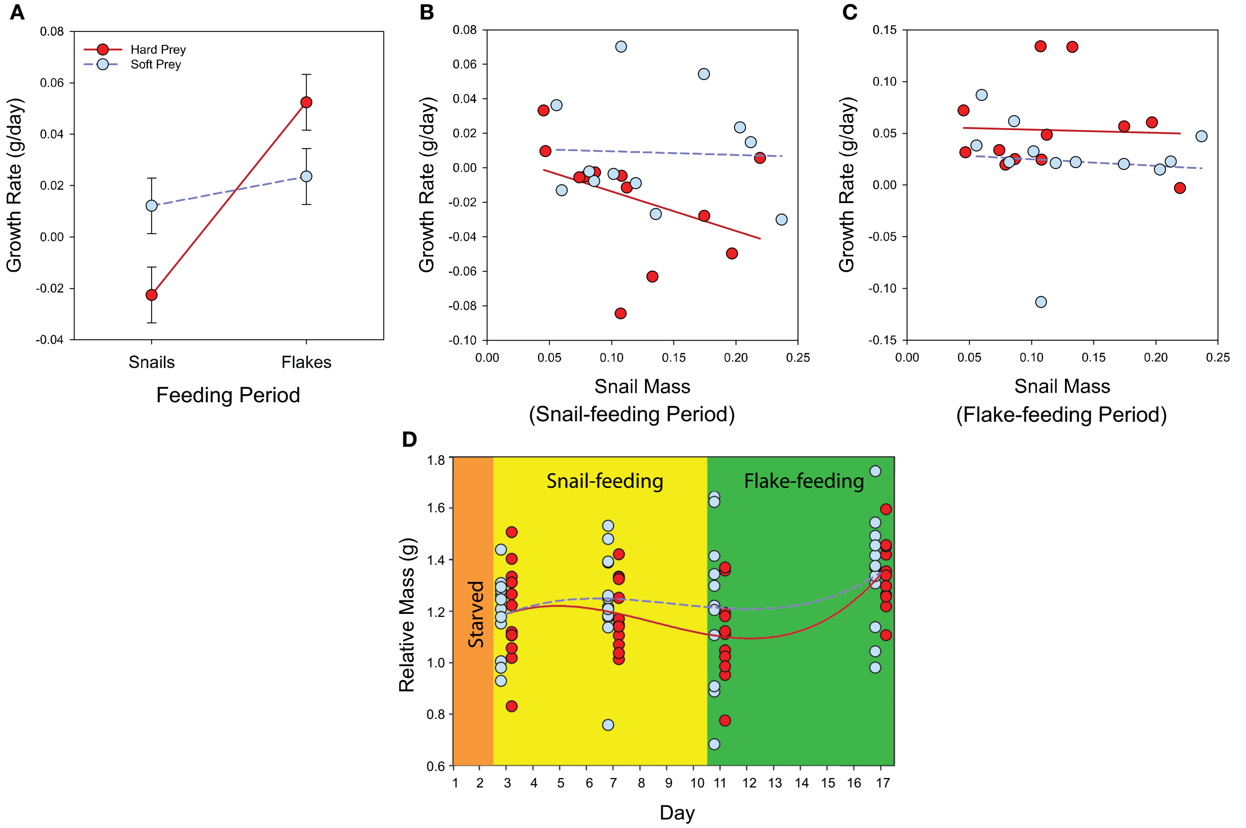

FIGURE 6 | Feeding experiment results in *Gambusia holbrooki* illustrating **(A)** differences in growth rate between hard- and soft-prey treatments during both snail-feeding and flake-feeding periods (least-squares means ± 1 standard error), **(B,C)** relationship between snail weight fed to fish and growth rate for both hard- and soft-prey treatments during **(B)** the snail-feeding period and **(C)** the flake-feeding period, and **(D)** overall pattern of mass change (relative to body length) during the experiment.

Overall, growth trajectories of the two treatments tended to diverge early in the experiment, reaching the maximal difference around the end of the snail-feeding period, but then converged by the end of the experiment due to compensatory growth during the flake-feeding period (**Figure 6D**). The estimated compensatory index was 93%, indicating a robust recovery of mass loss for fish in the hard-prey treatment via compensatory growth during the 6-day flake-feeding period.

DISCUSSION

Many animals eat a wide variety of prey, including items that likely elicit energetic, health, or reproductive costs, such as highly toxic or hard-shelled prey. Here we demonstrated that (1) durophagy is widespread in teleost fishes and that the majority of species that consume hard-shelled prey lack any relevant feeding specializations, (2) low levels of durophagy are prevalent throughout the range of *G. holbrooki* even though it results in reduced growth, (3) higher incidence of durophagy occurs under conditions of higher intraspecific competition for resources in Bahamian mosquitofish, and (4) compensatory growth can mitigate growth-related costs of durophagy. Overall, our results suggest that animals consume costly prey at a non-negligible frequency, increasing their consumption of these prey when high-quality prey become relatively scarce, and cope with the growth-depressing impacts of costly prey by exhibiting compensatory growth that offsets costs if alternative prey can be later acquired.

Costly Prey

While few would dispute the notion that many animals often consume suboptimal prey, an important question concerns the frequency with which animals consume truly *costly* prey in nature. To date, we have little relevant data to directly address this question, but much anecdotal evidence. Here we focused on durophagy, which is not only widely thought to entail considerable costs (see Introduction), but we experimentally confirmed a cost in terms of growth rate in the non-specialized fish *G. holbrooki* after eating molluscs for only 8 days. Our experimental design, which compared consumption of snails with intact shells to the consumption of only the snail bodies, allowed us to explicitly reveal growth-related costs of shell consumption *per se*, revealing the cost of consuming the hard, indigestible component of hard-shelled prey. Moreover, growth costs manifested rapidly even in adults, suggesting growth costs for juveniles or during longer-term durophagy would likely be more severe. In small livebearing fish, like *Gambusia* spp., gastropod shells can take up a significant portion of the gut, restricting the space available for other food or, in the case of pregnant females, for developing offspring within the body cavity. Therefore, molluscivory might affect growth, reproduction, and survival in *Gambusia* fishes. Hard-bodied prey are generally swallowed whole in non-specialized taxa, with little-to-no nutrition gained from the dense material. Thus, many, perhaps most, non-specialized animals likely experience a cost in terms of growth or fecundity when consuming hard-shelled prey relative to a soft-prey diet.

Regularity of Consumption of Costly Prey

Despite the apparent disadvantages, we found that costly-prey consumption appears widespread in nature. A majority of non-specialized species examined in our literature review exhibited durophagy, consuming ∼30–45% as much hard-shelled prey as specialists based on number, weight, and volume of diet items. Moreover, durophagy occurred throughout much of the native ranges of *G. holbrooki*, *G. hubbsi*, *G. manni*, and *G.* sp.—small, viviparous taxa that seem particularly vulnerable to durophagy-induced costs. Combined with previous work, this suggests that *Gambusia* fishes may regularly consume molluscs in the wild, sometimes at moderate to high frequencies [e.g., *G. affinis*: 58% occurrence (Walters and Legner, 1980), *G. nobilis*: 29% occurrence (Hubbs et al., 1978)]. Consumption of costly prey thus does not appear restricted to episodes of severe environmental harshness, but instead occurs, at least at low to moderate levels, throughout the year across many regions.

The apparent trend observed using x-ray methods where female *Gambusia* fishes often showed a higher frequency of molluscivory than males probably reflects the larger body size of females in these species, and their subsequent consumption of larger molluscs that are more dense and detectable with radiography. Indeed, our direct examination of stomach contents revealed more frequent consumption of smaller molluscs across all age and sex classes, with no differences between the sexes. That said, livebearing females may more regularly consume larger and denser snails than males due to their greater energy requirements—a pattern that could lead to considerable costs, as our experiment showed that consumption of larger snails had more negative consequences for growth. Interestingly, even though stomach-content analyses revealed higher absolute estimates of molluscivory than the x-ray methods, both methodological approaches pointed to the same associations with resource competition (see below). This suggests that x-ray methods underestimate total molluscivory, but provide reliable *relative* estimates of molluscivory, and reveal cases with strong likelihoods of fitness costs owing to the size and density of detectable shells. To determine the generality of our findings, future work should investigate the frequency of consumption of other types of costly prey, examine other taxa, and experimentally confirm the costliness of consumption of particular prey.

Costly Prey and Optimal Foraging Theory

According to optimal foraging theory, as high-quality foods become more difficult to acquire, animals should incorporate more suboptimal prey in their diet. Consistent with this prediction, the frequency of durophagy in our focal species appears related to resource competition—Bahamian *Gambusia* exhibited increased molluscivory in populations with higher density. This pattern was evident across three species spanning six islands inhabiting two different types of ecosystems (blue holes and tidal creeks), regardless of whether we used x-rays to detect molluscivory or stomach contents to quantify relative consumption of molluscs. This suggests that increased competition for food resources in these high-density populations elicits increased utilization of costly prey that provide little benefit relative to starving. While most of the mollusc species observed within *Gambusia* diets are present within nearly all study sites (RBL pers. obs.), and preliminary examination within a subset of these sites has found no covariation between mollusc abundance and *Gambusia* population density (RBL unpubl. data), future work should directly examine this topic. In the present study, predation risk may provide an additional contributing factor, as prey could alter activity levels or utilize alternative habitats with varying mollusc abundances in the presence of predators, and thus encounter mollusc prey at different frequencies in high-predation/low-density environments compared to low-predation/high-density environments. Regardless, swallowing whole snail shells that occupy considerable space in the digestive tract and body cavity, and eventually expelling them whole, poses a range of risks and potential costs with comparatively little energetic gain from the snail body. Thus, molluscivory should presumably be exceedingly rare except in extreme circumstances—but our findings in the literature survey and across the range of four *Gambusia* species indicate that it is not as rare as one might expect, suggesting an additional mechanism that mitigates its costs may be prevalent.

Costly Prey and Compensatory Growth

Compensatory growth is widespread across animal taxa (see **Box 1** and references therein) and could represent a common means of offsetting growth costs caused by eating costly prey. Compensatory growth has traditionally been viewed as an evolved mechanism that (at least partially) offsets fitness costs imposed by food shortage or a reduced growing season, but perhaps compensatory growth is more profitably viewed as a generalized life-history strategy to buffer adult body size against a wide range of environmental perturbations that could potentially reduce body size. Thus, compensatory growth might not only occur in response to food restriction or time constraints but also to generalized cues of a reduced probability of achieving an optimal body size. This means it might represent an important, previously unrecognized means of counteracting growth-depressing effects of the widespread phenomenon of costly-prey consumption.

The magnitude and rate of compensatory growth that we observed in *G. holbrooki* suggests this mechanism might greatly reduce, or even eliminate, potential fitness costs in the wild under a range of scenarios of low to moderate consumption of costly prey. Whether compensatory growth might vary with age, sex, stage of pregnancy, or duration of durophagy is currently unknown, but previous work has found some of these factors can influence the degree of compensatory growth [e.g., see Wilson and Osbourn (1960)]. Because the adaptive benefit of compensatory growth depends on the likelihood of surviving until high-quality food is re-acquired, this suggests that animals inhabiting environments with especially high mortality rates following costly-prey consumption should exhibit reduced levels of compensatory growth compared to organisms in low-mortality environments. Future work could test this hypothesis using comparative data across populations or species.

While compensatory growth can provide important benefits that mitigate costs of eating certain prey resources, it can also entail costs of its own, explaining why animals don't always exhibit the high growth rates observed during phases of compensatory growth (Arendt, 1997; Mangel and Stamps, 2001; Metcalfe and Monaghan, 2001; Johnsson and Bohlin, 2006; Royle et al., 2006; De Block and Stoks, 2008; Dmitriew, 2011; Hector and Nakagawa, 2012; Kahn et al., 2012). Faster than optimal growth can involve a range of costs, such as reductions in cell functioning efficiency, immune function, resistance to physiological stressors, fecundity, dominance rank, body size, locomotor performance, mating attractiveness, and lifespan. As long as the benefits outweigh the costs, compensatory growth provides an adaptive strategy for contending with costly prey. Considering that many of the purported costs of compensatory growth are delayed until later in life, often after (at least initial) reproduction (Metcalfe and Monaghan, 2001; Yearsley et al., 2004; Stoks et al., 2006; Ab Ghani and Merilä, 2014), combined with the ubiquity of compensatory growth in animals (e.g., Wilson and Osbourn, 1960; Tanner, 1963; Ali et al., 2003; Hector and Nakagawa, 2012), it may be that the fitness benefits indeed typically outweigh the costs in many natural systems. Nevertheless, to better understand the evolution of compensatory growth and its role in coping with the consumption of costly prey, future work should examine the potential costs of compensatory growth and their fitness consequences.

One area that seems to warrant future attention is how costly-prey consumption and compensatory growth might influence reproductive strategies. For instance, whether compensatory growth might often involve reproductive tissue, as opposed to somatic growth, has not yet been explored. Costly-prey consumption might affect reproductive traits such as egg development, yolk content, nutrient transfer to embryos, and embryo abortion. Moreover, while large body size often enhances fitness (Roff, 2002), elevated somatic growth can sometimes have smaller fitness consequences than reproductive traits that directly increase fecundity. Thus, we might expect to find compensatory *reproduction* following costly-prey consumption in some taxa. That is, if compensatory growth represents a life-history adaptation to achieve high fitness in the face of costly-prey consumption through rapid attainment of appropriate body size, then compensatory reproduction might represent an alternative, non-mutually exclusive strategy to maintain high reproductive output, especially later in life when somatic growth has less importance for fitness and future reproductive output is at a premium [essentially a special case of the terminal-investment hypothesis, (Charlesworth and Leon, 1976; Clutton-Brock, 1984)]. Therefore, animals might exhibit compensatory growth of reproductive tissues, such as larger or more numerous eggs or embryos, or invest more in reproductive behaviors, to compensate for lost reproductive output or mating opportunities. Future work should examine how costly-prey consumption might influence reproductive strategies.

CONCLUSION

Our results, combined with both the established predictive power of optimal foraging theory and the pervasiveness of compensatory growth in animals, point to these two mechanisms as factors of general importance in explaining why animals may regularly include costly prey in their diet. Thus, it appears that a profitable avenue for future investigation would center on optimal foraging and compensatory growth as major explanations for costly-prey consumption in nature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, and available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.mkkwh70z4 (Langerhans et al., 2020).

ETHICS STATEMENT

Animal research was reviewed and approved by the Institutional Animal Care and Use Committee of North Carolina State University (protocol 13-101-O).

AUTHOR CONTRIBUTIONS

RL conceived and coordinated the study, analyzed the data, and prepared the original manuscript. TG and KS performed the literature survey and laboratory experiment. RR led the field survey of *G. holbrooki*. RR, MA, and CL assisted with Bahamian mosquitofish components. RL and TG collected molluscivory data using x-rays. MA conducted the stomach-content analyses of Bahamian mosquitofish. CL made intellectual contributions to the work. All authors contributed significant edits to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.603387/full#supplementary-material

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REVIEW
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The Fish Family Poeciliidae as a Model to Study the Evolution and Diversification of Regenerative Capacity in Vertebrates

Diego Safian[1,2], Geert F. Wiegertjes[1] and Bart J. A. Pollux[2]*

[1] Aquaculture and Fisheries Group, Department of Animal Sciences, Wageningen University, Wageningen, Netherlands,
[2] Experimental Zoology Group, Department of Animal Sciences, Wageningen University, Wageningen, Netherlands

The capacity of regenerating a new structure after losing an old one is a major challenge in the animal kingdom. Fish have emerged as an interesting model to study regeneration due to their high and diverse regenerative capacity. To date, most efforts have focused on revealing the mechanisms underlying fin regeneration, but information on why and how this capacity evolves remains incomplete. Here, we propose the livebearing fish family Poeciliidae as a promising new model system to study the evolution of fin regeneration. First, we review the current state of knowledge on the evolution of regeneration in the animal kingdom, with a special emphasis on fish fins. Second, we summarize recent advances in our understanding of the mechanisms behind fin regeneration in fish. Third, we discuss potential evolutionary pressures that may modulate the regenerative capacity of fish fins and propose three new theories for how natural and sexual selection can lead to the evolution of fin regeneration: (1) signaling-driven fin regeneration, (2) predation-driven fin regeneration, and (3) matrotrophy-suppressed fin regeneration. Finally, we argue that fish from the family Poeciliidae are an excellent model system to test these theories, because they comprise of a large variety of species in a well-defined phylogenetic framework that inhabit very different environments and display remarkable variation in reproductive traits, allowing for comparative studies of fin regeneration among closely related species, among populations within species or among individuals within populations. This new model system has the potential to shed new light on the underlying genetic and molecular mechanisms driving the evolution and diversification of regeneration in vertebrates.

Keywords: Poeciliidae, evolution, fin, regeneration, diversification

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Wayne Iwan Lee Davies,
Umeå University, Sweden

Reviewed by:
Jennifer Rodger,
University of Western Australia,
Australia
David Bierbach,
Leibniz-Institute of Freshwater
Ecology and Inland Fisheries (IGB),
Germany

***Correspondence:**
Bart J. A. Pollux
bart.pollux@wur.nl

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INTRODUCTION

Regeneration is one of the most intriguing phenomena in nature. In the last decades, the capacity of regenerating a damaged or lost structure has been a central interest for scientists. Understanding this process may potentially lead to new treatments of medical problems involving tissue damage, such as injuries, cancer, aging and disease (Mao and Mooney, 2015), but also represents an interesting case of study for developmental and evolutionary research. Regeneration is a complex process that involves the regulation of different cell types and reports have shown that it can recapitulate cellular and molecular processes that take place during early development (Martin and Parkhurst, 2004; Ghosh et al., 2008). Remarkably, this capacity is widespread in different phyla, yet regenerative ability has diverged, being absent or restricted to specific tissues or short time-windows after birth in many animal lineages (Porrello et al., 2011).

Regeneration can be classified into three types: (1) Physiological regeneration, which is tissue regeneration that takes place under normal conditions, i.e., not in response to injury. This is a continuous process that supports the turnover of particular cells in a structure throughout the life of a given organism, maintaining the tissue homeostasis (e.g., the continuous replacement of cells in the epidermis and blood). (2) Reparative regeneration, which refers to an organism's capacity to repair organs or tissues after a minor injury (e.g., healing of blood vessels and skin cells after a cut). (3) Restorative regeneration (or "true regeneration"), which consists of the capacity to regrow a fully functional, scar-free structure after loss of that structure (e.g., regeneration of limbs, tail, external gills and fins). Several remarkable examples of restorative regeneration can be found in nature from invertebrates to vertebrates. Flatworms (*Planaria* sp.; Ivankovic et al., 2019) and polyps (*Hydra* sp.; Vogg et al., 2019) are highly regenerative invertebrates that can regenerate most of their structures after severe injuries. Some species can even form an entirely new individual from a small body fragment. This capacity is also present in vertebrates, but here the regenerative capability is often reduced in extent or restricted to specific structures (Galliot et al., 2017). For instance, many lizards can regenerate a missing tail but fail in regenerating other structures such as limbs (Alibardi and Toni, 2005). This review will only address restorative regeneration to which, for the sake of simplicity, we will hereafter refer to as "regeneration."

EVOLUTIONARY ORIGINS OF REGENERATION: A LONG-STANDING OPEN QUESTION

One of the more intriguing questions regarding regeneration is how this trait has evolved in different lineages. It is clear that regeneration ability is widely distributed in several phyla, but the origin of this capability remains to be determined. Based on the general similarity in the regenerative program such as induction of wound epidermis and involvement of similar cell signals controlling the process (e.g., Wnt, Fgf, and Bmps) in distant taxa, it has been hypothesized that regeneration perhaps arose in early animals as an epiphenomenon of development and was subsequently lost several times in different lineages (Bely and Nyberg, 2010; Galis et al., 2018). However, the cellular mechanisms that underlie the regeneration program can be remarkably diverse, suggesting that perhaps regeneration is not an ancestral trait but that instead it represents a novel innovation that has evolved independently many times in different phyla as an adaptive trait (Tiozzo and Copley, 2015).

Although it is not clear yet how regeneration evolves, three potential causes have been proposed to explain a lack of, or reduced, regenerative ability in animals (Bely, 2010): (1) The first is that the regenerative capacity becomes restricted as the individual keeps developing, due to a reduction of cellular plasticity. This is especially relevant for those regenerative processes that require dedifferentiation or transdifferentiation of local, post-mitotic cells, which again must enter into a new cell cycle to lose their identity, divide and differentiate. Galis et al. (2003) proposed that regeneration is only possible for structures that during embryogenesis develop independently of transient organs. They argued, for example, that limb regeneration in amniotes is not possible because embryonic limb development requires the support of interacting signals from the somites, which are no longer present during adulthood. (2) Another possibility is that other vital processes such as growth and reproduction become favored over regeneration, increasing the threshold for investment into regeneration. In these cases, regeneration could simply be too costly to occur (**Box 1**), especially if the missing tissue is dispensable for survival. In these animals, regeneration is a maladaptive trait that is selected against. (3) Finally, environmental conditions such as temperature and food availability may modulate the ability to regenerate a damaged tissue. Intriguingly, a recent study by Hirose et al. (2019) suggests that the ability to regenerate heart tissue might be modified during the evolutionary transition from exothermy to endothermy. This study showed that heart regeneration competence is associated with low number of polyploid cardiomyocyte in 41 vertebrate species (Hirose et al., 2019). In addition, it also revealed that thyroid hormone signaling (which is involved in regulating corporal temperature and is produced in higher levels in endotherms) increased cardiomyocyte polyploidization and inhibited heart regeneration in newborn mice. Remarkably, thyroid hormones conserved their inhibitory capacity in adult zebrafish, a species with high heart regenerative capacity but naturally low levels of thyroid hormones (Hirose et al., 2019). This study provides the first empirical evidence in support of the idea that habitat adaptation can shape the evolutionary trajectory of the regenerative capacity.

It is clear that internal and external cues converge to modulate an organism's regenerative ability and that these can be as diverse as the animal kingdom. Among regenerative species, fish have become an interesting system to study regeneration due to their restorative potential. Fins, in particular, are increasingly recognized as a relevant model to understand the factors shaping regenerative capacity, because next to their primary function (i.e., swimming) (i), fins are often fundamental for secondary functions that directly impact fitness (e.g., swimming, feeding, reproduction) and (ii) are (contrary to other regenerative tissues, such as the heart and spinal cord) often directly exposed to biotic and abiotic factors.

FISH FINS AS A MODEL SYSTEM TO STUDY REGENERATION

Traditionally, a limited number of model species have been used to study regeneration. Yet, in the recent years, important efforts have been made to expand the diversity of animals used in regeneration research, including species from distantly

BOX 1 | Immune system and fin regeneration.

After an injury, non-regenerative species respond by forming a scar that rapidly closes the wounded area without regenerating the lost structure. Regenerative organisms, on the other hand, are able to form a wound epidermis that is compatible with regeneration. It has been argued that (the absence of) an immune response is crucial to determine the type of repair response. Therefore, the evolution of a more efficient immune system (immune competence) has been linked to the loss of regeneration in several advanced taxonomic groups (Alibardi, 2017; Julier et al., 2017).

Injuries almost always induce a response from the immune system. First, cells of the innate immune system such as neutrophils and macrophages provide a rapid defense against potential pathogens invading the damaged tissue. Neutrophils are the first immune cell to respond to the open wound; however, these granulocytes appear to be dispensable for fin regeneration (Li et al., 2012). In contrast, macrophages play a predominant role in the restoration of tissue homeostasis and the timely presence of macrophage subtypes seem to be relevant during different stages of appendage regeneration (Godwin et al., 2013; Petrie et al., 2014; Paredes et al., 2019). In zebrafish, Nguyen-Chi et al. (2017) reported that the two most apparent subtypes of macrophages, pro-inflammatory (M1) and anti-inflammatory (M2), actively participate during fin regeneration and that caudal fin amputation in larval zebrafish induced an early inflammatory response characterized by an accumulation of both subtypes of macrophages. This accumulation would eventually result in an increase of macrophage-derived tumor necrosis factor alpha (Tnfa), which in turn further promotes the recruitment of more macrophages and participates in the initiation of blastema cell proliferation (Nguyen-Chi et al., 2017). Upon this early signal, M2 macrophages remained high in number while the M1 macrophage population decreased in the wound area, suggesting that M2 macrophages are likely involved in the remodeling of cells during fin regeneration (Nguyen-Chi et al., 2017). This idea is further supported by recent studies on adult zebrafish showing that macrophages are capable of attenuating inflammation after injuries (Hasegawa et al., 2017) and that macrophage accumulation within the regenerating tissue is needed for proper fin regeneration, in line with a pro-regenerative gene profile (i.e., expression of genes associated with blood vessel development, leukocyte migration, and regulation of the inflammatory response) (Sanz-Morejón et al., 2019). In addition, it has been proposed that a population of tissue-resident macrophages contributes in regenerating fin after injury (Morales and Allende, 2019). Interestingly, the responses of macrophages and neutrophils in zebrafish seem to be dependent on the type of fin injuries i.e., while thermally induced wounds were resolved by regeneration, infected wounds lead to persistent inflammation and minimal tissue repair, suggesting that a more robust immune response (as induced by bacterial infection) restricted the extent of regeneration (Miskolci et al., 2019).

The adaptative immune system also participates in the regenerative process, but this response is typically slower and probably associated with maintaining tissue homeostasis during the regenerative process. A specialized group of T cells, the regulatory T cells (T_{reg}), are particularly relevant to tissue regeneration, at least in mammals: T_{reg} stimulate self-tolerance, prevent autoimmune and autoinflammatory disorders, and contain excessive inflammatory responses to infection and tissue damage by modulating the activity of effector cells of the immune system (Josefowicz et al., 2012). Additionally, tissue-specific T_{reg} with proficiency in controlling tissue homeostasis and repair have been identified in mammals (Sharma and Rudra, 2018). In fish, T_{reg} have also been identified with apparent conservation of many of the functions reported for higher vertebrates (Sugimoto et al., 2017). Zebrafish T_{reg} rapidly migrate and infiltrate the damaged organs, while conditional ablation of this cell type in adult zebrafish blocked tissue regeneration by impairing proliferation of blastemal cells (Hui et al., 2017; Zwi et al., 2019). Different to its role in tissue/wound healing, other components of the adaptive immune system have not been directly linked to fin regeneration capacity, yet should not be disregarded as, in other vertebrates, several of these components are present in the blastema during limb regeneration (Leigh et al., 2018).

phyla such as Platyhelminthes (e.g., *Schmidtea mediterranea*; Ivankovic et al., 2019), Cnidaria (e.g., *Hydra vulgaris*; Vogg et al., 2019), Arthropoda (e.g., *Drosophila melanogaster*; Fox et al., 2020), Echinodermata (e.g., *Patiria miniata*; Cary et al., 2019) and Chordata, which include species from different class such as Actinopterygii (e.g., zebrafish; Marques et al., 2019), Amphibia (e.g., *Xenopus leavis and Ambystoma mexicanum*; Joven et al., 2019; Phipps et al., 2020), Reptilia (e.g., *Podarcis muralis*; Alibardi, 2017) and Mammalia (e.g., *Acomys cahirinus*; class Mammalia; Maden and Varholick, 2020).

Comparative studies on more closely related animals that have diverse regenerative capacities in a well-defined phylogenetic framework are likely to shed further light on the evolution of this trait and the selective pressures that favor it. In this context, fishes represent an excellent system to study the evolution of regeneration. Fishes are an extraordinary diverse group of animals (Hughes et al., 2018) and several studies have reported on the presence of regenerative properties of several different tissues, such as the heart, spinal cord, brain, liver, and fins (Gemberling et al., 2013). For example, all bony fishes (Superclass Osteichthyes) that have been studied so far have shown at least some degree of fin regeneration. However, while homomorphic (complete) fin regeneration has been reported in lobe-finned fishes (class Sarcopterygii) and the early ray-finned fish lineages (class Actinopterygii; e.g., Polypteriformes, Lepisosteiformes, Cypriniformes, Esociformes, and Salmoniformes; **Figure 1**, blue branches), heteromorphic or incomplete fin regeneration (i.e., resulting in an unfinished, abnormal fin shape) has additionally been found in the more recent lineages (Neotelesots, i.e., Cichliformes, Cyprinodontiformes, Perciformes, and Scorpaeniformes; **Figure 1**, red branches) (Wagner and Misof, 1992; Cuervo et al., 2012; Darnet et al., 2019), suggesting that the regenerative capacity has been reduced in these latter groups. Yet, a broader study addressing the causes and underlying molecular mechanism of homo- and heteromorphic fin regeneration is currently missing.

General Fin Organization in Fish

Fish fins fulfill key functions (e.g., during escaping from predators, capturing prey and reproduction) that directly impact fitness and, hence, are subject to strong natural and sexual selection (Fu et al., 2013; Pollux et al., 2014; Price et al., 2015). Fish fins can be grouped into single fins (i.e., dorsal, anal and caudal fin) and paired fins (i.e., pectoral and pelvic fins) (**Figure 2**). Fins are supported in the basal segment by an endoskeleton. Typically, this endoskeleton in paired fins consists of vertebra-independent skeletal muscle and bones, called proximal radials, arranged side by side along the anterior-posterior axis, followed by small nodular distal radials (**Figure 2**). In contrast, the endoskeleton of the single fins is coupled directly to bones of the vertebra or via neural spines that extend from the vertebral bones. Despite these differences in the endoskeleton, in both paired and single fins the dermal skeleton is composed of several segmented rays surrounded by soft inter-ray tissue, lacking muscle and cartilage (**Figure 2**). During fish growth, the dermal skeleton increases in length through the addition of new segments in the most distal part of the fin, which initially forms as a thin and soft tissue that becomes thicker and mineralized as the fin increases in length

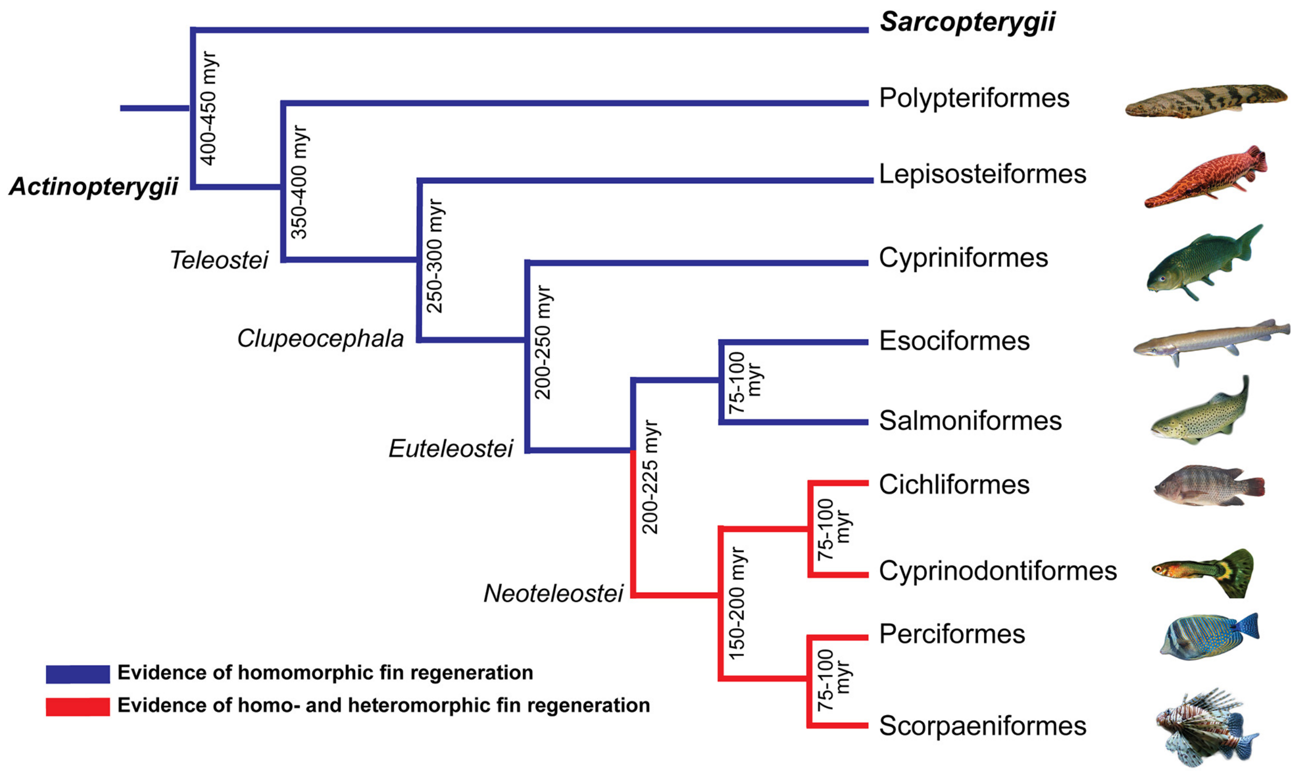

FIGURE 1 | Schematic representation of type of fin regeneration in different fish lineages. Topology of phylogenetic tree showing the distribution of homo- and heteromorphic fin regeneration in the class Sarcopterygii (lobe-finned fishes) and different orders of the class Actinopterygii (ray-finned fishes). Lineages with reports of homomorphic fin regeneration are highlighted in blue, while lineages with evidence for homo- and heteromorphic fin regeneration are highlighted in red (Wagner and Misof, 1992; Cuervo et al., 2012; Darnet et al., 2019). Numbers after nodes represent the estimated time (in millions of years, myr) since the emergence of the new lineage.

(Goldsmith et al., 2006). Remarkably, both the endoskeleton and dermal skeleton have shown regenerative capacity in fish.

Endoskeleton

Complete appendage regeneration has been observed in both lobe-finned fishes (class Sarcopterygii) and ray-finned fishes (class Actinopterygii) and recent studies suggest that these share a conserved regenerative program to repair the endoskeleton (Nogueira et al., 2016; Darnet et al., 2019). The early ray-finned *Polypterus senegalus* and *P. ornatipinnis* have a remarkable capacity to regenerate endoskeleton fins, showing complete regeneration within 1 month after proximal amputations (Cuervo et al., 2012). A blastema (mass of proliferating progenitor cells) is formed within 3 days post-amputation (dpa) to later give rise to a basal apical epidermis that borders the blastema in an anterior–posterior direction (Cuervo et al., 2012). This apical epidermis rapidly expands and bends toward the dorsal side, thereby giving rise to the fin fold that will develop into the endoskeleton (Cuervo et al., 2012). Cartilage differentiation is observed early in the expected bone formation area (9 dpa). A cartilaginous plate is formed, in which thickened margin predicts the differentiation of long bones, whereas radials emerge by splitting of the cartilaginous plate (Cuervo et al., 2012). Recent work has shown that other more recent ray-finned fish such as teleosts (e.g., *Amatitlania nigrofasciata, Astronotus ocellatus,* and *Carassius auratus*) can also regenerate the endoskeleton (Darnet et al., 2019).

Dermal Skeleton

Since the endoskeleton precedes dermal skeleton regeneration, one might predict that this latter capacity is a continuation of the former. However, a recent study showed that the capacity of regenerating the first does not necessarily guarantee dermal skeleton regeneration (Pápai et al., 2019), opening the possibility that different systems regulate the regeneration of both structures. The molecular mechanism underlying dermal skeleton regeneration has been extensively studied in zebrafish and its relevance and latest advances have been discussed recently by Marques et al. (2019) and Sehring and Weidinger (2020). Therefore, in this review we will only briefly describe this process. Similar to the regeneration of other appendages, dermal fin regeneration relies on a blastema to repair the lost tissue (Pfefferli and Jaźwińska, 2015; **Figure 3**). Cells in the apical segment of the new and growing appendage maintain their undifferentiated stages, while blastema descendent cells differentiate into the new tissue (Pfefferli and Jaźwińska, 2015). Therefore, a delicate balance between self-renewal and differentiation of progenitor cells is required to succeed. It has been proposed that this balance

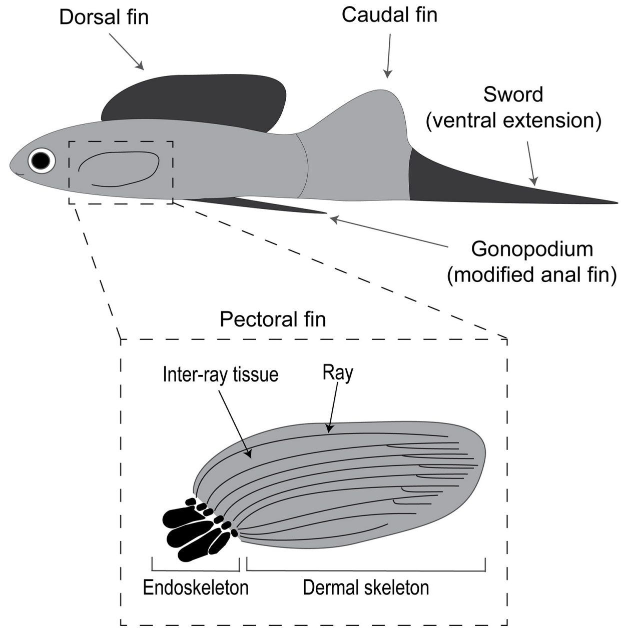

FIGURE 2 | Sexual and natural selection have driven modifications in male fin morphology and function in the fish family Poeciliidae. The different fins types include the paired pectoral fins and the single dorsal, caudal and anal (in males modified into a copulatory organ called the gonopodium) fins. The dashed square inset shows the structure of archetypical fins in Actinopterygii (ray-finned) fishes highlighting the endoskeleton (in black; only present in paired fins) and the dermal skeleton or exoskeleton (present in both paired and single fins).

is tightly regulated in the apical part of the blastema by Wnt signaling system, which coordinates blastemal cell proliferation and osteoblast maturation indirectly via downstream signals such as retinoic acid, Igf and Bmp in zebrafish (Wehner et al., 2014; Wehner and Weidinger, 2015; **Figure 3**).

As discussed earlier (under section "Fish fins as a model system to study regeneration" and **Figure 1**), in some fish groups regeneration has been reduced, showing heteromorphic fin regeneration. Yet, one other striking feature of appendage regeneration in the animal kingdom is the capacity of precisely reassembling the lost structure: i.e., giving rise to a new structure with precisely the same shape and size as the old structure after an injury (**Figure 3**). In zebrafish, both small wounds and major injuries, and even geometrically diverse cuts, in the fin result in a new structure that is indistinct from the old one. The predominant hypothesis to explain this process proposes that cells must evolve a positional memory, triggering the appropriate rate and extent of re-growth after injury. The remaining cells in the wound area would conserve memory and instruct how much and what type of cell programming will be required. Supporting this hypothesis, transplantation experiments of fin rays from the lobular caudal fin into a new position within the caudal fin showed that fin rays grow to reach their expected length, irrespective of the position of insertion (Shibata et al., 2018). Additionally, despite regeneration being faster in injuries close to a proximal position rather than in a distal position, both types of injuries reached the original growth length in similar time (Lee et al., 2005). The genetic and molecular mechanism controlling positional memory during fin regeneration is still unclear, but two main models have been proposed. (i) The "growth factor-base model" indicates that the levels of morphogens control fin length growth (Lee et al., 2005), while (ii) the recently proposed "niche size model" suggests that the numbers of niches harboring progenitor cells that support the blastema progressively decrease from the most proximal to the most distal part of the fins (Stewart et al., 2019). Although more work needs to be done in order to fully understand the mechanism of positional memory (Wang et al., 2019), it is clear that regenerative capacity relies on blastema cell proliferation and locally produced growth factors, which in turn can modulate the growth rate and accuracy of fin regeneration (Lee et al., 2005; Blum and Begemann, 2012; Stewart et al., 2014, 2019).

HYPOTHESES FOR THE EVOLUTION OF FIN REGENERATION IN THE FISH FAMILY POECILIIDAE

The Family Poeciliidae as a Model to Study Regeneration

The fish family Poeciliidae (order Cyprinodontiformes) is a diverse group of neotropical fish that consists of approximately 299 species in 27 genera (Lucinda, 2003). All species in the family exhibit internal fertilization and all, save for one, are viviparous (Furness et al., 2019), giving live-birth to fully developed precocial offspring (Lankheet et al., 2016). Members of this family, e.g., the guppy (*Poecilia reticulata*), mollies (subgenus *Mollienesia*), swordtails and platies (genus *Xiphophorus*), are commonly used in a wide variety of biological studies, ranging from cancer research and toxicology to sexual selection, life history evolution, genetics, ecology, and behavior (Ramsey et al., 2011; Pollux et al., 2014; Culumber and Tobler, 2017; Tobler et al., 2018; de Carvalho et al., 2019; McGowan et al., 2019; Thomaz et al., 2019; Hagmayer et al., 2020). This is in part because these fishes are easy to keep and breed in laboratory conditions and partly because this family provides a well-defined phylogenetic framework (Furness et al., 2019), allowing for comparative studies among closely related species with contrasting lifestyles (e.g., inhabiting different environments, displaying different forms of sexual selection, having different modes of reproduction).

The evolution of this family has been strongly marked by sexual and natural selection, resulting in great morphological and behavioral diversity evidenced by the evolution of ornamentation, specialized fins, courtship behavior and dichromatism among others (Pollux et al., 2014; Furness et al., 2019). Fins are a particularly important morphological feature fulfilling many different secondary functions that directly influence fitness (e.g., capturing prey, escaping predators, attracting mates), and as such are also subject to strong selective pressure. As a consequence, there is an extreme variation in fin size and shape within and among poeciliid species (**Figure 2**). Here, we argue that the regenerative capacity of fins is another,

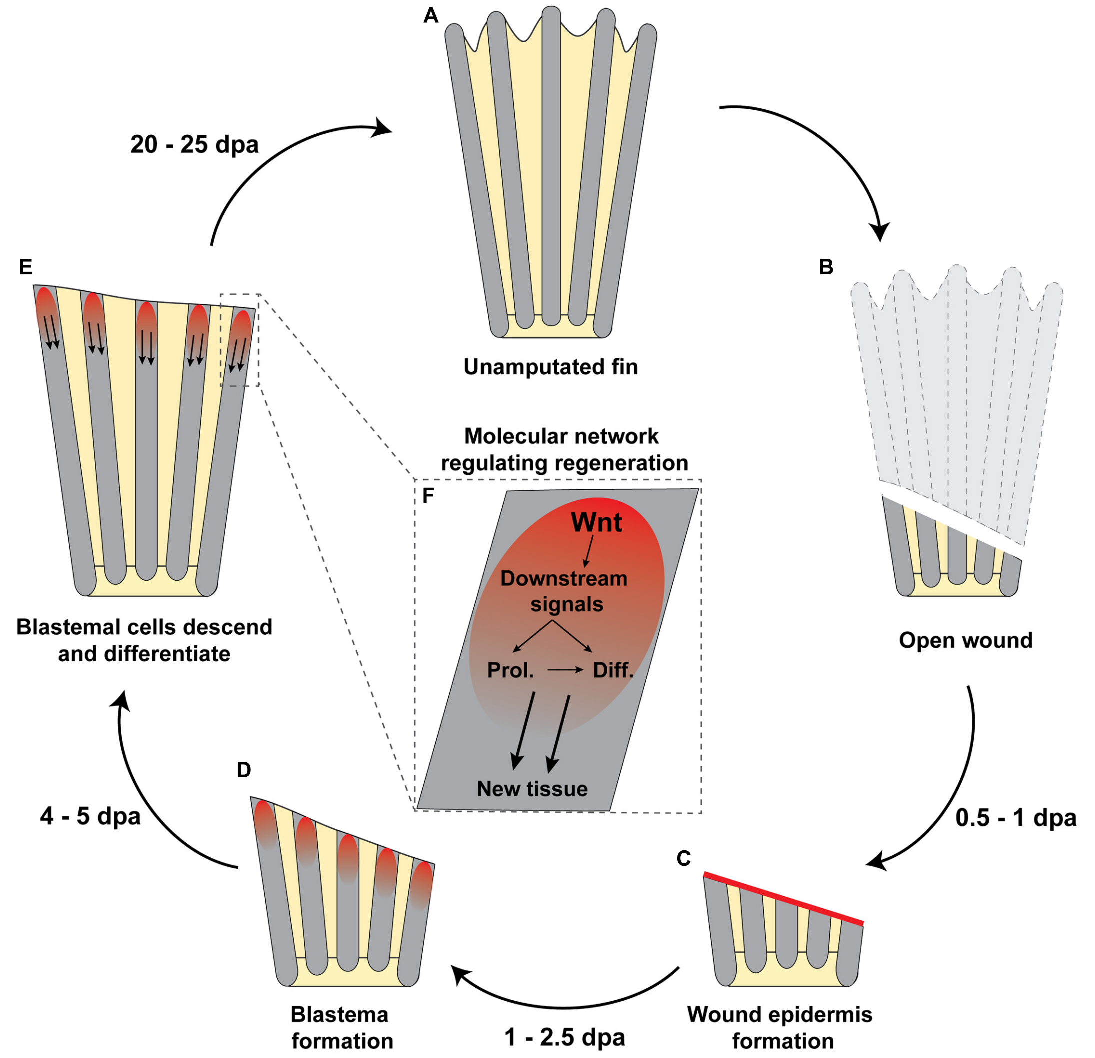

FIGURE 3 | Regeneration of the dermal skeleton in fish fins after injury. **(A)** Uninjured fin. **(B)** The regenerative process starts with an open wound from a partially amputated fin. **(C)** The open wound is rapidly closed by a wound epidermis (red line) within 1 day post amputation (dpa). **(D)** It is thought that wound epidermis initiates the blastema formation (red area in the most distal area of the fin), where **(E,F)** blastema cells descend upon Wnt and downstream signaling, which induce post-mitotic cells entering the cell cycle to proliferate (Prol.) and then differentiate (Diff.) to form the new tissue. **(A)** After 20–25 dpa, the newly regenerated fin is finished, completely reassembling the original structure. The outgrowth rate and accuracy of regeneration (homo- or heteromorphic) can vary depending on the species.

still overlooked feature of fins that is also subject to stringent natural and sexual selection. Specifically, we predict that the regenerative capability of a fin should be positively associated with its functional importance.

If true, then we expect to find significant variation in regenerative capability within or among Poeciliid species. Indeed, the few studies that have been performed to date show differences in the timing of blastema formation among Poeciliid species, ranging from 1 to 2.5 dpa (Zauner et al., 2003; Murawala et al., 2017), and full-length regeneration, ranging from 21 to 25 dpa (Kolluru et al., 2006; Offen et al., 2008; Patel et al., 2019). However, a systematic investigation of the regenerative abilities across the Poeciliidae is not yet available. Moreover, comparative studies that juxtapose closely related species (or populations within species) inhabiting different environments (e.g., predation *vs* predation-free environments) or displaying different mating strategies (e.g., courtship *vs* sexual harassment) with the aim of identifying factors that drive the evolution of fin regeneration are currently lacking. In the next sections, we propose three new, empirically testable hypotheses for the evolution of fin regeneration and offer ideas for how to test these using fish from the family Poeciliidae (**Figure 4**).

Hypothesis 1. Signaling-Driven Fin Regeneration

Sexual selection is an important evolutionary force influencing phenotypes across the animal kingdom (Panhuis et al., 2001).

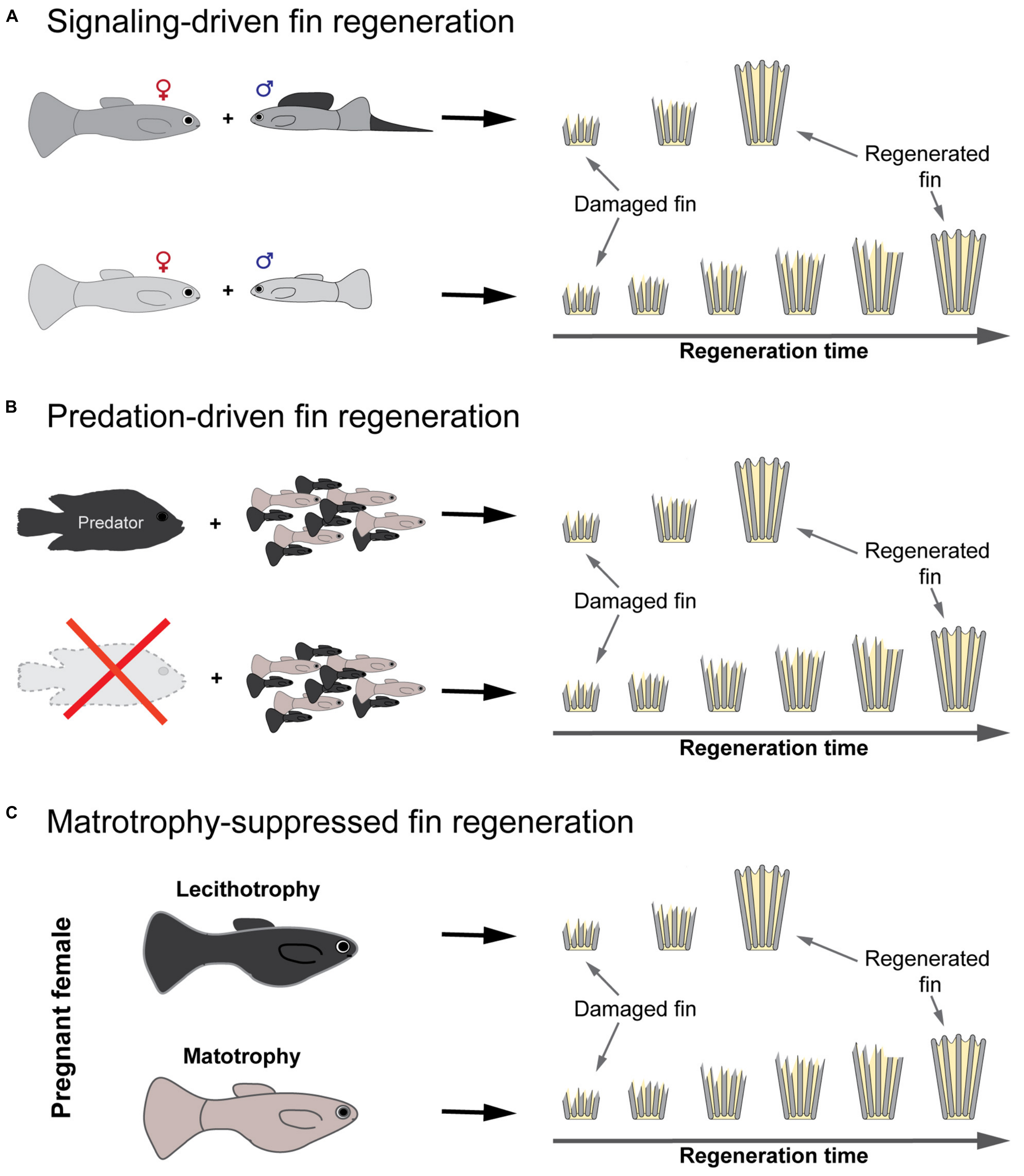

FIGURE 4 | Hypotheses for the evolution of fin regeneration in the fish family Poeciliidae. **(A)** If fins play an important role in precopulatory sexual selection, then they should be selected to have a faster and more accurate regeneration in males that rely in courtship. **(B)** If fins play an important role in predator evasion, then they should be selected to have a faster and more complete regeneration when living in a high predation environment. **(C)** If reproduction and regeneration enter an energetic conflict during pregnancy and a matrotrophic mode of reproduction is energetically more demanding than lecithotrophy, then matrotrophy should be associated with a suppressed female capacity for fin regeneration during gestation.

In the Poeciliidae, male phenotypic traits play an important role during precopulatory sexual selection. It is thought that sexual selection may be responsible for the extraordinary diversity of male morphological traits in this family (Pollux et al., 2014; Culumber and Tobler, 2017; Furness et al., 2019), explaining the existence and elaboration of conspicuous sexually selected traits

(**Figure 2**). Fins are thought to play a particularly important role during mate selection (either during female mate choice or male-male conflict) and mating tactics in several species (Jordan et al., 2006; Kang et al., 2013; Goldberg et al., 2019). We propose that in species where fins play a key role in courtship display, sexual selection can drive the evolution of faster fin regeneration. The idea is that if a particular fin plays an important role in a species' mate selection, then that fin should have a faster and more complete (i.e., homomorphic) fin regeneration after being damaged, compared to a species in which that fin does not play a role in sexual selection (**Figure 4A**). Three fins in particular may be subject to this signaling-driven fin regeneration: the caudal fin (sword), dorsal fin and anal fin (gonopodium) (**Figure 2**).

Caudal Fin

Some males in the genus *Xiphophorus* develop a pigmented and elongated ventral extension of the caudal fin, forming a sword-like structure (Meyer, 1997). These species are fittingly referred to as the Swordtails. An early study showed that female *Xiphophorus helleri* preferred males with longer or intact fin tails over male with smaller or surgically removed portion of caudal fins (Basolo, 1990a). These results were latter supported in another study by Rosenthal and Evans (1998), in which females were presented with computer-altered videos of males, showed that courting males with intact swords were strongly preferred over courting males in which portions of the sword had been computationally erased frame-by-frame. Their study demonstrates the importance of intact swords for a male's mating success and, hence, highlights the need for a fast and accurate fin regeneration program. Given the importance of these swords in female mate choice one could argue that swordtails should have a higher regenerative capacity of the caudal fin compared to species that do not carry swords, such as the closely related platies within the genus *Xiphophorus*.

Dorsal Fin

The males of several Poeciliid species use a conspicuous elongated dorsal fin (**Figure 2A**) in erect-fin displays to females in the context of courtship behavior (Farr et al., 1986; Travis and Woodward, 1989; Ptacek and Travis, 1996; Goldberg et al., 2019) or to conspecific males as an intimidating signal during male-male conflict (Bildsøe, 1988; Benson and Basolo, 2006; Prenter et al., 2008; Goldberg et al., 2019). Recently, Goldberg et al. (2019) studied the evolution of enlongated dorsal fins in males of the subgenera *Mollienesia* and *Limia* (genus *Poecilia*). They argued that these ornamental dorsal fins initially emerged as a tool for male-male contests, but that they were later co-opted for male displays during courtship. Regardless of the origin, it clear that these dorsal fins are crucial for a male's mating success (Basolo, 1990b, 1995; Jordan et al., 2006). This raises the possibility that in species where the male's dorsal fin plays an important role in either courtship behavior or male-male conflict, sexual selection may have favored the evolution of a faster and more complete fin regeneration.

Anal Fin

All Poeciliid species exhibit internal fertilization, which is achieved by males inserting their intromittent organ (referred to as the gonopodium, **Figure 2**) into the female gonopore and releasing spermatophores (Bisazza, 1993; Greven, 2005). In some species, males use a conspicuously colored gonopodium during courtship displays (e.g., *Girardinus metallicus*; Kolluru et al., 2015). In other species, the length of the gonopodium is linked to alternative mating tactics, with the combination of a small body size and long gonopodium thought to facilitate sneak or coercive mating (Bisazza, 1993; Bisazza and Pilastro, 1997; Pilastro et al., 1997; Greven, 2005; Pollux et al., 2014). However, regardless of their precise role in the mating process an intact gonopodium is crucial for the successful transfer of spermatophores in every poeciliid species (Evans et al., 2011; Devigili et al., 2015; Head et al., 2017), suggesting that the regenerative capacity of the gonopodium should be equally high in all species (and all alternative mating phenotypes within species).

Approaches to Test These Predictions

The fish family Poeciliidae offers an excellent model system to test the idea that signaling driven sexual selection has led to the evolution of a faster regeneration of caudal and dorsal fins (but not gonopodia) in species where they play a key role in female mate choice. There are at least two well established approaches that can be used to test these ideas in the Poeciliidae. The first is by adopting a comparative phylogenetic approach in which species with conspicuously swords or enlarged dorsal fins used in courtship are compared to closely related species in which these fins do not play a major role in female mate choice. In particular, comparative studies with platies and swordtails in the genus *Xiphophorus* (Meyer, 1997; Jones et al., 2013; Kang et al., 2013), or "short-finned and sail-finned" (*P. latpinna*, *P. petenensis,* and *P. velifera*) species in the genus *Poecilia* (subgenus *Mollienesia*; Ptacek, 2005), could reveal interesting differences in fin regeneration among species in association with the function of these fins in courtship behavior. A second potentially powerful approach available in the Poecillidae is by comparing males within a single population that exhibit different alternative mating tactics. Many poeciliid species show intra-specific alternative mating tactics, with larger males displaying a different, often contrasting set of phenotypes and mating strategies than smaller males (Constanz, 1975; Farr et al., 1986; Ryan and Causey, 1989). Generally, larger males have a brighter coloration, more conspicuous ornamental traits (e.g., dorsal fins or swords) and courtship behavior to elicit cooperative mating, while smaller males have a drab coloration (mimicking female coloration), relatively smaller and less conspicuous ornaments and a mating system characterized by coercive or sneak mating (Farr et al., 1986; Ryan and Causey, 1989; Travis and Woodward, 1989; Ryan et al., 1992; Bisazza, 1993; Ptacek and Travis, 1996; Becker et al., 2012; Furness et al., 2020). If the dorsal fin of courters and sneakers indeed fulfills a different function, then one could argue that the larger ornamental fin of courting males should have a higher regenerative capability compared to the less conspicuous fin of sneaker males.

Hypothesis 2. Predation-Driven Fin Regeneration

The evolution of species is driven by a combination of selection pressures which vary across spatio-temporal scales shaping their morphological, physiological and behavioral traits. It has been argued that predation may be an important evolutionary force driving enhanced regeneration, because sublethal predation is one of the most common reasons for experiencing structural loss in nature (e.g., loss of limbs, tail, external gills, or fins). While studies in invertebrates seem to provide some support to this hypothesis (Baumiller and Gahn, 2004; Gahn and Baumiller, 2005; Berke et al., 2009), studies in vertebrates have not directly evaluated the effect of predation on the regenerative competence (e.g., regeneration rates, blastema formation and molecular mechanisms controlling this process). Studies on the lizard family Lacertidae for example have focused on the frequency of tail autotomy (i.e., self-amputation), which is an interesting phenomena that is enhanced under high predation risk (Fox et al., 1994; Pafilis et al., 2009), but the effect of predation on the subsequent regeneration of tails still remains to be evaluated.

Caudal and Pectoral Fins

In fish, swimming performance is a key component of many common activities that are directly related to fitness, such as avoiding predators, capturing prey, fighting, living in a fast-flowing environment. The fish's fins play a crucial role in steering and thrust generation during locomotion. Poeciliid fishes perform highly complex 3-dimensional escape maneuvers, which are primarily generated by the caudal peduncle and fin (Fleuren et al., 2018a,b, 2019). During fine maneuvering (e.g., while feeding or steering through a complex environment) they further rely on the synchronous use of the paired pectoral fins to control speed and direction (Lankheet et al., 2016). The importance of caudal and pectoral fins during swimming suggests that they should be subject to stringent evolutionary selection (Langerhans and Reznick, 2010; Higham et al., 2016).

Predation Risk

Predation in particular is well-known for affecting numerous behavioral, morphological and life-history traits of Poeciliid fishes (Reznick and Endler, 1982; Reznick et al., 1990, 2004; O'Steen et al., 2002; Burns et al., 2009; Kotrschal et al., 2017; Hagmayer et al., 2020) and several studies have shown that the presence of predators can influence the shape and size of the caudal peduncle and fin (Gross, 1978; Winemiller, 1990; Langerhans et al., 2004; Hendry et al., 2006; Weber et al., 2012; Price et al., 2015; Hammerschlag et al., 2018). In nature fins are frequently damaged, for example as a result of disease, predator attacks, aggressive male-male conflicts or severe floods and storms (Ziskowski et al., 2008; Sinclair et al., 2011; Furness et al., 2020). Such damages to, or loss of, fins is known to dramatically reduce the fish' escape performance, burst speed and sustained swimming ability (Webb, 1973, 1977; Plaut, 2000; Fu et al., 2013; Cai et al., 2020), likely negatively affecting its fitness in high predation environments. Given the importance of intact fins for the swimming performance of fish and hence their ability to escape from predators, we propose that predation risk may not only affect shape and size of fins, but also their regenerative capacity. Specifically, we propose that if fins play an important role in escaping predators and fin damage negatively affects the ability to escape predators, then a high predation risk should select for a faster and more complete fin regeneration (**Figure 4B**).

Approaches to Test This Hypothesis

To date, studies investigating a possible relationship between predation risk and fin regeneration are absent in fish. Interestingly, an early study by Broussonet (1789) reported that the caudal fin of the goldfish (*Carassius auratus*) regenerates faster than the ventral, pectoral and dorsal fins, which might lead one to speculate that this is related to the functional relevance of the caudal fin in swimming. Clearly, however, further studies are needed. One way in which this idea can be empirically tested in the Poeciliidae is by comparing the regenerative capacity of the different fins in poeciliid populations that have historically been exposed to different predation levels. The Trinidadian guppy (*Poecilia reticulata*) undoubtedly represents the best-known example of a study system that includes clearly defined natural populations that live with and without predators (e.g., Reznick, 1982, 1983; Reznick et al., 1990, 1996, 1997, 2004; Magurran, 2005; Olendorf et al., 2006), but similar "high and low predation populations" are found in many other poeciliid species, e.g., in the genera *Brachyrhapis* (Johnson and Belk, 2001; Johnson et al., 2009; Monterroso et al., 2014), *Gambusia* (Langerhans et al., 2004; Langerhans, 2009), *Poeciliopsis* (Hagmayer et al., 2020), and *Xiphophorus* (Basolo and Wagner, 2004). If predation is driving the evolution of a faster fin regeneration, then comparing wild-caught specimens and their F_1 and F_2 progeny in a common-garden laboratory environment will reveal consistent differences in fin regeneration as a function of predation regime and the extent to which these differences are heritable (Reznick, 1982; Reznick et al., 1990; Reznick and Bryga, 1996).

Hypothesis 3. Matrotrophy-Suppressed Fin Regeneration

Regeneration is an energetically demanding cell-based process that requires the activation of the immune system (**Box 1**), wound healing, blastema formation and, the subsequent restitution of a fully functional structure. Bely (2010) proposed that a (temporarily) reduced regenerative capacity might result from a trade-off with other fundamental processes which are equally or energetically more demanding than regeneration. Indeed, evidence suggests that the regenerative capacity can be negatively affected by other energetically expensive processes throughout the lifespan, including reproduction, one of the energetically most demanding periods in an animal's lifetime (Bernardo and Agosta, 2005; Maginnis, 2006; Seifert et al., 2012). Upon reaching sexual maturity, much of an animal's energy is often reallocated from somatic growth to reproduction (Taranger et al., 2010). The idea of a costly reproduction has led to the hypothesis that gamete production and germ cell maintenance compromise long-term somatic cell maintenance and repair (Maklakov and Immler, 2016), which means that when energy availability is limited, regeneration and reproduction might come in conflict with each other. Indirect support for this hypothesis comes from a recent study that found that the caudal fin of zebrafish when exposed to a challenging condition (sublethal low-dose of ionizing radiation, with genotoxic stress affecting the soma and the germline) regenerated faster in germline-free zebrafish than in germ-cell-carrying fish (Chen et al., 2020), suggesting that regeneration is curbed in individuals that produce energetically costly germ cells.

Different Reproductive Modes

The family Poeciliidae provides a unique system to study trade-offs between regenerative capacity and reproduction, by studying one of maternally most demanding reproductive periods, the pregnancy. All members of this family, except one (*Tomeurus gracilis*), are live-bearing fish. Moreover, several independent evolutionary transitions from lecithotrophy (a form of maternal provisioning in which nutrients are provided to the eggs before fertilization) to matroptrophy (a type of maternal provisioning in which nutrients are provided after fertilization, i.e., throughout embryonic development *in utero*) have occurred in this live-bearing family (Pollux et al., 2009). These transitions coincide with a shift in the timing of the allocation of maternal nutrients to the offspring from pre- to postfertilization. The amount of resources a female can allocate to her offspring is the result of a delicate balance between maternal energy uptake (via feeding), her own caloric utilization (maintenance) and the amount of excess energy that is subsequently available for other functions such as reproduction and/or regeneration (e.g., Reznick et al., 1996; Trexler, 1997; Banet et al., 2010; Pollux and Reznick, 2011; Hagmayer et al., 2018).

In poeciliids, a pregnancy may impose high energetic demands on females, due to: (i) the physical burden of having to propel an increasingly larger body volume and higher body mass through the water during locomotion (Plaut, 2002; Ghalambor et al., 2004; Fleuren et al., 2018b, 2019; Quicazan-Rubio et al., 2019) and (ii) the physiological burden of having to provide oxygen and, in some species, nutrients to the developing offspring. This physiological burden, however, is not equal for all Poeciliid species and may differ greatly depending on their mode of maternal provisioning. Specifically, the energy requirements during pregnancy are significantly higher for matrotrophic species compared to lecithotrophic ones, because of the additional energy costs of having to: (i) nourish their developing embryos, i.e., the acquirement, consumption, digestion and absorption of additional food plus the subsequent transport of converted nutrients to the embryos, and (ii) develop a very costly tissue, i.e., the placenta, that regulates many complex and energetically expensive immunological and endocrine maternal-fetal interactions (Pollux et al., 2009). One could argue that the higher energetic demands associated with placentation, a special form of matrotrophy found in the Poeciliidae in which the female transfers nutrient to the fetus *in utero* via a placenta, make it more likely that females experience conflicting demands between maintenance, reproduction and regeneration during pregnancy. We therefore posit that the mode of maternal provisioning should influence a female's regenerative capacity during gestation. Specifically, we propose that since (i) the pregnancy is energetically more demanding in matrotrophic than lecithotrophic females and (ii) matrotrophic provisioning and regeneration are both very costly processes, matrotrophy should be associated with a suppressed female capacity for fin regeneration during gestation (**Figure 4C**).

Approaches to Test This Hypothesis

There is a well-supported phylogenetic framework for the evolution of the placenta in the Poeciliidae that can be used for comparative studies on lecithotrophic and matrotrophic species to test these predictions (Pollux et al., 2014; Furness et al., 2019). These studies show that (1) the placenta independently evolved multiple times in this family and (2) that there are closely related species that either lack a placenta or have intermediate stages in the evolution of the placenta. This remarkable diversity allows for the selection of closely related species, particularly in the genus *Poecilia* (Meredith et al., 2010, 2011) and *Poeciliopsis* (Reznick et al., 2002), for comparative experimental studies that address fundamental questions about how female fin regeneration during pregnancy co-evolves with the evolution of the placenta. Future studies should optimally look for differences in fin regeneration throughout a female's reproductive cycle, i.e., prior to sexual maturation and egg fertilization, during pregnancy and after parturition, between lecithotrophic and placental matrotrophic females. These studies will shed new light on the factors that could potentially limit the regenerative competence of species.

CONCLUSION

Important progress has been made on understanding the cellular and molecular mechanisms underlying appendage regeneration. Unfortunately, however, most of these insights are based on only a few model species (e.g., the Zebrafish). Moreover, many fundamental questions regarding why or how regeneration evolved remain poorly understood. Future studies should therefore include new animal models and compare species that may have evolved divergent regenerative capacities in response to different selection pressures. Here, we propose 3 novel hypotheses for the evolution of fin regeneration in fishes and argue that the fish family Poeciliidae offers an interesting new study system for comparative studies at different phylogenetic levels (among species, among populations within species and among individuals within populations) to empirically test these hypotheses. Such comparative studies in the fish family Poeciliidae may help identify the factors that drive or influence the evolution of regeneration and help elucidate the underlaying mechanisms of evolution (e.g., changes in the cellular processes, gene expression, molecular pathways, immunology). The era of next-generation sequencing and the recent advances in genome research in the Poeciliidae (Schartl et al., 2013; Kunstner et al., 2016; Shen et al., 2016; Warren et al., 2018; Mateos et al., 2019; Van Kruistum et al., 2019, 2020) add further to the appeal of this family.

AUTHOR CONTRIBUTIONS

DS drafted the manuscript and design the figures. GW and BP contributed to content and editing of the manuscript and figures. All authors contributed to the article and approved the submitted version.

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Predation and Resource Availability Interact to Drive Life-History Evolution in an Adaptive Radiation of Livebearing Fish

Kaj Hulthén[†]*, Jacob S. Hill, Matthew R. Jenkins and Randall Brian Langerhans**

Department of Biological Sciences, W.M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, United States

Predation risk and resource availability are two primary factors predicted by theory to drive the evolution of life histories. Yet, disentangling their roles in life-history evolution in the wild is challenging because (1) the two factors often co-vary across environments, and (2) environmental effects on phenotypes can mask patterns of genotypic evolution. Here, we use the model system of the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting blue holes to provide a strong test of the roles of predation and resources in life-history evolution, as the two factors do not co-vary in this system and we attempted to minimize environmental effects by raising eight populations under common laboratory conditions. We tested *a priori* predictions of predation- and resource-driven evolution in five life-history traits. We found that life-history evolution in Bahamas mosquitofish largely reflected complex interactions in the effects of predation and resource availability. High predation risk has driven the evolution of higher fecundity, smaller offspring size, more frequent reproduction, and slower growth rate—but this predation-driven divergence primarily occurred in environments with relatively high resource availability, and the effects of resources on life-history evolution was generally greater within environments having high predation risk. This implies that resource-driven selection on life histories overrides selection from predators when resources are particularly scarce. While several results matched *a priori* predictions, with the added nuance of interdependence among selective agents, some did not. For instance, only resource levels, not predation risk, explained evolutionary change in male age at maturity, with more rapid sexual maturation in higher-resource environments. We also found faster (not slower) juvenile growth rates within low-resource and low-predation environments, probably caused by selection in these high-competition scenarios favoring greater growth efficiency. Our approach, using common-garden experiments with a natural system of low- and high-predation populations that span a continuum of resource availability, provides a powerful way to

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Jerald B. Johnson,
Brigham Young University,
United States

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Mark C. Belk,
Brigham Young University,
United States
Cameron K. Ghalambor,
Colorado State University,
United States

***Correspondence:**
Randall Brian Langerhans
langerhans@ncsu.edu

[†]Present address:
Kaj Hulthén,
Department of Biology, Aquatic Ecology Unit, Lund University, Lund, Sweden

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deepen our understanding of life-history evolution. Overall, it appears that life-history evolution in this adaptive radiation has resulted from a complex interplay between predation and resources, underscoring the need for increased attention on more sophisticated interactions among selective agents in driving phenotypic diversification.

Keywords: common-garden experiment, growth rate, life histories, mortality rates, predator-prey, resource availability, divergent natural selection, Poeciliidae

INTRODUCTION

Growth rates and reproductive traits, such as age at sexual maturity and offspring size and number, can have strong effects on fitness and yet vary notably among and within species (Partridge and Harvey, 1988; Stearns, 1992; Roff, 2002). Life-history theory comprises a broad and diverse analytical framework that attempts to understand the causes and consequences of this variation. With their intimate relationship with fitness, combined with their importance in shaping the structure and dynamics of populations, communities, and ecosystems, life-history traits have attracted voluminous and long-standing research in evolutionary ecology (e.g., Lack, 1947; Reznick and Endler, 1982; Stearns, 1992; Roff, 2002; Bassar et al., 2010; Walsh et al., 2012). A fundamental goal of this work is to predict how life-history traits will evolve under particular environmental conditions (e.g., Stearns, 1976, 2000; Reznick and Endler, 1982; Reznick et al., 1997; Riesch et al., 2013; Moore et al., 2016).

A central axiom of life-history theory is that during an organism's life, time and energy are finite, and thus organisms have evolved adaptive allocation of a limited energy budget across multiple competing tasks and functions (Stearns, 1992; Zera and Harshman, 2001; Roff, 2002; Flatt and Heyland, 2011). For instance, organisms must acquire necessary resources and allocate energy toward somatic growth, reproductive tissue, and other features. Trade-offs imposed by time and energy are proposed to drive phenotypic variation among different environmental conditions and across the lifespan of organisms (Van Noordwijk and de Jong, 1986; Stearns, 1989; Roff, 1992; Charnov, 1993; Roff, 2002). This trade-off framework forms a key foundation for the modern view of life-history evolution, yet we still have much to learn about how specific agents of selection directly and indirectly contribute to shape variation in key life-history traits in the wild (e.g., Riesch et al., 2020). For example, we have a wealth of research documenting statistical associations between important life-history traits and an array of potentially influential environmental variables (e.g., Ballinger, 1979; Reznick and Endler, 1982; Spitze, 1991; Martin, 1995; Riesch et al., 2014). Although informative, such associations are frequently difficult to interpret (due to covariation among ecological selective pressures) and thus, a contemporary challenge is to isolate and disentangle the role of specific ecological agents in the evolution of life histories.

Two of the most-studied ecological factors affecting time and energy allocation are predation risk and resource availability. These factors are generally considered the primary agents driving the evolution of life-history traits (e.g., Reznick and Endler, 1982; Martin, 1987; Lynch, 1989; Vanni and Lampert, 1992; Boggs and Ross, 1993; Martin, 1995; Reznick et al., 2001; Arendt and Reznick, 2005; Riesch et al., 2020). However, two key obstacles have so far hindered our ability to uncover their importance in shaping life histories in the wild. First, differences in resource availability often covary with predation regime: e.g., more productive and relatively nutrient-rich environments often support a larger number of interacting species (including predators), and predators often reduce prey population densities and indirectly elevate resource levels (Walsh and Reznick, 2008; Rudman et al., 2016). Thus, determining the relative roles of predation and resources in influencing life histories is statistically daunting, to say the least. Second, environmental effects on life-history phenotypes can obscure or even mask patterns of underlying genotypic life-history evolution—it may be naïve to assume that evolutionary responses to environmental factors mirror the phenotypic responses (e.g., Conover and Schultz, 1995). Hence, we need more than phenotype-environment associations observed in the field to understand genetic evolution of life histories (Berven, 1982; Reznick, 1982; Reznick and Bryga, 1996). One approach to address these obstacles is to measure evolutionary change subsequent to experimental manipulation of environmental factors (Reznick et al., 1990, 2019; Walsh and Reznick, 2011; Wathne et al., 2020). While certainly informative, this approach does not directly address how life histories actually evolve under natural conditions, and generally pertains to relatively short time scales. An alternative and powerful approach is to use model systems that offer "natural experiments," where populations/species have evolved in distinct and well-characterized environments in the wild (Diamond, 1986). Ideally, we would like to investigate natural systems with (1) strong variation in, but little covariation among, predation risk and resource availability, (2) relatively few potentially confounding factors, and (3) the ability to raise the organism in a common environment to assess the genetic basis to trait divergence among populations.

Livebearing fishes (family Poeciliidae), such as guppies, mollies, swordtails, and mosquitofish, have been at the forefront of the development of life-history theory (e.g., Reznick and Endler, 1982; Reznick et al., 1990, 2002; Johnson, 2001; Johnson and Belk, 2001; Bronikowski et al., 2002; Jennions and Telford, 2002; Jennions et al., 2006; Riesch et al., 2014, 2015; Moore et al., 2016; Belk et al., 2020; Santi et al., 2020). One especially powerful model system of livebearing fish is the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*), as the system offers the opportunity to isolate the effects of predation risk and resource availability on life-history trait evolution. These small, livebearing fish inhabit blue holes that differ considerably in predation risk (presence/absence of predatory fish) and resource availability (continuous variation in density of zooplankton resources)—both factors are temporally consistent but do not covary with one another in this system (Heinen et al., 2013). Moreover, no known environmental factor should obscure the effects of these two agents on phenotypic evolution because previous research has investigated numerous environmental features of blue holes—e.g., temperature, salinity, turbidity, dissolved oxygen, pH, total phosphorus, total nitrogen, total organic carbon, surface area, water depth, and densities of chlorophyll *a*, phycocyanin, and phytoplankton—and documented a lack of covariation between these variables and either the presence of predatory fish or zooplankton density (e.g., Langerhans et al., 2007; Heinen et al., 2013; Björnerås et al., 2020). The fish are also highly amenable to laboratory rearing under common-garden conditions, enabling us to test for evolutionary divergence of life-histories in response to these ecological agents while minimizing direct environmental effects on the phenotypes (i.e., phenotypic plasticity).

Here we examined Bahamas mosquitofish raised in a common laboratory environment derived from eight populations varying in predation risk and resource availability. Based on an extensive body of theoretical and empirical studies, we tested *a priori* predictions of adaptive life-history divergence in response to these two selective agents (e.g., Gadgil and Bossert, 1970; Stearns, 1976; Law, 1979; Michod, 1979; Reznick et al., 1990, 1996, 2001, 2019; Charlesworth, 1994; Abrams and Rowe, 1996; Downhower et al., 2000; Gasser et al., 2000; Johnson, 2001; Dmitriew, 2011; Riesch et al., 2013, 2020). While further mathematical development of underlying theory is desirable for making clear and unambiguous predictions, our predictions here derive from research that links mortality rates (across all age classes), density regulation (e.g., resource competition, juvenile survival), and food availability to the adaptive evolution of life histories. First, we predicted that elevated extrinsic mortality rates, combined with reduced population densities experienced in populations with high predation risk would select for (1) higher fecundity, (2) smaller offspring size, (3) more frequent reproduction, (4) earlier age at maturity, and (5) more rapid growth. These predictions derive from both the direct effects of predator-induced mortality and the potential indirect effects of elevated resource competition in the absence of predators where population densities are much higher. Second, we predicted that relatively high resource availability would select for (1) higher fecundity, (2) smaller offspring size, (3) more frequent reproduction, (4) earlier age at maturity, and (5) slower growth due to lower growth efficiency. We note that an alternative prediction for the effect of predation risk on growth rate exists and may apply in this system: lower mortality rates and high densities in low-predation environments could select for higher growth efficiency in these highly competitive environments, leading to the prediction of more rapid growth in populations with low predation risk (Arendt and Wilson, 1999; Robinson and Partridge, 2001; Lindgren and Laurila, 2005). While all environmental/transgenerational effects cannot be fully excluded from our study, we attempted to minimize these sources by raising multiple generations under common conditions. Thus, we interpret phenotype-environment correlations that emerge after common-garden rearing to reflect signals of local adaptation, and thus, largely of genetic origin.

MATERIALS AND METHODS

Study System and Environmental Measurements

Inland blue holes (water-filled, vertical caves) on Andros Island, The Bahamas were colonized by Bahamas mosquitofish during the past ∼15,000 years, harbor relatively simple fish and plankton communities (e.g., typically 1–3 fish species, 1–3 dominant zooplankton species), appear very stable through time, and are quite isolated from one another in most cases (Fairbanks, 1989; Langerhans and Gifford, 2009; Heinen et al., 2013; Heinen-Kay and Langerhans, 2013; Riesch et al., 2013; Björnerås et al., 2020; Sha et al., 2021). The post-Pleistocene radiation of Bahamas mosquitofish in these blue holes is largely characterized by predator-driven multi-trait adaptation (e.g., Langerhans et al., 2007; Heinen-Kay and Langerhans, 2013; Anderson and Langerhans, 2015; Heinen-Kay et al., 2015; Fowler et al., 2018; Langerhans, 2018), although resource availability appears to also play some role in phenotypic differentiation (e.g., Heinen et al., 2013; Martin et al., 2014; Riesch et al., 2020). In some blue holes, Bahamas mosquitofish coexist with a major predatory fish (bigmouth sleeper, *Gobiomorus dormitor*), resulting in a clear dichotomy between "high-predation" blue holes, where predators impose strong mortality and effectively reduce Bahamas mosquitofish population densities, and "low-predation" blue holes with no major fish predators and consequently relatively low mortality rates (e.g., Langerhans et al., 2007; Heinen et al., 2013; Martin et al., 2015; Riesch et al., 2020). While high-predation localities clearly have greater adult mortality rates, prior analyses of population densities and age structure suggest that all age classes of Bahamas mosquitofish suffer higher mortality in the presence of bigmouth sleepers, with population densities considerably lower than low-predation localities (Heinen et al., 2013; Riesch et al., 2020; see **Supplementary Material**).

Blue holes also range widely in primary productivity and resource availability, spanning over an order of magnitude in the density of their key prey, zooplankton (primarily copepods; Gluckman and Hartney, 2000; Heinen et al., 2013; Araujo et al., 2014; Sha et al., 2021). Following previous work, in this study we treated predation regime as a categorical variable based on the presence/absence of bigmouth sleeper, and measured resource availability as the density of zooplankton. Zooplankton densities were taken from a previous study (Heinen et al., 2013). Briefly, we estimated zooplankton densities using a 60-m tow of a zooplankton net (20-cm diameter, 153-μm mesh) at 0.5-m depth. Thus, zooplankton densities were estimated in habitats where Bahamas mosquitofish are abundant within all sites (Heinen et al., 2013). We counted all zooplankton within a 2.5-ml subsample of each plankton collection using a stereo microscope. Resource estimates in blue holes show high repeatability across seasons and years (intraclass correlation coefficients: 0.88–0.98; Heinen et al., 2013), and recent detailed investigations of zooplankton densities and depth distributions in a subset of six blue holes have confirmed the utility of these measurements in capturing consistent, among-population variation in resource levels (intraclass correlation coefficient of standardized zooplankton densities measured 7–8 years apart: 0.95; Sha et al., 2021). Zooplankton density does not covary with predation regime across blue holes (n = 19 blue holes, P = 0.71), nor with *G. hubbsi* density (n = 17 blue holes, P = 0.69); a pattern consistent within the subset of eight blue holes examined here (both $P > 0.38$). Zooplankton density appears to represent a strong and robust estimate of resource availability for Bahamas mosquitofish in blue holes (see **Supplementary Material**). However, owing to the large differences in *G. hubbsi* population densities between predation regimes caused by predator-induced mortality, some of the phenotypic differences between these predatory environments could reflect density-dependent selection. That is, the higher population densities within low-predation environments likely result in more intense resource competition compared to high-predation environments (see **Supplementary Material**). Therefore, when we refer to effects of predation risk in this study, those effects could arise from direct effects of mortality rates or indirect effects of altered population density (we assess the possible causes in the Discussion). Throughout, we use "predation regime" or "predation risk" to refer to the presence/absence of bigmouth sleepers in the population of origin, and "resource availability" or "resource level" to refer to the zooplankton density in the population of origin.

Source Populations and Laboratory Rearing

Wild *G. hubbsi* were collected under snorkel using hand-held dip nets from eight inland blue holes that differ in predation regime (4 low-predation, 4 high-predation; **Supplementary Figure S1**) during two occasions in 2016 (8–20 June, 30 September–3 October) and transported to experimental facilities at North Carolina State University. We selected these blue holes *a priori* as representative of the larger set of blue holes on Andros Island (e.g., Langerhans, 2018). The fish that formed the wild-caught parental generation (F0) were collected as newborns (in an attempt to minimize maternal and environmental effects) and subsequently reared to adulthood in single-sex groups (to keep fish virgin until breeding) in re-circulating systems providing biological, mechanical and UV filtration. Municipal source water was filtered using a 5-stage filtration system (Aqua FX Mako 5-stage RO/DI system, Aqua Engineering and Equipment, Inc., Winter Park, Florida), treated to reclaim electrolytes and general hardness (R/O Right, KENT Marine, Franklin, Wisconsin), buffered to a pH of approximately 8.3 (Marine Buffer, Seachem Laboratories, Madison, Georgia), and maintained at approximately 1.5 ppt salinity (~2,850 μS, Instant Ocean, Blacksburg, Virginia). Digital networked controllers (ReefKeeper Lite, Digital Aquatics, Everett, Wisconsin; Remote Operator, Unitronics, Quincy, Massachusetts) maintained water temperature at approximately 25°C, photoperiod at 14-h light/10-h dark, and monitored salinity and pH. Fish were fed *ad libitum* daily with a mixture of TetraPro Tropical Crisps (Tetra, Blacksburg, Virginia) and freeze-dried daphnia, bloodworms and brine shrimp (Hikari, Hayward, California). F0 fish were raised in 115-L tanks with artificial vegetation (6 tanks per population, ~10 fish per tank).

To measure fecundity, offspring size, and interbrood interval of lab-raised F0 fish (see below), and to acquire F1 fish, sexually mature F0 fish were uniquely mated: each female mated with a single male, with no fish used more than once. We isolated mated females within the re-circulating systems in 38-L chambers, and after approximately 4 weeks (or when visibly showing signs of late-stage pregnancy) we provided very dense artificial vegetation as safe refuge for newborn offspring (to minimize cannibalism from females). We visually screened for newborn offspring at least once per day. Mated females remained isolated after parturition of their first brood so we could potentially measure fecundity and offspring size across multiple broods per female.

To measure fecundity and the size of offspring delivered by F1 fish, and to acquire F2 fish for our growth-rate and maturation experiment, we raised F1 fish in 115-L rearing tanks within the re-circulating systems and subsequently mated them after sexual maturity. We pooled outbred families of similar age within populations (152 total full-sibling F1 families, ~9 115-L tanks per population) and separated sexes upon first sign of male maturation. We mated F1 females to multiple unrelated males from the same population (to maintain high genetic diversity), and held mated females in groups of ~12 per 115-L tank with dense artificial vegetation. We screened tanks daily for newborn offspring.

Life-History Measurements

Prior work examining numerous blue hole populations of Bahamas mosquitofish has shown higher fecundity and smaller offspring size in Bahamas mosquitofish inhabiting high-predation blue holes (Downhower et al., 2000; Riesch et al., 2013; Riesch et al., 2020). However, prior tests for genetically based divergence in these traits have been extremely limited (three populations), with no previous study measuring interbrood interval, age at maturity, or growth rate, or directly testing the roles of predation regime or resource levels on life histories of common-garden raised individuals. Here, we quantified fecundity (number of offspring per brood), offspring size (body size at birth), interbrood interval (number of days between reproductive events), age at maturity in males, and juvenile growth rate for fish derived from eight populations and raised under common laboratory conditions.

To measure fecundity and offspring size for F1 and F2 offspring broods, we examined a total of 471 mated lab-raised females derived from the eight populations (145 F0, 326 F1 females), comprising a total of 866 broods for fecundity (270 F1 broods, 596 F2 broods) and 747 broods for offspring size at birth (199 F1 broods, 548 F2 broods) (see **Supplementary Table S1** for details). For F1 broods only, we measured the interbrood interval between 113 pairs of broods—we could not measure this in F2 broods because we did not individually track females across multiple broods in that case.

Each time newborn offspring were observed in a tank, we immediately removed all offspring, counted them, placed them in a 1-L beaker filled with <2-cm deep water, and photographed them from above using a tripod-mounted DSLR camera (Canon T3i; Canon Inc., Tokyo, Japan) equipped with a macro lens (Sigma 50 mm f/2.8 EX DG Macro; Sigma Corp., Ronkonkoma, New York). For F1 broods only, we additionally removed and photographed the female that had given birth following the same methodology. Using the digital photos, we used tpsDig2 (Rohlf, 2017) to measure the standard length (SL) of three offspring (or all offspring if brood size ≤ 3). The average offspring SL served as our estimate of offspring size at birth for a given brood. We additionally used the digital photos to measure the SL of each female after each parturition to include as a potential covariate for F1 broods, as larger females were expected to produce larger broods (e.g., Riesch et al., 2013). Although female body size was unknown for F2 broods (multiple females pooled together), female body size does not differ between predation regimes in the wild or in our common-garden experiment (see **Supplementary Material**), and we found that female size did not influence offspring size in F1 broods (see "Results").

To examine age at maturity and juvenile growth rate, we conducted an experiment using 51 F2 broods (**Supplementary Table S2**). Each brood was photographed the day of birth as described above, raised in a separate 10-L tank within a recirculating system for approximately 61 days (61.18 ± 0.87, mean ± std. dev.), photographed again using the same methods, and then continued to be raised in the same conditions until all fish reached sexual maturity. Because external identification of sexual maturity in poeciliid females is not straightforward (sexual maturation largely occurs internally), but sexual maturity is readily identifiable for males, we only measured the age at maturity for males. Males were determined as sexually mature when their gonopodium had fully completed its entire morphological development (Turner, 1941). We removed males from the tanks when mature to minimize any possible effects of social interactions with adults on age at maturity in males (e.g., Borowsky, 1973; Sohn, 1977a,b; Hughes, 1985; Borowsky, 1987; Magellan and Magurran, 2009). To measure growth rate, we used the digital photos to measure SL of five fish per brood (or all fish if brood size ≤ 5) at each time point to measure the average body size at birth and after approximately 2 months of rearing. Growth rate was estimated as change in mm SL per day = $\mathrm{SL}_{2\,\mathrm{months}} - \mathrm{SL}_{\mathrm{birth}}/\#$ of days. This 2-month duration captured the vast majority of juvenile growth. No fish initiated sexual differentiation or reached sexual maturity during this period, but males began external sexual differentiation within 2 weeks after this period.

Each day, we fed fish *ad libitum* as described above, checked for sexual maturity, and confirmed health and responsiveness to feeding for each fish to help ensure they all received adequate food daily. Water conditions followed that described above, with the addition that we recorded water temperature every 10 min throughout the experiment using a HOBO UA-002-64 temperature data logger for potential use as a covariate in statistical analysis (average 2-month water temperature across all broods: 24.90°C ± 0.12). A low level of mortality occurred early in the experiment in five populations (8 fish, 3.8% mortality), and thus effects of mortality or selection should be minimal. Because the experiment was run in two temporal blocks, with the second block starting immediately after the first block reached 2 months of age, we included a random Block term in our statistical analyses.

Statistical Analysis

For fecundity, we first tested for effects of laboratory rearing on size-specific fecundity by comparing F1 brood sizes in our common-garden experiment to wild-caught females from the same eight populations (n = 100, data from Riesch et al., 2013). We conducted a generalized linear model using a Poisson distribution with a log link, with fecundity as the dependent variable, population, rearing environment (wild vs. lab), and their interaction as independent variables, and $\log_{10}$-transformed female SL as a covariate. We then tested whether predation regime or resource availability influenced among-population variation in fecundity of F1 and F2 broods. We conducted a generalized linear mixed-model with a Poisson distribution and log link using the number of offspring per brood as the dependent variable and predation regime, $\log_{10}$-transformed zooplankton density, generation (F1 vs. F2 broods), and their interactions as independent variables. Population was included as a random effect. Because female ID was unknown for F2 broods, we could not include female body size as a covariate. However, we performed an analogous statistical analysis for F1 brood sizes alone that did statistically adjust for any effects of female body size and brood number, and found qualitatively similar results (**Supplementary Table S3**). Because F1 females were smaller (younger) than F0 females (33.4 mm SL vs. 37.9 mm SL, on average), we expected smaller fecundity in F2 broods compared to F1 broods but were interested in the potential interactions between generation and predation regime or resource availability.

To test for effects of predation regime and resource availability on offspring size at birth, we conducted a general linear mixed-model with $\log_{10}$-transformed average offspring SL at birth as the dependent variable and predation regime, $\log_{10}$-transformed zooplankton density, generation (F1 vs. F2 broods), and their interactions as independent variables. Population was again included as a random effect. Once again, and for the same reason, we could not include female body size as a covariate but analysis of F1 offspring body size at birth that did statistically adjust for any effects of female body size and brood number found qualitatively similar results (and no effects of female body size or brood number; see **Supplementary Table S3**). Moreover, results were unchanged if we included fecundity as a covariate in the model.

To test for effects of predation regime and resource availability on the frequency with which females deliver broods, we conducted a generalized linear mixed-model with a Poisson distribution and log link using the interbrood interval (number of days between consecutive delivery of broods) as the dependent variable and predation regime, $\log_{10}$-transformed zooplankton density, and their interactions as independent variables. Log_{10}-transformed female SL and brood number served as covariates, with population and female ID treated as random effects.

We tested for effects of predation regime and resource availability on male age at sexual maturity in lab-raised F2s using a generalized linear mixed-model with a Poisson distribution and log link. Age at maturity (# of days) served as the dependent variable, and predation regime, $\log_{10}$-transformed zooplankton density, and their interaction served as independent variables. We included population and brood ID as random effects. We initially included average water temperature as a covariate, but excluded it due lack of influence ($P = 0.96$).

To test for effects of predation regime and resource availability on juvenile growth rate in lab-raised F2s, we conducted a general linear mixed-model with average growth rate (mm per day) as the dependent variable, and predation regime, $\log_{10}$-transformed zooplankton density, and their interaction as independent variables. Population and block were included as random effects. We further included tank density and average water temperature as covariates, as these influenced average growth rate (see section "Results").

To provide a multivariate overview of life-history variation among populations, we calculated population means for all 5 traits using values for F2 broods in all cases except interbrood interval (only F1 brood data available), and performed a principal component analysis (PCA) using the correlation matrix. We retained PC axes with eigenvalues greater than 1 for inspection. We plotted relevant PC scores for populations in each predation regime against resource availability to assess overall multivariate patterns of life-history divergence.

Analyses were performed using the R statistical program (R Development Core Team, 2009) with the *lme4* and *afex* packages (for generalized linear mixed models) and the JMP statistical program (v. 14.2, 2018, SAS Institute Inc.; for general linear mixed models and generalized linear models). In all cases, to interpret interaction terms involving predation regime and resource availability, we provided two visual depictions: (1) average trait values for each predation regime at both low and high resource levels (values of 0.2 and 0.75 $\log_{10}$–transformed zooplankton density), and (2) plotted relevant population means within each predation regime against resource availability. To provide an intuitive metric of effect sizes, we present percent differences between groups.

RESULTS

Comparing wild-caught and lab-raised F0 females, we found that the eight populations examined showed strongly consistent size-specific fecundities, with no evidence of differences between the rearing environments in brood sizes (**Supplementary Table S4**). Thus, the laboratory rearing environment for F0 females did not result in altered size-specific fecundity for any population compared to that observed in the wild.

In our tests of the effects of predation regime and resource availability on fecundity in lab-raised Bahamas mosquitofish, we found clear evidence for a three-way interaction between predation regime, zooplankton density, and generation (**Table 1**). This finding reflected two main patterns. First, high-predation populations exhibited higher fecundity than low-predation populations within both F1 and F2 broods (**Figure 1A**; matching predictions). Second, the effects of resource availability depended on the predation regime and generation: a positive association between resource availability and fecundity was evident in most cases (albeit weakly in F1 broods of high-predation females) except for F2 broods of low-predation females (**Figure 1B**; largely matching predictions). Thus, we found evidence for a role of both predation risk and resource availability in the evolution of fecundity in Bahamas mosquitofish, however the influence of predation risk was stronger and more consistent than resource levels (**Supplementary Table S5**). On average, high-predation females had 46.2% higher fecundity in F1 broods than low-predation females, statistically controlling for body size, brood number, and resource availability. For F2 broods, high-predation females had a fecundity 37.7% higher on average than low-predation females, statistically controlling for effects of resource availability. Fecundity of F2 broods was smaller than F1 broods, but this likely reflected the smaller body size of females in the second generation.

Across two generations of lab-raised fish, we found opposite effects of resource availability on offspring size in the different predation regimes (**Table 1**, "Pred × Res" term). In low-predation populations, offspring size tended to increase in sites with increasing levels of resources (opposite to *a priori* prediction), but offspring size in high-predation populations strongly decreased with higher resource availability in their native populations (matching *a priori* prediction) (**Figure 1D** and **Supplementary Table S5**). At low resource levels, we found no differences in offspring size between predation regimes, but at moderate to high resource levels, *G. hubbsi* showed larger offspring size at birth in the absence of predators (**Figure 1C** and **Supplementary Table S5**). The larger offspring size in F2 broods at least partially reflects the smaller fecundity in F2 broods.

For interbrood interval, we found evidence for an interaction between predation regime and resource availability, but no effects of female body size or brood number (**Table 2**). Reproductive frequency was more rapid in populations having higher resource availability (matching *a priori* prediction), but only within high-predation populations (**Figure 1F** and **Supplementary Table S5**). Within low-resource environments, predation risk has not led to any difference in interbrood interval, but at moderate-high resource levels, high-predation populations reproduce more frequently, as predicted (**Figure 1E** and **Supplementary Table S5**).

We found that predation regime had no influence on the age at maturity in lab-raised F2 males (contrary to *a priori* prediction), but resource availability had a strong effect matching our *a priori* prediction (**Table 3**). Male Bahamas mosquitofish have apparently evolved to mature more quickly in populations with higher resource levels, regardless of predation risk (**Figures 2A,B**). This resulted in a 13.9% later age at maturity, on average, in males derived from low-resource compared to high-resource environments (**Supplementary Table S5**).

We observed independent effects of resource availability and predation regime on juvenile growth rate in lab-raised F2 Bahamas mosquitofish (**Table 4**). Matching our *a priori* prediction, populations that have evolved in blue holes with higher resource availability showed slower growth rates, regardless of whether they have evolved with or without

TABLE 1 | Statistical results examining variation in fecundity and offspring size of F1 and F2 broods of lab-raised Bahamas mosquitofish derived from eight blue holes varying in predation regime and resource availability.

	Fecundity			Offspring size		
Source	***df***	**χ^2**	***P***	***df***	***F***	***P***
Predation regime (Pred)	1	16.09	<0.0001	1, 4.55	15.09	0.0139
Log zooplankton density (Res)	1	3.63	0.0568	1, 4.56	4.69	0.0880
Generation (Gen)	1	1,186.75	<0.0001	1, 737.7	261.74	<0.0001
Pred × Res	1	3.67	0.0554	1, 4.56	15.61	0.0130
Pred × Gen	1	1.13	0.2880	1, 737.7	0.44	0.5096
Res × Gen	1	0.35	0.5536	1, 736.6	0.92	0.3379
Pred × Res × Gen	1	22.30	<0.0001	1, 736.6	3.08	0.0795

predators (**Figure 2D**). At low resource levels, predation risk has not led to divergence in growth rates, but at moderate-high resource levels, populations without predatory fish have evolved higher growth rates than high-predation populations (**Figure 2C** and **Supplementary Table S5**). Our covariates revealed higher juvenile growth rates were associated with lower tank densities and higher water temperature.

We retained two PC axes in our PCA of population mean life-history trait values. The first axis explained 60% of the among-population variance in life-history traits, capturing variation in four of the five traits, while the second axis explained 24% of the variance and captured variation only in male age at maturity (**Supplementary Table S6**). PC 1 illustrated that the major gradient in life-history divergence of Bahamas mosquitofish involves predation-regime dependent effects of resource availability (stronger in high-predation populations) that results in strong divergence between predation regimes only within environments having moderate to high resource availability (**Figure 3**). Positive PC 1 scores are associated with faster juvenile growth rates, larger offspring size, longer interbrood intervals, and lower fecundity (**Supplementary Table S6**). Because PC 2 largely described variation in male age at maturity (**Supplementary Table S6**), it essentially re-described variation already captured in **Figure 2B**.

DISCUSSION

In this study, we tested a set of *a priori* predictions of adaptive life-history divergence in response to predation and resource availability. To do so, we took advantage of a natural system where these primary agents of ecological selection vary considerably among populations, but do not covary with one another. Raising eight populations under common laboratory conditions, we uncovered important insights into the nature of evolutionary responses of life-history traits to predation and resource availability. Taken together, our findings strongly suggest that life-history evolution in this post-Pleistocene radiation has largely been shaped by interactions between these two factors. Not only did we reveal clear evidence that both predation and resource levels are important in driving life-history evolution, but the effect of predation regime mostly depended on resource availability, while the effects of resource availability often differed among predation regimes—results observed whether using either univariate, trait-by-trait analyses or multivariate, PCA analyses. While this radiation has garnered attention for the predictability of adaptive phenotypic evolution (Cain et al., 2014; Herron and Freeman, 2014; Reece et al., 2014; Langerhans, 2018), we instead found here that observed evolutionary patterns only met our simple predictions in 2 out of 10 cases (resource effects on male age at maturity and juvenile growth rate). Meanwhile, 7 of our *a priori* predictions were upheld only in a nuanced manner (e.g., smaller offspring size in high-resource environments, but only in high-predation localities; higher reproductive frequency in high-predation environments, but only in high-resource populations). These results suggest that variation in predation and resource availability can lead to relatively complex evolutionary patterns of life-history divergence, and that accurately predicting these trajectories may require the consideration of interactions among selective agents.

First, we found that high predation risk has generally resulted in the evolution of higher fecundity, smaller offspring size, and more frequent reproduction (shorter interbrood intervals), at least within populations having moderate-high resource levels. These trait changes concur with theoretical expectations and prior empirical evidence for how predator-induced extrinsic mortality, and its subsequent reduction in population density, should drive shifts in these life-history traits (e.g., Reznick and Endler, 1982; Reznick et al., 1990, 2002; Charlesworth, 1994; Gasser et al., 2000; Moore et al., 2016). In environments where predators cause elevated adult mortality rates in prey populations, natural selection should favor maternal investment to produce larger numbers of offspring at a relatively rapid pace, compared to environments with low mortality rates where populations are more limited by density-dependent mechanisms, such as resource competition. Further, larger offspring often have competitive advantages in low-predation environments, whereas smaller offspring can often better elude predators (Brockelman, 1975; Bashey, 2008; Jørgensen et al., 2011; Rollinson and Hutchings, 2013). Because of energy constraints, as well as space constraints in the case of livebearing organisms like *G. hubbsi*, offspring number and size often show a trade-off, where higher fecundity comes at the cost of smaller offspring size (Smith and Fretwell, 1974; Stearns, 1992; Qualls and Shine, 1995; Roff, 2002). Most prior work on these key life-history traits, including

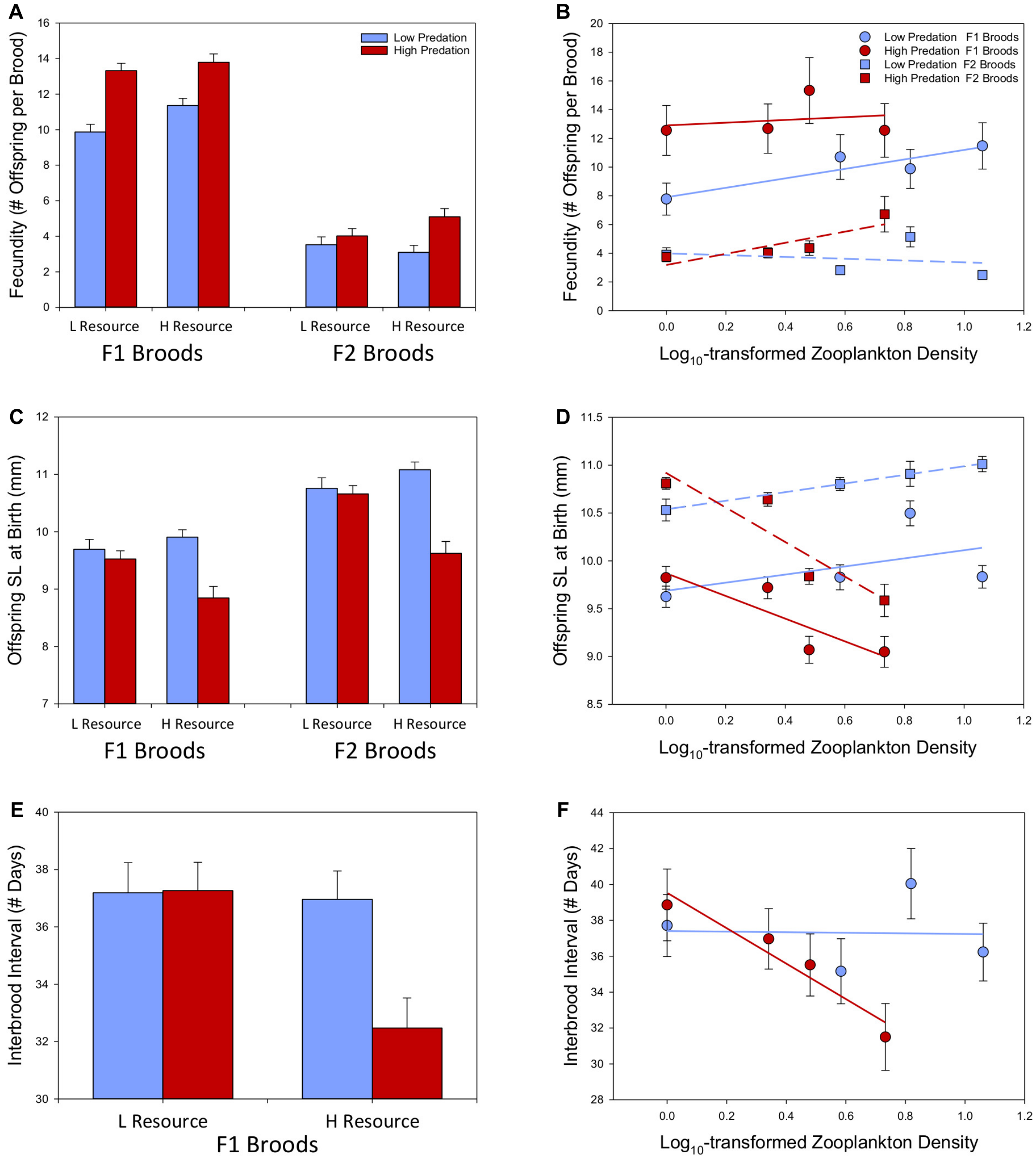

FIGURE 1 | Variation in life-history traits between predation regimes and across varying levels of native resource availability based on eight populations of lab-raised F1 and F2 broods of Bahamas mosquitofish reared under common conditions (estimated marginal means ± SE depicted). We illustrate variation between low-resource (0.2 $\log_{10}$ zooplankton density) and high-resource environments (0.75 $\log_{10}$ zooplankton density) **(A,C,E)**, as well as across the continuous range of resource availability **(B,D,F)**.

those on Bahamas mosquitofish (Riesch et al., 2013; Riesch et al., 2020) have primarily focused on wild-caught specimens, but only through common-garden studies can we establish evolutionary inferences of life-history divergence in the wild. The life-history shifts observed here may largely reflect genetic evolution across populations, as these life-history patterns occurred in animals raised under common conditions, and we found no effects of laboratory rearing on size-specific fecundity. These results contrast with prior arguments that life-history variation in *G. hubbsi* largely reflects phenotypic plasticity in response to food availability (Downhower et al., 2009). While comparatively few studies have so far examined how predation or resource levels may influence the evolution of reproductive frequency—which often requires captive rearing of reproductive females through multiple brood cycles—our findings do match those of previous studies which have found greater frequency of reproductive events in high-predation environments (Reznick and Endler, 1982). A larger number of prior studies have examined predation's role in the evolution of offspring number and size, and our results generally match those previously reported for other livebearing fishes, including *P. reticulata* (Reznick and Endler, 1982), *Brachyrhaphis episcopi*

TABLE 2 | Statistical results of a generalized linear mixed-model examining variation in interbrood interval of F1 broods in Bahamas mosquitofish derived from eight populations.

Source	*df*	χ^2	*P*
Predation regime (Pred)	1	3.63	0.0568
Log zooplankton density (Res)	1	4.93	0.0264
Pred × Res	1	3.87	0.0493
Log female SL	1	0.07	0.7892
Brood number	2	0.14	0.9313

TABLE 3 | Statistical results of a generalized linear mixed-model examining variation in male age at maturity of F2 Bahamas mosquitofish derived from eight populations.

Source	*df*	χ^2	*P*
Predation regime (Pred)	1	0.00	0.9848
Log zooplankton density (Res)	1	6.86	0.0088
Pred × Res	1	0.15	0.6972

(Jennions and Telford, 2002), *Brachyrhaphis rhabdophora* (Johnson and Belk, 2001), as well as a recent meta-analysis concluding that life-history divergence in response to predation has been consistent and predictable among multiple disparate species of livebearing fishes (Moore et al., 2016). This study adds to the growing evidence for predation's direct and indirect roles in the evolution of these life-history traits and our findings also point to a previously underappreciated dependence of these effects on resource availability (see below).

The faster juvenile growth rates observed in low-predation populations of Bahamas mosquitofish compared to those evolving in the presence of predators contradict theoretical predictions that high extrinsic mortality rates and low density dependence should favor higher growth rates. Prior work has suggested that faster growth to adulthood should increase fitness in high-mortality environments, and some work in livebearing fishes has found evidence for more rapid growth in high-predation localities (Johnson, 2001; Johnson and Belk, 2001; Reznick et al., 2001; Arendt and Reznick, 2005). However, this research has sometimes observed no evolved differences in growth rates between predatory environments (Gale et al., 2013; Reznick et al., 2019), or found inconsistent patterns of divergence that appear to reflect the influence of resource availability (Arendt and Reznick, 2005), which covaried with predation risk in those systems. Here, we suggest that the more intense resource competition in low-predation populations (low mortality, high density) has placed a greater premium on juvenile growth efficiency, because quickly reaching a larger, more competitive body size is more important in the absence of predators than in their presence. A key difference between prior work and the current study is that we simultaneously considered both predation risk and resource availability—had we ignored the resource levels that populations have historically experienced, we would not have found any differences in juvenile growth rates between predation regimes.

In contrast with theoretical predictions, and with most prior work in similar taxa (e.g., Reznick and Bryga, 1987; Reznick et al., 1990; Reznick et al., 1997; Johnson, 2001), we found no evidence that predation risk has led to evolutionary changes in age at sexual maturity in male Bahamas mosquitofish. However, in previous work predation regime and resource availability are known to covary among populations, making it difficult to discern their relative impacts on changes in this life-history trait. Here we found that resource availability, not predation regime, has strongly influenced male age at maturity in a manner that could potentially explain prior findings of an earlier age at maturity in those high-predation environments that also had higher resource availability than their low-predation counterparts. With strong resource-driven selection, resource levels may constrain the evolution of male age at maturity so that any predator-driven selection has little influence on its evolution. Our findings concur with recent work in Trinidadian guppies where replicate populations introduced from a high-predation source to low-predation environments only evolved a later age at maturity after reaching high densities and reducing the resource levels of their streams (Reznick et al., 2019). Those results suggest that reduced resource levels, not altered mortality rates *per se*, drive the evolution of age at maturity in guppies, at least in the absence of major predatory fish. Results of our study suggest resource levels, not predation risk, drive the evolution of age at maturity across all study sites in Bahamas mosquitofish. In addition, fish in different predation regimes may experience similar selection for adult male body size owing to factors such as resource acquisition and female mating preferences (Reynolds and Gross, 1992; Rosenthal and Evans, 1998). If so, this could limit the fitness benefits of younger age at maturity in high-mortality environments because an earlier age at maturity typically comes at the cost of a smaller body size—male poeciliids virtually stop growth after sexual maturity (Turner, 1941; Schultz, 1961). Of course, small body size could have advantages in surviving predatory encounters (Langerhans, 2009), although prior work in this system has found no evidence of age/size-specific mortality or differences in adult body size between predation regimes (e.g., Heinen et al., 2013; Riesch et al., 2013, 2020; Langerhans, 2018). Further work on predation's direct and indirect role in the evolution of age at maturity is required.

While predation risk has clearly influenced the evolutionary trajectories of a diverse set of life-history traits in this system, predation-driven divergence primarily occurred in environments with moderate to high resource availability. But why? Statistically speaking, a key reason that we observed greater divergence between predation regimes within environments having relatively higher resource availability is that the effects of resource levels on several life-history traits differed between predation regimes. Specifically, resource availability had highly consistent and predictable effects on all traits within high-predation environments, but had heterogenous effects within low-predation environments, ranging from strong and predictable (age at maturity, juvenile growth rate) to variable/weak (fecundity, interbrood interval) to opposite of predictions (offspring size). Thus, if we can understand the causes of the effects of resource levels on life histories

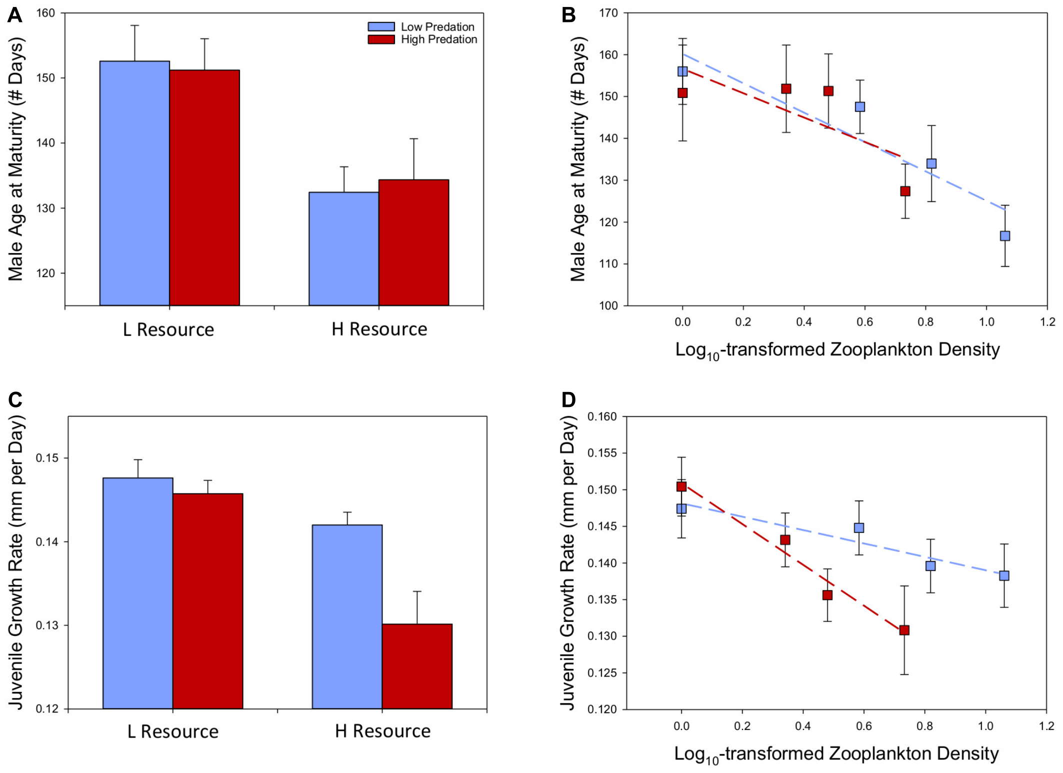

FIGURE 2 | Variation in life-history traits between predation regimes and across varying levels of native resource availability based on eight populations of lab-raised F2 Bahamas mosquitofish reared under common conditions (estimated marginal means ± SE depicted). Following **Figure 1**, we illustrate variation between low-resource (0.2 $\log_{10}$ zooplankton density) and high-resource environments (0.75 $\log_{10}$ zooplankton density) **(A,C)**, as well as across the continuous range of resource availability **(B,D)**.

within each predation regime, then we should uncover the underlying causes of why predator-driven divergence primarily occurred in sites with moderate to high resource availability. There are two patterns to be explained: effects of resource availability within (1) low-predation environments, and (2) high-predation environments.

In low-predation environments, we argue that the observed patterns of resource-driven variation in life histories are consistent with the notion that natural selection for the rapid attainment of a competitively superior large body size is generally stronger in these high-competition scenarios compared to high-predation environments. That is, low-predation populations may exhibit stronger resource limitation than high-predation populations at all levels of resources, with stronger density-dependent selection from resource competition in these predator-free, high-density environments

TABLE 4 | Statistical results of a generalized linear mixed-model examining variation in juvenile growth rate of F2 Bahamas mosquitofish derived from eight populations.

Source	*df*	*F*	*P*
Predation regime (Pred)	1, 5.19	6.90	0.0450
Log zooplankton density (Res)	1, 5.19	20.49	0.0057
Pred × Res	1, 4.58	3.67	0.1188
Tank density	1, 45.53	36.72	<0.0001
Water temperature	1, 10.39	11.64	0.0063

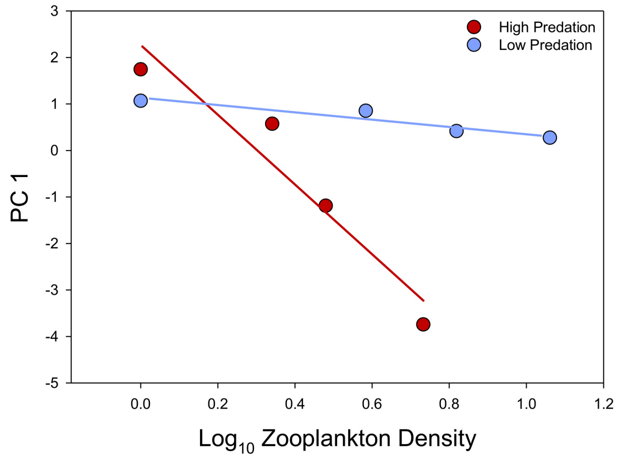

FIGURE 3 | Variation in PC1, illustrating that low-predation populations inhabiting environments with moderate to high resource availability have evolved a relatively faster juvenile growth rate, larger offspring size, a longer interbrood interval, and lower fecundity than high-predation counterparts.

(see **Supplementary Material**). In the absence of predators, Bahamas mosquitofish have evolved larger offspring in environments with higher resource levels, opposite to theoretical predictions (Pianka, 1970; Brockelman, 1975; Stearns, 1976, 1977). This could reflect the combination of (1) strong selection for larger juvenile body size due to its advantages in resource competition in low-predation environments, (2) the fact that higher resource availability, and thus the ability to rely on higher energy acquisition, allows the evolution of even larger offspring size, and (3) that optimal offspring size increases with decreasing growth rates, as observed in higher-resource environments (Jørgensen et al., 2011). With the evolution of larger offspring size in high-resource environments, this constrains the evolution of fecundity (due to space constraints in livebearing animals) and reproductive frequency (i.e., gestation period, due to developmental time constraints), potentially explaining why these low-predation populations have not evolved higher fecundity or more frequent reproductive events in localities with higher resource availability. Moreover, this selection for rapid attainment of an appropriate body size could additionally help explain why greater resource levels have resulted in earlier age at maturity, as individuals can more quickly reach a target body size under higher resource levels. Finally, this could explain the premium placed on rapid growth efficiency in low-predation populations, where fish showed more rapid juvenile growth than high-predation populations, and also showed elevated growth rates in low-resource environments where selection should strongly favor efficiency of converting energy from food to somatic growth.

In high-predation populations, the strong and predictable effects of resource availability on life-history traits might reflect two different causes. First, it could simply result from the theoretical effects of resource availability on life histories. For example, the earlier age at maturity, more frequent reproduction, and higher fecundity in populations with higher resource availability matches classic life-history predictions based on food levels. However, life-history theory does not necessarily predict smaller offspring size independent of fecundity in high-resource environments (which we observed) because theory focuses more on overall reproductive effort. Similarly, the finding of slower juvenile growth rates in populations with higher resource availability does not match predictions based on resource levels *per se*, but is consistent with selection on growth efficiency. Further, because optimal offspring size should increase with lower growth rates, these patterns are a bit perplexing. So, simple direct effects of resource availability may partially, but not fully, explain these findings. A second explanation is that higher resource levels somehow reflect stronger predator-driven selection. Under high resource levels, selection arising from extrinsic mortality rates can be exacerbated, where even stronger life-history shifts are favored with the reduction of energetic constraints. Moreover, even in this system where ecosystem productivity (e.g., chlorophyll *a*, phytoplankton density, zooplankton density) is decoupled from overall predation regime (presence/absence of predatory fish; e.g., Heinen et al., 2013), perhaps within high-predation environments resource availability still covaries with predation intensity. That is, where present, the density of the top predator in these systems (bigmouth sleepers) might increase with increasing levels of overall productivity, and thus exert greater predator-driven mortality or selection on Bahamas mosquitofish within those localities. Remarkably, bigmouth sleeper density does indeed correlate positively with zooplankton density across blue holes, although these variables are independent of *G. hubbsi* density (see **Supplementary Material**). This means that multiple factors may cause predator-driven selection to be stronger in environments with higher resource availability.

Altogether, the most likely explanation for the resource-dependent effects of predation regime on life-history divergence is the (1) strong selection for large juvenile body size and a reduction of energetic constraints with higher resource levels within low-predation populations, potentially combined with (2) relatively stronger selection from predator-induced mortality with higher resource availability within high-predation populations. That is, in low-resource environments resource-driven selection can be intense both in the absence and presence of predators, explaining the low levels of life-history divergence observed between those environments in this study. On the other hand, in environments with relatively abundant resources, predator-driven selection could overwhelm energy constraints in high-predation localities, while reduced energetic constraints can allow greater evolutionary responses to strong resource competition in the absence of predators, explaining the strong life-history divergence between predation regimes observed in those environments.

An important caveat regarding the findings here is that we only estimated patterns of evolutionary divergence in life histories under a single common environment of *ad libitum* food levels and the absence of predators. A more thorough understanding of life-history evolution requires assessments of phenotypes under multiple environmental conditions. Measuring multiple life-history traits across multiple rearing treatments for eight vertebrate populations presents strong logistical challenges—but such an approach can provide important insights into the evolution of phenotypic plasticity and possible population differences in plasticity (e.g., Arendt and Reznick, 2005; Gale et al., 2013). Future work should address this topic.

The extent to which the observed life-history shifts among populations reflect independent trait evolution or joint shifts of correlated traits remains largely unknown. Our multivariate assessment suggests that male age at maturity evolves independently of the rest of the life-history traits examined, but future work is needed to determine whether population differences in the other traits mostly reflect independent responses to direct selection or include substantial effects of indirect selection on correlated traits. Regardless, this study has uncovered intriguing patterns of life-history evolution across populations varying in predation risk and resource availability.

CONCLUSION

Because of the obvious connection between life-history traits and fitness, a large volume of comparative studies have investigated the roles of particular ecological variables, especially predation and resource availability, in driving population differences in these phenotypes. While informative, phenotype-environment associations derived from field studies can be difficult to interpret when strong covariation among ecological factors exists, and cannot directly address evolutionary variation in traits. Therefore, a contemporary challenge is to unravel the effects of individual selective agents on genetic divergence of key life-history traits (e.g., Reznick et al., 2019; Reznick and Travis, 2019). Our study adds new insights to this literature, suggesting that both predation risk and resource availability simultaneously and interactively influence the genetic evolution of life-history strategies in the wild. This knowledge deepens our understanding of adaptive life-history evolution, and explicitly points to the need of future work to address interactions among selective agents in the evolution of life histories.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.pc866t1nt (Langerhans and Hulthén, 2021).

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of North Carolina State University (protocols 16-193-O, 19-756-O).

AUTHOR CONTRIBUTIONS

RBL conceived and designed the study with major contributions from KH. RBL, KH, JH, and MJ collected the data. RBL analyzed the data and prepared the figures. RBL and KH coordinated the study and led the writing and revisions, to which all authors contributed. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.619277/full#supplementary-material

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Prioritizing Sex Recognition Over Learned Species Recognition: Hierarchical Mate Recognition in an Invasive Fish

Kit Magellan*† and Emili García-Berthou

Institute of Aquatic Ecology, University of Girona, Girona, Spain

Mate recognition is the process of identifying and assessing the appropriate species, sex or population of another individual for their suitability as a potential mate. Recognition may be innate or learned. Learning, the acquisition of knowledge or skills through experience, involves a relatively long-term change in behavioral responses. In this study we examined learned and innate mate recognition in invasive male mosquitofish, *Gambusia holbrooki*, interacting with female conspecifics and male and female native toothcarp, *Aphanius iberus*. Male mosquitofish directed no mating attempts at male toothcarp whereas numerous attempts were made toward female toothcarp. Male mosquitofish therefore differentiated between males and females, but initially did not distinguish between con- and heterospecific females. Neither the presence of a male toothcarp, nor the presence of a refugia affected the number of mating attempts received by females. However, by the second day males appeared to learn to more accurately direct their mating attempts, with larger female mosquitofish receiving the most attention, though smaller toothcarp females were still harassed. We propose that male mosquitofish, with a coercive mating system, are selected for persistence despite rejection by potential mates. In this scenario, the pool of potential mates may include heterospecifics whose avoidance of mating attempts may be ignored by male mosquitofish. It may thus be adaptive for male mosquitofish to prioritize sex recognition over species recognition: if one sex is recognized as a "non-mate" this will cut 50% from the pool of potential mates whereas recognition of a single species will remove many fewer potential mates from the pool. This innate sex recognition together with rapid learning of species identity may be a factor in the invasive success of mosquitofish.

Keywords: behavior, biological invasions, mosquitofish, toothcarp, *Gambusia*, *Aphanius*

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Andrea S. Aspbury,
Texas State University, United States

Reviewed by:
Mary E. Ramsey,
The University of Texas at Austin, United States
Ana Ruiz-Navarro,
University of Murcia, Spain

****Correspondence:***
Kit Magellan
kitmagellan@gmail.com

†Present address:
Kit Magellan,
University of Battambang, Battambang, Cambodia

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INTRODUCTION

Mate recognition is the process of identifying and assessing another individual for their suitability as a potential mate (Ryan and Rand, 1993; Pfennig, 1998; Mendelson and Shaw, 2012) and can be applied to recognition of an individual of the appropriate species, sex, or population (Ryan and Rand, 1993). Mate recognition is part of a process in which an individual is identified as a compatible mate (mate recognition), and assessed for quality with respect to the potential fitness benefits that may be conferred relative to other compatible mates (mate preference) (Ryan and Rand, 1993; Pfennig, 1998; Mendelson and Shaw, 2012). Individuals may use multiple traits to assess potential mates (Pfennig, 1998; Candolin, 2003; Mendelson and Shaw, 2012) and different traits may be involved in mate recognition and mate preference (Pfennig, 1998).

Learning, the acquisition of knowledge or skills through experience, is widespread in nature. It involves a change in behavioral responses, which should be neither transient (changing with almost every presentation of a stimulus) nor permanent (once knowledge or skill has been learned it is unchangeable), but must have at least the capacity to change. Learning may be individual or trial-and-error (i.e., learning through experience), or social (i.e., learning from others) (Galef and Laland, 2005; Campobello and Sealy, 2011). The requirements for learning may change throughout an individual's life. For example, learning to recognize predators is critical at all life stages so should be learned at the earliest opportunity. On the other hand, learned mate recognition does not become essential until sexual maturity, although interestingly mate recognition may be facilitated early in development through sexual imprinting (e.g., Irwin and Price, 1999; ten Cate and Rowe, 2007; Verzijden et al., 2012). However, recognition may be innate or learned (Kamo et al., 2002), and often both processes are incorporated into recognition of a single type of stimulus (e.g., Epp and Gabor, 2008; Milet-Pinheiro et al., 2012; Mueller et al., 2013). If recognition is innate, all (or most) members of a species or population will share the same recognition ability, whereas if recognition is learned, each individual must undertake the learning process so recognition abilities will vary between individuals. Learned recognition is therefore more likely to be subject to recognition mistakes (Kamo et al., 2002).

In this study we examined learned mate recognition through the interactions between invasive mosquitofish, *Gambusia holbrooki*, and native toothcarp, *Aphanius iberus*. Among invasive species, the Poeciliid *G. holbrooki* (and the closely related *Gambusia affinis*) are perfect models for this type of investigation. This native of North America (Parenti, 1981) has been introduced to all continents except Antarctica for mosquito control (Lever, 1996; García-Berthou et al., 2005; Pyke, 2008), so encountering many novel situations, and has subsequently successfully adapted to and colonized new habitats (e.g., Pyke, 2008; Benejam et al., 2009). Indeed, they are considered to be among the 100 worst invasive species worldwide (Lowe et al., 2000). One factor in their invasion success may be their rapid learning ability (Magellan et al., 2019). Moreover, the relatively recent contact between mosquitofish and toothcarp (since 1921 or later, García-Berthou et al., 2005; Benejam et al., 2009) suggests that, unless invasion has induced rapid selection for adaptive recognition (Strauss et al., 2006; Sih et al., 2010), recognition mechanisms are unlikely to have developed (Sherman et al., 1997; Payne et al., 2004).

Although both mosquitofish and toothcarp are Cyprinodontiform fishes (Parenti, 1981) these species differ in several respects. In contrast to the wide mosquitofish distribution, the toothcarp is endemic to the Iberian Peninsula (Ruiz Navarro and Oliva Paterna, 2012) and is classed as endangered and decreasing under the IUCN Red List and by the Bern Convention on the Conservation of European Wildlife and Natural Habitats (Freyhof and Brooks, 2011). For example, at the last count, of the 38 original Mediterranean populations 15 were known to be extinct (Doadrio, 2002) and this situation is likely to have worsened. In addition, while both species are sexually dimorphic (mosquitofish: Meffe and Snelson, 1989; Evans et al., 2011; toothcarp: Ruiz Navarro and Oliva Paterna, 2012), mosquitofish have internal fertilization and live birth (Meffe and Snelson, 1989) while toothcarp is an egg-laying species with external fertilization (Ruiz Navarro and Oliva Paterna, 2012) precluding the possibility of hybridization between these two species. However, as both sex and species recognition is necessary for successful copulation, these species provide the interesting prospect of investigating the potential for learning of different aspects of recognition.

Our aims were threefold. First, as male mosquitofish are voracious in pursuit of mating opportunities (Meffe and Snelson, 1989; Evans et al., 2011), and we are interested in the mechanisms by which invasive mosquitofish impact on native species, we examined male mosquitofish mate recognition ability. We predicted that mosquitofish males would attempt to mate preferentially with their own species and would be able to distinguish between sexes such that any misdirected mating attempts would be mainly toward female toothcarp. Second, we evaluated three alternative hypotheses for male mosquitofish mating preferences: (i) as larger female poeciliids are more fecund (Meffe and Snelson, 1989; Evans et al., 2011), and therefore should be preferred (Andersson, 1994), male mosquitofish would attempt to mate preferentially with larger females of either species; (ii) that the extent of harassment experienced by female toothcarp would depend on whether a companion toothcarp was male or female; and (iii) that females of either species would be able to use a refuge to escape male harassment (e.g., Magellan and García-Berthou, 2016), i.e., that male mosquitofish mating preferences are a function of female availability. Finally, we investigated male mosquitofish capacity to learn mate recognition predicting that male mosquitofish would be able to direct their mating attempts more accurately within a short time scale.

MATERIALS AND METHODS

Fish were collected, using dip nets, from the Ter (42.0451°N, 3.1960°E), Fluvià (42.1875°N, 3.0851°E), and Muga (42.2527°N, 3.0756°E) rivers (mosquitofish) and from Fra Ramon lagoon (42.0149°N, 3.1129°E), Baix Empordà salt marshes (toothcarp). The mosquitofish from these populations had no prior contact with toothcarp and while toothcarp individuals may have previously encountered mosquitofish this contact was minimal. Fish were transported to the laboratory and placed in six 60 L species-specific (three per species) stock tanks (60 × 30 × 32 cm). Mosquitofish from all three rivers were housed together. Each tank contained a gravel substrate, conditioned water and a filtered air supply. Tanks were illuminated with 6 W bulbs and maintained at a constant photoperiod (12:12 h light:dark cycle) and temperature (25 ± 1°C). Fish were fed to satiation once per day with commercial food flakes or frozen bloodworms (*Chironomus* spp.). Fish were allowed to acclimate to laboratory conditions for at least 2 months prior to the start of experimentation.

The experiment was conducted in six 26 L tanks (45 × 22 × 28 cm), each containing a gravel substrate, an air supply, an aquarium heater and water filled to a depth of 20 cm. A light source was positioned above each tank. The evening before a set of observations, six pairs of toothcarp were selected at random with the proviso that at least one was a female and that they could be easily identified. In cases where there were two females this meant that one was larger than the other. This resulted in 10 female–female toothcarp pairs and 13 female–male pairs. One pair of fish was added to each of the experimental tanks, fed, and left overnight to acclimate. The following morning six sets of one male and one female mosquitofish were selected at random and added to the experimental tanks. A refuge, consisting of a clear plastic jar, 5 cm diameter and 12.5 cm tall covered with a gray mesh, was added to half of the tanks (treatment B: refugia). The refugia were oriented so that the open end faced the front of the tank. The other three tanks were left without refugia (treatment A: control).

All fish were fed and left for 1 h after which 10-min observations were conducted. All observations were videoed and scored later. After each trial, a refuge was added (treatment A) or removed (treatment B) as required. Fish were again left for 1 h to acclimate, after which a second 10-min observation was carried out as before. The order of treatments A and B was thus randomized. At the end of all trials for a day, refugia were added to each of the tanks as needed, and fish were again fed and left overnight. The following morning fish were fed and left for 1 h as before and the 10-min trials (treatment C: day 2, with refugia) were videoed. After all trials, total length (measured to the nearest millimeter using a ruler) and sex of each fish were recorded and fish were placed in separate stock tanks so that they would not be reused. In total 23 sets of fish were observed in all three treatments.

Each video was analyzed recording the number of mating attempts directed at each individual in a group by the male mosquitofish and the time each fish spent in the refugia. Male mosquitofish mating attempts were defined as a gonopodial thrust, with or without a preceding chase. For statistical analyses the female in male–female toothcarp pairs or the larger female in female–female pairs was designated T1 (i.e., toothcarp 1). The second toothcarp in each group was designated T2 and the female and male mosquitofish were designated M1 and M2, respectively.

First the difference in total length between T1 and M1 in each group of fish was assessed using a paired t-test.

Then, mating attempts received from the mosquitofish male were examined in three analyses. As toothcarp 2 (T2) received no or very few mating attempts (see section "Results") this data set was excluded from analyses and only the mating attempts received by T1 and M1 were included. For these analyses we used Generalized Estimating Equations (GEEs), an extension of Generalized Linear Models developed for situations where response variables are non-independent, to account for the within-subjects factors (e.g., Magellan and García-Berthou, 2015). For all analyses, fish group was the experimental unit (i.e., the two toothcarp and two mosquitofish that were examined together) and the dependent variable, frequency of mating attempts received, was analyzed with Poisson distribution and a log-link function. First, to assess the influence of the species and length of females, and the capacity of male mosquitofish to learn mate recognition (i.e., the effect of treatment) on the number of mating attempts received, the analysis comprised two within-subjects factors (treatment and species) and one covariate (female length) as the independent variables. Second, as examination of graphs showed a large difference between treatments A and B and treatment C (see **Figure 1**), the data from the second day was examined separately using one within-subjects factor (female species) and one covariate (female length). Third, the effect of the presence of a male toothcarp (i.e., when T2 was male) on the frequency of mating attempts received by T1 was assessed using mating attempts received by T1 as the dependent variable, with one within-subjects factor (treatment) one between subjects factor (T2 sex) and one continuous factor (T1 length).

Finally, the potential effects of the presence of a refuge were examined. As these data were zero inflated, non-parametric analyses were used. First the data for time spent in refugia by each individual were collapsed across each independent variable of interest and differences were assessed using Wilcoxon signed rank tests for the related data sets (time and species) and a Mann–Whitney U-test for differences between sexes. Then the relationships between refugia use and mating attempts received were assessed using separate Spearman rank correlations for the females of each species (i.e., T1 and M1) on each day.

RESULTS

In the assessment of female size, the female mosquitofish were significantly longer than the (larger) female toothcarp in each group (T1: 31.0 ± 2.56 mm, M1: 36.7 ± 4.61 mm, mean ± s.d.; paired-$t_{(22)} = -4.85$, $p < 0.001$).

All but three of the male mosquitofish attempted to mate in at least one of the treatments. The vast majority of mating attempts were directed at the female or larger female toothcarp (T1) and the female mosquitofish (M1). No male toothcarp received any mating attempts from the male mosquitofish in any of the trials while only three of the small females in the female–female toothcarp pairs received a single mating attempt.

Importantly there was no difference between female mosquitofish and female toothcarp in the frequency of mating attempts received (**Table 1**: Analysis 1). However, there was a significant difference between treatments in the frequency of mating attempts received (**Table 1**: Analysis 1). Moreover, while female length itself showed no significant pattern there was a significant interaction between length and treatment, with larger females receiving more mating attempts, particularly on day 2 (Treatment C) (**Table 1**: Analysis 1, **Figure 1**).

When data from day 2 were considered separately the relationships between species were modified. There was a significant interaction between species and length of females.

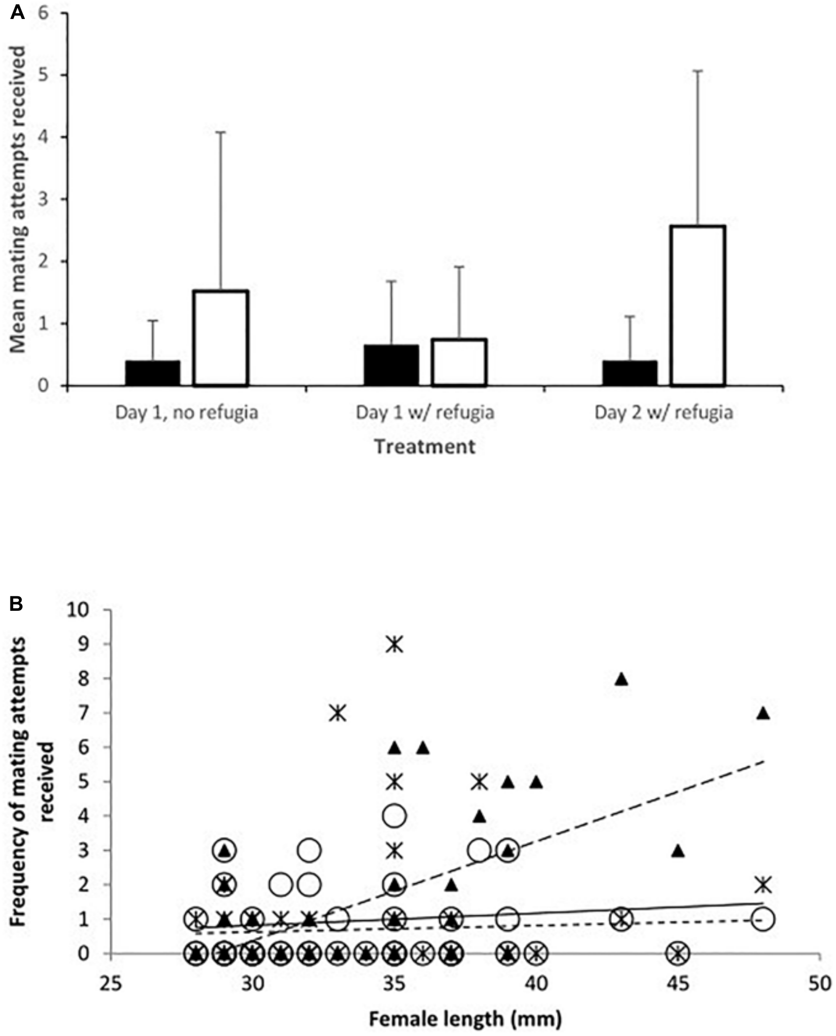

FIGURE 1 | (A) Mean mating attempts received by toothcarp (black bars) and mosquitofish (white bars) females from mosquitofish males. Error bars show standard deviation; and **(B)** The frequency of mating attempts received by mosquitofish and toothcarp females combined in relation to their total length. Trendlines have been added for illustration. Treatment A: black crosses, solid line; B: white circles, small dashed line; C: black triangles, large dashed line.

The frequency of mating attempts received increased with female size for mosquitofish, but for toothcarp females mating attempts received showed a slight decline with increasing length (**Table 1**: Analysis 2, **Figure 2**). Although the main effect of species was still not significant a trend in species differentiation was apparent (**Table 1**: Analysis 2).

When considering the effect of the presence of a male T2, the sex of the conspecific individual (T2) showed no significant effect and treatment was likewise not significant (**Table 1**: Analysis 3). However, the frequency of mating attempts across all treatments decreased significantly with increasing length of T1 (**Table 1**: Analysis 3, **Figure 3**).

Fish of both species and sexes entered refugia although use was overall limited with only 15 of the 23 trials on day 1 and 14/23 on day 2 including any refugia use. While both con- and heterospecific fish used refugia concurrently on occasion, in most trials in which refugia use occurred only one of the four fish entered (1 fish: 19 out of 46 trials; 2 fish: 9/46; 3 fish: 1/46; 4 fish: 0/46). Refugia use did not vary between sexes ($Z = -0.252$, $p = 0.801$; **Figure 4**) or between days ($Z = -0.692$, $p = 0.489$; **Figure 4**), though toothcarp made greater use of refugia than mosquitofish ($Z = -3.894$, $p < 0.001$; **Figure 4**). In addition, there was no relationship between refugia use and mating attempts received by females of either species on either day (Day 1: toothcarp: $r = 0.089$, $p = 0.686$, mosquitofish: $r = -0.165$, $p = 0.451$; Day 2: toothcarp: $r = -0.192$, $p = 0.381$, mosquitofish: $r = -0.215$, $p = 0.324$).

DISCUSSION

Male mosquitofish were evidently able to differentiate between males and females. In the 15 h of observations analyzed for this study no mating attempts were directed at male toothcarp

TABLE 1 | Results from the three Generalized Estimating Equations.

Variable	Wald χ^2	df	p
Analysis 1: Both species, Day 1 and 2			
Treatment (T)	8.106	2	**0.017**
Species (Sp)	0.309	1	0.578
Length (L)	0.754	1	0.385
T × Sp	1.686	2	0.430
T × L	10.956	2	**0.004**
Sp × L	0.738	1	0.390
T × Sp × L	3.533	2	0.171
Analysis 2: Both species, Day 2 only			
Species (Sp)	3.454	1	0.063
Length (L)	0.355	1	0.551
Sp * L	4.949	1	**0.026**
Analysis 3: Toothcarp only, Day 1 and 2			
Treatment (T)	4.487	2	0.106
T2 Sex (Sx)	0.435	1	0.510
T1 Length (L)	5.741	1	**0.017**
T × Sx	3.197	2	0.202
T × L	4.452	2	0.108
Sx × L	0.357	1	0.550
T × Sx × L	2.555	2	0.279

Analysis 1: the effects of treatment, species, and total length of females on the frequency of mating attempts received by females of both species; Analysis 2: the effects of species and female length on the number of mating attempts received by females of both species on the second day; Analysis 3: the effects of treatment, conspecific sex, and female length on the frequency of mating attempts received by toothcarp 1. Significant results are highlighted.

whereas numerous attempts were made toward female toothcarp. However, male mosquitofish initially did not distinguish between con- and heterospecific females. Our alternative hypotheses to explain the relative frequency of male mosquitofish mating attempts also received limited or no support. First, although female mosquitofish were larger, and overall larger females were preferred, the exact relationship between male mosquitofish mating attempts and female size depended on both species and time. Male mosquitofish harassed smaller toothcarp females more than larger females, whereas the reverse was true for mosquitofish females, but this did not become notable until the second day. The exact role of female length in male mosquitofish mating preferences in the current study is therefore unclear. Second, whether the second toothcarp was male or female made no difference to the frequency of mating attempts received by female toothcarp, indicating that male mosquitofish did not divide their efforts between all the available females; that neither male nor female presence provides an adequate deterrent or stimulus; and that mate guarding, or other forms of aggression, from male toothcarp are absent or at least ineffective against male mosquitofish. Third, the presence or absence of refugia made no difference to mating attempts received by females, implying that females of neither species used refugia to escape male attention. This is in contrast to previous work in which toothcarp appeared to utilize refugia to escape aggression from mosquitofish (Magellan and García-Berthou, 2016). However, in this case it is clear that male mosquitofish mating attempts are not a function of female availability.

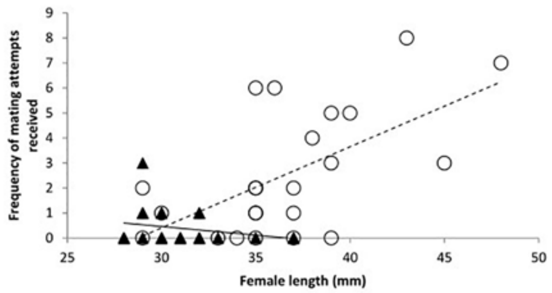

FIGURE 2 | The frequency of mating attempts received by females on day 2 in relation to their total length. Trendlines have been added for illustration. Toothcarp: black triangles, solid line; mosquitofish: white circles, small dashed line.

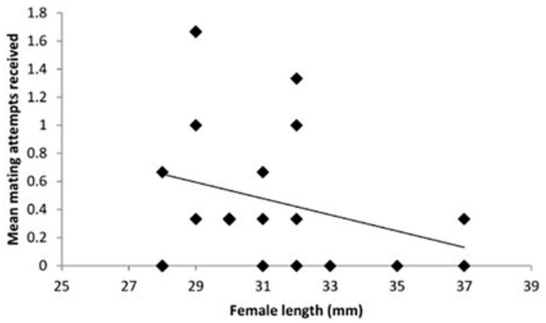

FIGURE 3 | The mean frequency of mating attempts received by toothcarp females paired with conspecific males and females across all three treatments in relation to their total length (n = 23). A trendline has been added for illustration.

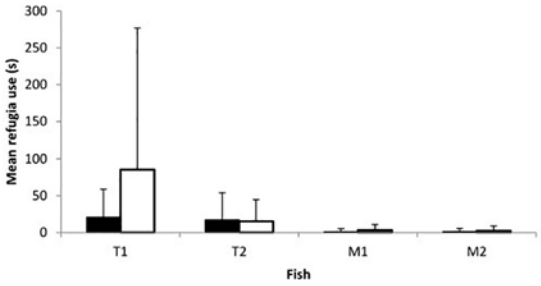

FIGURE 4 | Mean refugia use by fish of both species on day 1 (black bars) and day 2 (white bars). T1 = female or larger female toothcarp; T2 = second toothcarp; M1 = female mosquitofish; M2 = male mosquitofish. Error bars represent standard deviation.

All male mosquitofish in this study were "normal" males in that all were sexually mature and had previous access to females of their own species so were presumably sexually experienced. Therefore, these results cannot be explained by sexual naivety. However, as mosquitofish males and females were housed together but separately from toothcarp, it is conceivable that this is an extreme case of preference for novel or unfamiliar individuals (Kelley et al., 1999) but even if this was the case, male toothcarp were still treated differently. It is also possible that female toothcarp were not as proficient at avoiding mating attempts as male toothcarp and female mosquitofish. However, male toothcarp also harass females of their species and females are adept at avoiding this harassment either through escape or aggression (KM pers. obs.) so this is unlikely. Finally, fish may shoal together based on similarities in, e.g., activity types or habitat preferences as much as through conspecific attraction (Ward et al., 2020). As conspecifics are likely to be most similar in these respects, male mosquitofish may have considered any individual in their vicinity to be a conspecific, but again, male toothcarp did not receive the same attention as females. Whatever factors are involved it is clear that male mosquitofish differentiated between male and female toothcarp and initially attempted to interact with female toothcarp as they would females of their own species.

Male mosquitofish did, however, direct their mating attempts more accurately by the second day. Neither morphology (Meffe and Snelson, 1989; Evans et al., 2011) nor pheromone production (Burnard et al., 2008), both factors in mate preference, are likely to have changed substantially within this time, and while female behavior may have been modified this was not particularly apparent (KM pers. obs.). Although, mating attempts received by females of each species still did not differ significantly, there was an obvious trend in the data and it is likely that with slightly more time mosquitofish males would learn to direct their mating attempts exclusively toward mosquitofish females. Recognition of both sexes within an individual's species as a unified category separate from heterospecifics, and differentiating between species within a single sex, are distinct concepts (Mendelson and Shaw, 2012). Our results indicate that for male mosquitofish either sex differentiation takes priority over species differentiation or they are able to differentiate between the sexes more readily than they are able to differentiate between species. Mating decisions are hierarchical processes in which mate choice is based first on compatibility and then evaluation of other quality indicators (Mendelson and Shaw, 2012). For mosquitofish males it appears that compatible is synonymous with female, and quality assessment entails selecting the correct type of female. A similar delay in species recognition has been shown in another Poeciliid fish, the guppy, *Poecilia reticulata*, in which males initially attempted cross species matings with the swamp guppy, *Poecilia picta* (Magurran and Ramnarine, 2004, 2005) and the Goodeid, *Skiffia bilineata* (Valero et al., 2008, 2009) before targeting their own species. However, in these studies sex recognition was assumed rather than tested. Whatever the exact chronological recognition sequence, male mosquitofish did begin to discriminate between con- and heterospecific females by the second day. It is interesting that there appeared to be an increase in discrimination against larger toothcarp females that are probably more morphologically similar to mosquitofish females than smaller toothcarp, suggesting that male mosquitofish were learning to differentiate between these comparable phenotypes.

How do these results elucidate the mechanisms by which mosquitofish males select suitable mates? Mate acceptance thresholds may be adjusted based on the costs of heterospecific matings, the frequency of interactions with heterospecifics and the benefits of conspecific matings (Pfennig, 1998) so an individual may attempt to mate with individuals of several different species. In the normal course of events, a (usually) male will solicitate a mating with a female, the female will reciprocate resulting in positive reinforcement (Krebs and Davies, 1993), and the male will associate that phenotype with successful mating, thus learning positive recognition of potential mates. Avoidance of heterospecifics will be facilitated by negative experiences such as rejection by heterospecific females or aggression from males (Verzijden et al., 2012). However, for mosquitofish with a coercive mating system (Meffe and Snelson, 1989; Evans et al., 2011), the usual female response to a solicited mating attempt is rejection. Indeed, in this study there were no observations of consensual mating between male and female mosquitofish. Moreover, males do not engage in energetically expensive displays, provide no nuptial gifts, and undergo no sperm transfer without full copulation (Evans et al., 2011), so the costs of mating attempts comprise only energy and time used in pursuit of females. Male mosquitofish may therefore be selected for persistence despite rejection, thus negating heterospecific avoidance though negative experiences.

For male mosquitofish it may be more cost effective to learn who not to mate with, i.e., negative recognition. Mate recognition is a behavioral response indicating that one individual considers another an appropriate mate, even if mistakenly (Ryan and Rand, 1993) and there are many cases of adaptive hybridization (Arnold and Hodges, 1995; Mallet, 2007; Mendelson and Shaw, 2012) so other species may also form part of the available pool of potential mates. A single species may overlap with a mosaic of other species across its entire geographic range (Hoskin and Higgie, 2010; Camacho-Cervantes et al., 2014) and for the highly invasive mosquitofish this state is intensified. As potential mates are a subset of the taxonomic species of an organism, but also of the whole ecological community (Mendelson and Shaw, 2013), for mosquitofish the pool of potential mates can be considered to be global. The form of recognition that is prioritized (in this case sex or species) depends on the likelihood of making a mistake in recognition and the fitness costs of doing so (Pfennig, 1998). If mosquitofish males can recognize any single species as being unsuitable mates, this cuts out just a small proportion of the potential mate pool. However, if males can distinguish sexes and recognize females as potential mates, fully 50% of the potential mate pool will be excluded. Thus, for mosquitofish it may be more beneficial to prioritize sex recognition, and subsequently learn to avoid the particular species with which it interacts at any given location. This further suggests that sex recognition is innate in this species, whereas species recognition is learned. These findings may play a role in mosquitofish success as an invader.

The initiation of learned recognition was remarkably rapid in this study, occurring within 24 h. Rapid learning has also been demonstrated in an invasive population of the closely related *G. affinis*, which showed significant improvement in food location ability within 3 days (Magellan et al., 2019). However, previous mate recognition studies using the guppy have shown no recognition of heterospecific *S. bilineata* females within 3 days (Valero et al., 2008); recognition of *P. picta* females after four (Magurran and Ramnarine, 2004) and six (Magurran and Ramnarine, 2005) days; and individual recognition of conspecific (Griffiths and Magurran, 1997) and heterospecific (Valero et al., 2009) individuals after 12 and 14 days, respectively. It may be that the distance between these species is a factor in the speed of learning (Mendelson and Shaw, 2012). In comparison to the mosquitofish-toothcarp model system, the species used in the above studies are much more similar. In common with mosquitofish, the Poeciliids (*P. reticulata* and *P. picta*) and *S. bilineata* are native to nearby areas and have comparable mating systems (Parenti, 1981; Meffe and Snelson, 1989; Evans et al., 2011), whereas toothcarp belongs to a group which diverged much earlier (Parenti, 1981). The marked differences between mosquitofish and toothcarp, particularly in mating system, may provide superior information by which recognition learning can occur. This capacity for rapid learning may be a factor in (e.g., Roudez et al., 2008), or an effect of Payne et al. (2004), the invasive success of mosquitofish (Magellan et al., 2019). They have been introduced to many new environments and therefore potentially exposed to many new heterospecifics. As discussed above, the costs to mosquitofish of misplaced mating attempts are relatively minor. Injury through aggressive interactions, while not likely with toothcarp, may be an additional cost in other situations. The ability to rapidly learn recognition may help to minimize these costs and so predispose mosquitofish to be a successful invader.

These findings may have wider implications. Recognition is defined as the ability to distinguish something, such as another individual, and to produce a distinct and quantifiable response. Recognition of a diverse variety of entities may be necessary during an individual's lifetime, from recognition of mates, through predator recognition, to recognition of suitable habitats or environmental features. In all cases, recognition necessitates the abilities to remember particular signals and to discriminate them from other signals (ten Cate and Rowe, 2007); and the same or different signals may be recognized in different contexts (Okamoto and Grether, 2013). This suggests a number of questions. For example, how long is learned recognition by mosquitofish retained? What recognition mechanisms are used to ensure recognition is accurate? Is recognition specific to a single species or context or can it be generalized to other species or contexts, such as from mating to competition? These questions provide potential areas of future research in studies of learning, recognition and particularly for invasive mosquitofish.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KM conceived, designed, and carried out the experiment, and took the lead on data analysis and manuscript preparation. EG-B consulted throughout. Both authors collaborated on writing and approved the submission.

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Reproductive Mode and Conflict Shape the Evolution of Male Attributes and Rate of Speciation in the Fish Family Poeciliidae

David N. Reznick[1]*, **Joseph Travis**[2], **Bart J. A. Pollux**[3] **and Andrew I. Furness**[4]

[1] Department of Biology, University of California, Riverside, Riverside, CA, United States, [2] Department of Biological Science, Florida State University, Tallahassee, FL, United States, [3] Experimental Zoology Group, Wageningen University & Research, Wageningen, Netherlands, [4] Department of Biological and Marine Sciences, University of Hull, Hull, United Kingdom

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***Correspondence:**
David N. Reznick
david.reznick@ucr.edu

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Sexual conflict is caused by differences between the sexes in how fitness is maximized. These differences are shaped by the discrepancy in the investment in gametes, how mates are chosen and how embryos and young are provided for. Fish in the family Poeciliidae vary from completely provisioning eggs before they are fertilized to providing virtually all resources after fertilization via the functional equivalent of a mammalian placenta. This shift in when females provision their young relative to when an egg is fertilized is predicted to cause a fundamental change in when and how sexual conflict is manifested. If eggs are provisioned before fertilization, there should be strong selection for females to choose with whom they mate. Maternal provisioning after fertilization should promote a shift to post-copulatory mate choice. The evolution of maternal provisioning may in turn have cascading effects on the evolution of diverse features of the biology of these fish because of this shift in when mates are chosen. Here we summarize what these consequences are and show that the evolution of maternal provisioning is indeed associated with and appears to govern the evolution of male traits associated with sexual selection. The evolution of placentas and associated conflict does not cause accelerated speciation, contrary to predictions. Accelerated speciation rate is instead correlated with the evolution of male traits associated with sexual selection, which implies a more prominent role of pre-copulatory reproductive isolation in causing speciation in this family.

Keywords: viviparity, sexual selection, intergenomic conflict, placenta, Poeciliidae

INTRODUCTION

Intergenomic Conflict

Trivers (1974) presented the first formal exposition of intergenomic conflict. He inferred the existence of parent-offspring conflict from the genetic relatedness of mothers and offspring and among siblings. Conflict arises because the quantity of resources that is in the best interest of offspring to get from their mothers is greater than is in the best interest of mothers to give to their offspring. Conflict creates a tug of war in which selection favors maternal control over the allocation of resources to offspring at the same time that it favors offspring who acquire more from their mothers (Haig, 1993).

Trivers (1974) argued that natural selection will favor mothers that invest equally in all offspring because all offspring carry an equal complement of her genes into the next generation. Each offspring shares 100% of its genes with itself, 50% with its mother and 50% with its full siblings. A consequence of these proportions of genes in common with self-versus siblings is that selection favors offspring who are able to glean more resources from their mothers, even at the expense of their mothers and siblings, because the benefit of such selfishness is paired with a cost that is discounted by 50% for the mother and siblings. A corollary of this argument is that polygamy will increase the intensity of the conflict because an individual offspring shares only 25% of its genes with siblings sired by a different father, so the cost associated with selfishness is discounted by 75% rather than 50%.

Parent-offspring conflict can be viewed as a special case of sexual conflict because offspring are surrogates for the paternal genome. The reason each offspring shares only 50% of its genes with its mother and full siblings is that the other 50% is derived from their father. The root of the conflict between the mother and offspring is thus the presence of the paternal genome and what the father's genes gain at the expense of the mother. Trivers (1974) argument has since been expanded in diverse ways. For example, Haig (1990) shows that if offspring differ in quality and the mother is able to assess their quality, then she will maximize her fitness by allocating resources preferentially to those offspring likely to have higher fitness, rather than treat all offspring equally.

How sexual conflict is manifested is influenced by the mode of reproduction (Furness et al., 2015). If reproduction is via broadcast spawning, as in sea urchins, in which multiple individuals release eggs and sperm into the water column, then the main venue for conflict is at the sperm-egg interface. Sperm are under strong selection to be effective at penetrating eggs at the same time that eggs are under strong selection to admit one and only one sperm because polyspermy is lethal to the egg. The rapid evolution of the bindin gene on sperm is a signature of such conflict (Levitan and Ferrell, 2006).

When organisms are mobile, enabling the creation of nesting sites, as in salmon, or participating in amplexus, as in frogs, then the avenues of conflict multiply. Either form of reproduction adds some measure of paternity assurance, enhances the potential for female choice of mates, but also opens the door to male-male competition for access to females and alternative male mating strategies (Gross, 1985; Taborsky et al., 2008). The advent of pre-copulatory sexual selection, in the form of female mate choice and male-male competition, brings with it the evolution of enhanced sexual dimorphism in morphology and behavior (Anderson, 1994; Zeh and Zeh, 2003).

The evolution of internal fertilization adds new dimensions to sexual conflict; males in some lineages evolve ever more elaborate courtship to attract mates or may evolve more elaborate morphology for grasping and inseminating them, countered by female morphology to maintain control (Arnqvist and Rowe, 1995, 2002). We see the advent of an internal struggle between sperm and semen among competing males to gain access to eggs or between the male and female to fertilize eggs and influence the tendency of females to re-mate(Heifetz et al., 2000; Wolfner, 2002; Ram and Wolfner, 2007). Internal fertilization also enhances the potential for cryptic female choice, or the ability of females to influence which sperm fertilize the eggs (Eberhard, 1996). These same conditions apply to species that retain fertilized eggs, then give birth to live born young.

The evolution of matrotrophy, or the continued provisioning of young after the egg is fertilized, expands the arenas in which conflict occurs because the timing of maternal provisioning overlaps with the expression of the paternal genome in the developing embryo (Furness et al., 2015). It is either at this stage, or during post-natal provisioning of young, that Trivers (1974) concept of parent-offspring conflict comes into play. In the case of matrotrophy, prenatal conflict is predicted to be mediated by "chemical means," such as the secretion of hormones produced by the embryo into the mother's bloodstream that elevate the delivery of nutrients to the baby (Haig, 1993). Manifestation of conflict during post-natal care instead often takes the form of begging behavior or aggression among siblings (Mock and Parker, 1997).

The Viviparity-Driven Conflict Hypothesis

Zeh and Zeh (2000) proposed the viviparity-driven conflict hypothesis (VDCH) to address the evolutionary consequences of the shift from egg laying to live bearing, but their logic applies well to the shift from lecithotrophy to matrotrophy. Their hypothesis addresses how the evolution of maternal provisioning changes how sexual conflict is manifested. Lecithotrophic females make their full investment in offspring before the egg is fertilized. If such a female is to influence who sires her offspring, then she must be choosy about who she mates with. If lecithotrophic females mate with inferior males, they risk squandering their large pre-copulatory investment. Since female appraisal of male quality is based on male behavior and/or phenotype, female choice can lead to the evolution of elaborate male morphology and courtship behavior associated with sexual selection (Arnqvist and Rowe, 2005). In contrast, placental females produce tiny, inexpensive eggs (Pires et al., 2010; Pires and Reznick, 2018). They can reduce the risk of poor mate choice by mating with multiple males, enabling them to influence which sperm fertilize eggs or through post-zygotic mechanisms of sexual selection, such as selective embryo abortion or the differential allocation of maternal resources to embryos based on genotype (Haig, 1990; Zeh and Zeh, 2000, 2001; Crespi and Semeniuk, 2004). This shift in when mates are selected shifts the emphasis from the importance of choosing with whom to mate with to instead mating with multiple males to access genetic diversity in the sperm gene pool. A consequence is that we should see less evidence of sexual selection on male morphology and courtship behavior in species with matrotrophy. Furthermore, Zeh and Zeh (2008) predicted that the evolution of matrotrophy will increase the rate of evolution of post-zygotic reproductive isolation and the rate of speciation. The basis for this prediction is that conflict may be resolved in different ways by different populations. Conflict resolution differs from adaptation because it involves each sex evolving in response to the other in a tit for tat fashion. The paths followed by different populations may thus diverge from one another such that, when the two are brought back into contact, there may be a mismatch in how conflict is resolved. The evidence in support of Zeh and Zeh's hypothesis at the time

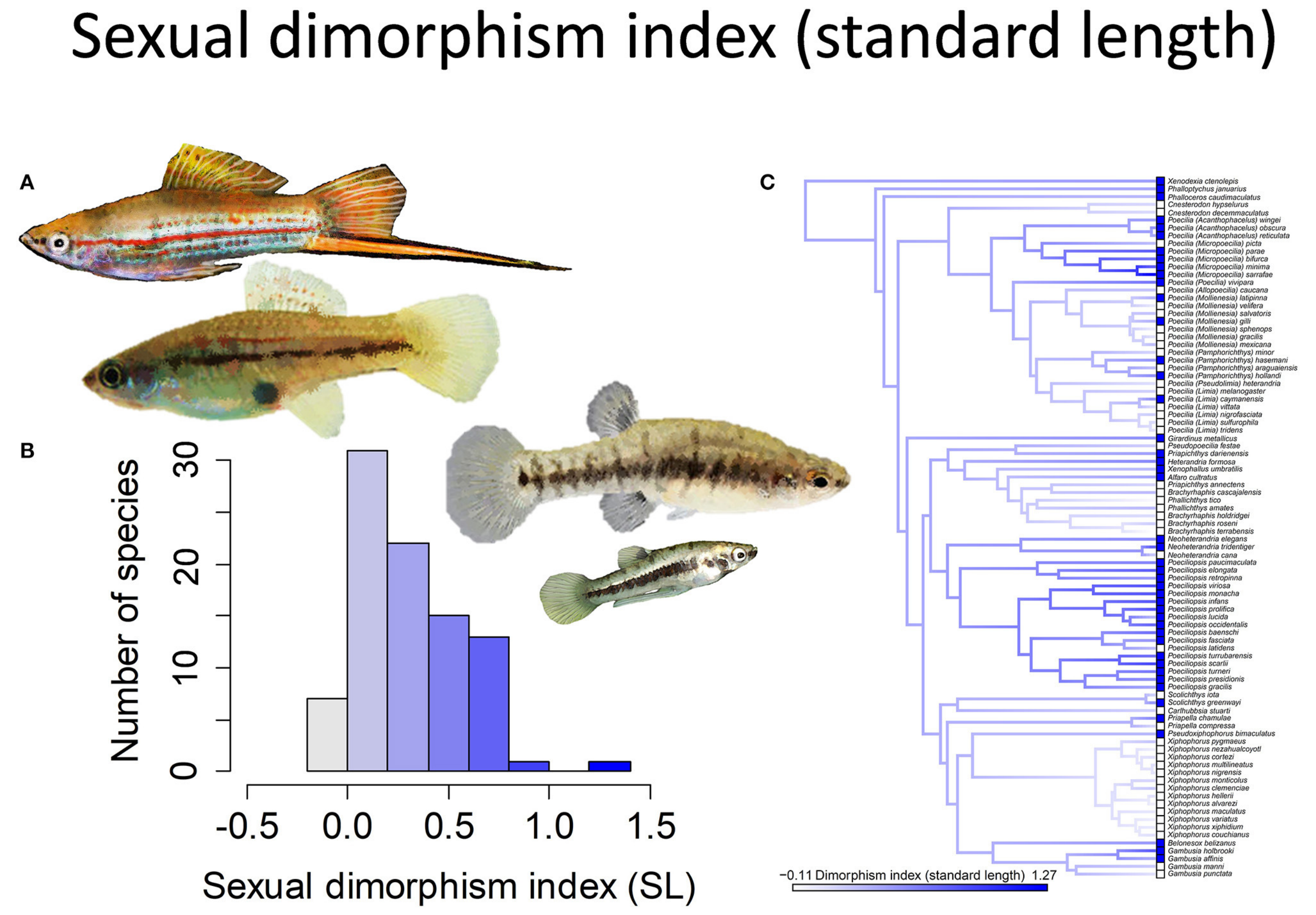

FIGURE 1 | The magnitude of sexual size dimorphism (standard length) in the family Poeciliidae ranges from −0.11 to 1.27. Zero indicates males and females are of equal size, negative values males larger than females, and positive values females larger than males. **(A)** *Xiphophorus hellerii* males and females are of approximately equal size (pictured left) while males are considerably smaller than females in *Heterandria formosa* (pictured right). **(B)** Frequency histogram of the sexual dimorphism index ($n = 90$). **(C)** Ancestral state reconstruction of the sexual dimorphism index as a continuous character using the contMap function in the R package phytools, which estimates the Maximum likelihood ancestral states at all internal nodes and interpolates the states along each edge. The phylogeny tips depict a binary categorization of the size dimorphism index (i.e., low vs. high). The most likely ancestral state of the family, when treated as a binary character, is that of a high degree of size dimorphism (Furness et al., 2019).

of writing was based on broad comparisons, such as between vertebrate classes. For example, mammals evolve post-zygotic RI 5–10 times faster than birds or amphibians (Prager and Wilson, 1975; Fitzpatrick, 2004). The rate at which mammals evolve post-zygotic RI depends on the structure of the placenta, possibly as a function of the scope of immunological interaction between mother and fetus (Elliot and Crespi, 2006).

THE FISH FAMILY POECILIIDAE

The Poeciliidae present special opportunities for learning about how conflict shapes evolution because of the diversity within and among species in male attributes associated with sexual selection, male mating strategies, and the diversity among species in the mode of maternal provisioning (Bisazza, 1993; Pollux et al., 2009, 2014). The males of some species have bright coloration, ornamentation and elaborate courtship behavior (Bisazza, 1993; Bisazza and Pilastro, 1997). The females of some species have matrotrophy in the form of the functional equivalent of a mammalian placenta (Turner, 1937, 1940; Wourms, 1981; Pollux et al., 2014; Olivera-Tlahuel et al., 2019). Furthermore, the Poeciliidae also vary in the presence or absence of superfetation, or the ability to carry multiple broods of young in different stages of development. Superfetation is of interest because its presence is highly correlated with the presence of placentation (Pollux et al., 2014). The VDCH predicts that the evolution of the mode of maternal provisioning will shape the evolution of male attributes associated with sexual selection. We have tested these predictions by characterizing male attributes and female mode of reproduction throughout the family, constructing a DNA-based family tree, then integrating these data to make inferences about the interdependencies between male and female evolution (Pollux et al., 2014; Furness et al., 2019). Specifically, we have asked:

- How often have male attributes associated with pre-copulatory sexual selection evolved?
- How often has the equivalent of a mammalian placenta evolved?
- How often has superfetation evolved?
- How often are these traits lost? Specifically, are there differences in the evolutionary lability of these male and female attributes? If the male attributes are the product of sexual conflict, then this means that there may be tension between natural and sexual selection, making the male attributes as likely to be lost as gained.
- Are there associations between the presence of male attributes associated with pre-copulatory sexual selection and the mode of maternal provisioning? The VDCH predicts that male attributes associated with sexual selection should be concentrated in lineages that lack placentas.
- Are there patterns in the order of evolution of male traits and the mode of maternal provisioning that suggest a cause-and-effect relationship? If maternal provisioning governs the evolution of male attributes, then male attributes associated with pre-copulatory sexual selection are expected to evolve in lineages that lack placentas and, furthermore, the evolution of the placenta should be associated with the subsequent loss of such male attributes if they had been present.
- Finally, is the evolution of a placenta associated with the accelerated evolution of post-copulatory reproductive isolation and speciation, as predicted by the VDCH? Addressing this question invokes deeper issues with regard to what form of reproductive isolation dominates in governing the rate of speciation. It has been argued that pre-copulatory reproductive isolation plays a more important role in speciation than does post-copulatory reproductive isolation (Coyne and Orr, 2004; Grant and Grant, 2008). It has also been argued that sexual selection can accelerate the rate of evolution of pre-copulatory reproductive isolation (Coyne and Orr, 2004). If this is true, then we might instead expect to see higher rates of speciation in lineages with enhanced male traits associated with sexual selection, which may also mean higher rates of speciation in lineages that lack placentas. The situation in this family thus provides the opportunity to discriminate among alternative hypotheses for what form of reproductive isolation governs the rate of speciation.

The summary of results that follows is derived from our prior publications. The female reproductive data on which these analyses are based are entirely our own, generated under a uniform protocol, rather than being derived from the literature. Male data were derived from measurements on preserved specimens, aquarium observations and the literature. Details can be found in the methods and supplementary materials associated with Pollux et al. (2014). In brief, we show that male attributes associated with sexual selection and the equivalent of the mammalian placenta have evolved multiple times within the family, that there is a strong association between the presence of these male attributes and the absence of placentation, and that the evolution of maternal provisioning appears to govern the evolution of male traits, as opposed to the other way around (Pollux et al., 2014; Furness et al., 2019). All of these results are consistent with predictions derived from the VDCH (Zeh and Zeh, 2000). Finally, we show, contrary to Zeh and Zeh's prediction, that it is the presence of enhanced sexual selection, rather than placentation, that is associated with accelerated rates of speciation (Furness et al., 2019).

Sexual Dimorphism

All species in this family are sexually dimorphic in terms of size and morphology, but there is immense variation in the scope and nature of sexual dimorphism (**Figure 1**) (Bisazza, 1993). In most species, males are smaller than females because male growth rate decelerates dramatically or even ceases at maturity while female growth trajectories continue to follow a normal, decelerating growth trajectory. In a small number of species the largest mature males can be larger than females (Pollux et al., 2014). These are also species in which the males are highly ornamented and have elaborate courtship.

The sexes differ in shape because the male anal fin metamorphoses into the gonopodium, or intromittent organ. The anal fin is fan-shaped in females and immature males. As males mature, the third through fifth rays of the anal fin become elongated, the fin stiffens and hooks, barbs and other species-specific morphological features develop at the tip of the gonopodium (**Figure 2**). At the same time, males develop the gonapodial suspensorium, which is a complex of bones and muscles in the abdomen associated with the mobility of the gonopodium. Males are able to rotate the gonopodium by up to 180 degrees so that it points toward their heads, rather than pointing toward the rear of the body. This change in position enables males to approach and inseminate the female from behind. The body shapes of the males of some species with the longest gonopodia appear modified in a fashion that shifts the insertion of the gonopodium toward the head. It appears that this shift plus the elongation of the gonopodium enables males to see the tip when the gonopodium is rotated 180 degrees to point anteriorly, which could in turn enable them to aim for the females gonopore as they approach them from behind (**Figure 3**) (Greven, 2005). Females develop an enlarged, unpaired ovary that becomes the site in which offspring develop. The differences between the confinement of the profile of the abdomen by the gonapodial suspensorium vs. the expansion of the abdomen in association with pregnancy creates a difference in the overall shape of males and females (Rosen and Bailey, 1963; Greven, 2005).

Variation Among Males and Associated Female Mating Preferences

In many species, males lack attributes associated with sexual selection. They have the same color pattern as females, lack any form of ornamentation and lack courtship. They tend to have moderately long gonopodia (30+% of the standard body length) and are much smaller than females. In a relatively small subset of species, males develop ornamentation in the form of enlarged dorsal fins, laterally compressed bodies and/or the lengthening of a subset of the caudal fin rays to form a "sword" (**Figure 4**). Some species also have sexual dichromatism, with males being

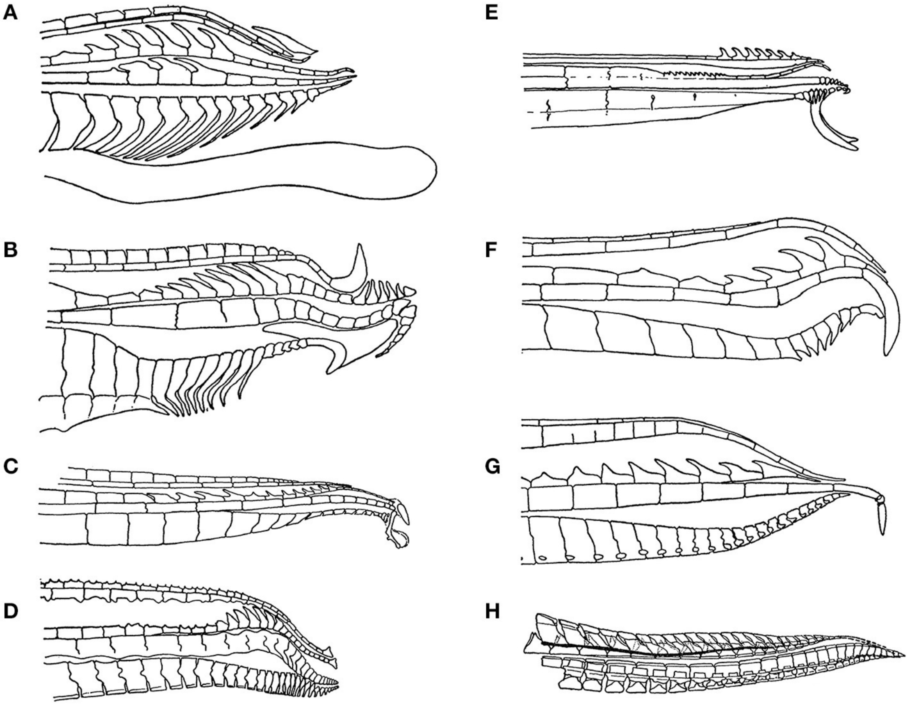

FIGURE 2 | The tip of the male gonopodium, the intromittent organ, exhibits a great deal of variation across the family Poecillidae: **(A)** *Poecilia reticulata*, **(B)** *Xiphophorus helleri*, **(C)** *Phalloceros caudimaculatus*, **(D)** *Belonesox belizanus*, **(E)** *Girardinus cubensis*, **(F)** *Neoheterandria elegans*, **(G)** *Heterandria formosa*, and **(H)** *Poeciliopsis elongata*. Figures redrawn from Rosen and Bailey (1963).

much more brightly colored than females (**Figure 5**). Amongst species, the length of the gonopodium ranges from 15 to nearly 50% of the standard length of the body (**Figure 6**). Langerhans (2011) found, on the basis of a phylogenetic analysis, that genera in which males typically had short gonopodia were more likely to exhibit courtship displays.

Species also vary in mating strategies. Most species lack courtship. Males instead approach females from behind and attempt copulation (**Figure 7**). Some species have elaborate courtship displays, but males of these species often employ alternative reproductive tactics, meaning they may either court or attempt forced copulation. When mature males vary in size, larger males tend to deploy courtship displays while smaller males instead attempt forced copulations (Erbelding-Denk et al., 1994; Rios-Cardenas and Morris, 2011).

Female mating preferences have been studied in many of these species. In some cases, absolute preference is measured by quantifying the attention given by a female to an individual male (Basolo, 1998), while relative preference is measured by offering females dichotomous choices between live males (Ryan and Wagner, 1987; Erbelding-Denk et al., 1994; Basolo, 1995; Ptacek and Travis, 1997; Morris, 1998), video projections of different male phenotypes (Trainor and Basolo, 2000), or dummies representing different male phenotypes (MacLaren et al., 2004). Relative preference can also be measured by quantifying association patterns among individuals in free-swimming groups (Houde, 1987, 1988b). Despite differences in methods, these studies have demonstrated consistently that females prefer larger, more elaborately ornamented, and more colorful males. Female preferences are strongest when different males are presented simultaneously to a female; when the choices are presented sequentially, the preference for the larger, more ornamental male remains but is much weaker (MacLaren and Rowland, 2006).

Do these preferred males sire more offspring under realistic situations when males can interact with each other as well as with females? Several studies have answered "yes" (Houde, 1988a; Ryan et al., 1990; Kodric-Brown, 1993; Morris et al., 2010). In *Limia perguiae,* large, preferred males sired all of the offspring in trials in which they were paired with a smaller male but sired no offspring when placed in a group with three smaller males and six females (Erbelding-Denk et al., 1994). It is difficult to interpret these contradictory results because there were few details of the experiment provided in Erbelding-Denk et al. (1994). In particular, it is unclear whether any or all of those females were receptive; male behaviors and interactions among males change dramatically when females are receptive (Farr and Travis, 1986) and only receptive females make clear, decisive choices among males (Ptacek and Travis, 1997). Two studies suggest that preferred males do not always have more reproductive success than other males (Bisazza and Pilastro, 1997; Asphury and Basolo, 2002). However, these studies quantified only copulations, not the siring of offspring, and both studies focused on species without courtship or male ornamentation.

FIGURE 3 | Examples of male mating behavior in different poeciliid species. Courtship in **(A)** *Girardinus metallicus* and **(B)** *Limia nigrofasciata*. **(C)** A male *Heterandria formosa* approaches a female from behind, in this non-courting species. Mating attempts in the form of gonopodial thrusts in **(D)** *Poecilia parae*, **(E)** *Phallichthys quadripuctatus*, and **(F)** *Poeciliopsis occidentalis*. Photo credits: **(A)** https://acquariofiliaconsapevole.info/pesci_girardinus_metallicus, **(B)** Katie Holz, **(C)** TFH Archives, **(D)** Godfrey Bourne, www.discoverlife.org, **(E)** http://aquaria.palo-alto.ca.us/, **(F)** Dennis Caldwell.

A number of additional studies have examined female preferences in species without courtship (McPeek, 1992; Basolo, 1998; Bisazza and Pilastro, 2000; MacLaren and Rowland, 2006; Casner et al., 2016). In nearly every case, females prefer a larger, more ornamented male, even when that male is not the same species as the female (Basolo, 1998). Several of these authors have interpreted these results to indicate a pre-existing preference for larger size and ornamentation that males in species with courtship have evolved to exploit.

The size distribution of mature males of many species is often polymodal, highly skewed, or widely variable (Farr et al., 1986; Kolluru and Reznick, 1996; Ptacek and Travis, 1996; Arias and Reznick, 2000; Reznick et al., 2007a; Regus et al., 2013; Cohen et al., 2015; Pires and Reznick, 2018). While this size variation has been associated with species in which males are highly ornamented and different sized males deploy different mating strategies (Zimmerer and Kallman, 1989; Rios-Cardenas and Morris, 2011), our comparative analyses reveal that such variation in male size at maturity is ancestral in the family Poeciliidae (unpublished observation). It is commonly seen in species that lack courtship behavior. In a few species, investigators have found that there is a genetic basis for differences in male size at maturity. The strongest research on the genetics of male size at maturity has been applied to species in the genus *Xiphophorus* which have a well-described P-locus with alternative alleles that determine age and size at maturity (Kallman, 1989; Lampert et al., 2010). An analogous system of Y-linked inheritance has been reported in sailfin mollies (Travis, 1994).

Variation Among Females

The diversity we see among species in male attributes associated with mating is matched by diversity in female reproductive attributes. The ancestral mode of reproduction is most likely egg retention, meaning that eggs are fully provisioned before fertilization then retained by the female throughout development (Furness et al., 2019). This is the mode of maternl provisioning called "lecithotrophy," or yolk feeding. From this starting

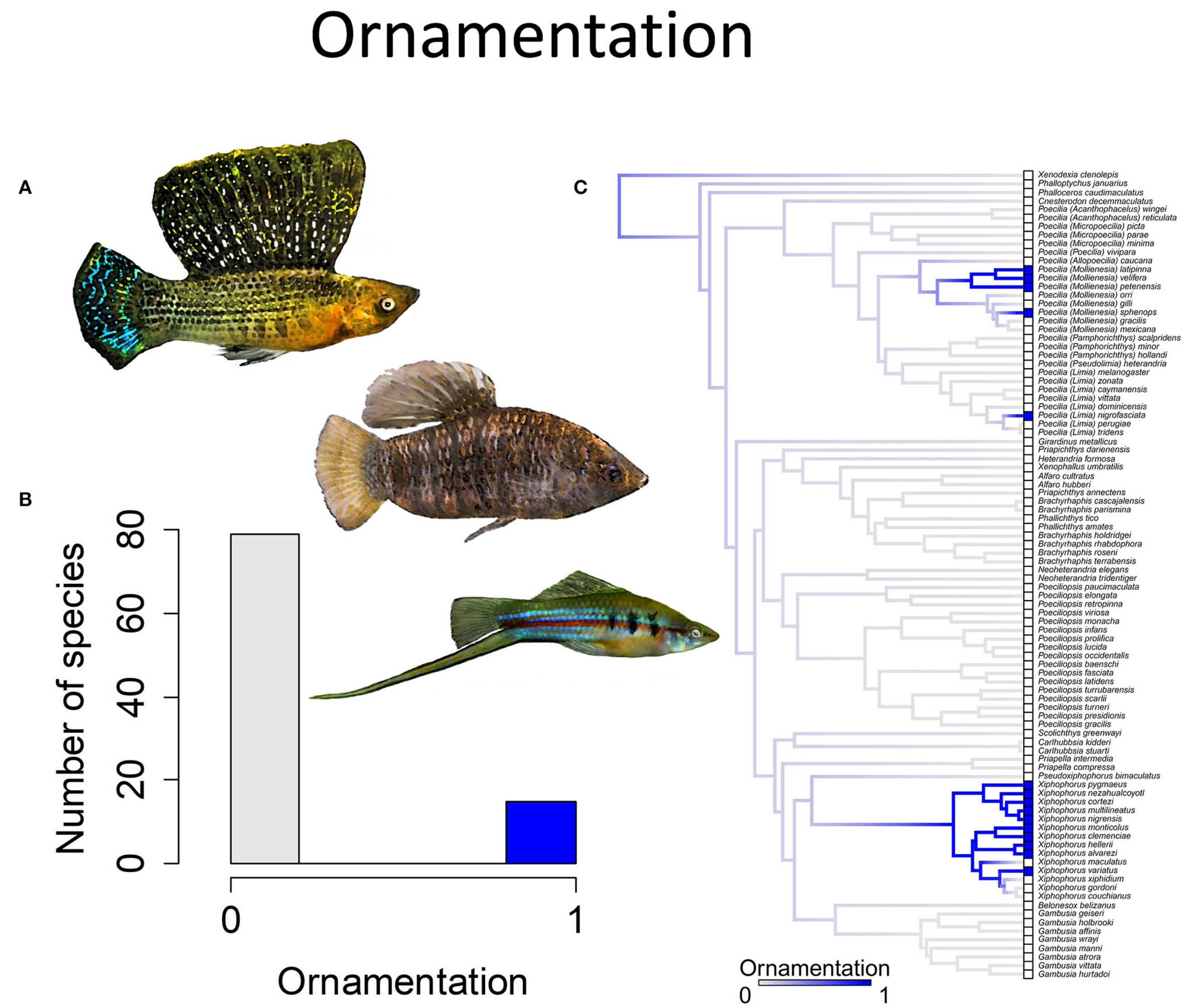

FIGURE 4 | Ornamentation in the family Poeciliidae is when males exhibit ornamental display traits such as **(A)** a sail-like dorsal fin, as depicted in *Poecilia velifera* (top) and *Limia nigrofasciata* (middle), or a sword-like extension of the caudal fin, as depicted in *Xiphophorus hellerii* (bottom). **(B)** Frequency histogram of presence or absence of male ornamentation (*n* = 94). **(C)** Ancestral state reconstruction of ornamentation as a binary character was performed using stochastic character mapping in the R package phytools. Branch colors represent posterior densities of edge states based on 1000 stochastic character maps of each reconstruction. The phylogeny tips depict a binary categorization of ornamentation (i.e., absence vs. presence). Figure redrawn after Furness et al. (2019). The most likely ancestral state of the family is the absence of ornamentation (Furness et al., 2019).

point, some species have acquired two novelties—matrotrophy (placentation) and superfetation.

Morrison et al. (2017) characterized the properties of the eggs of egg-laying Cyprinodont killifish (*Nothobranchius fuerzi, Rivulus hartii, Cyprinodon variegatus*, plus the Atherinomorpha *Oryzias latipes*) in the same order as the livebearing poeciliids, in an effort to illuminate the transition from egg laying to egg retention. They found that the egg that was internalized at the inception of livebearing probably already had limited capacity to acquire resources from its mother. They showed that eggs can concentrate radiolabeled amino acids against a concentration gradient, but also that they can absorb fluorescent microspheres. The totality of their results suggest that these substances were acquired from the surrounding medium via a non-specific form of active transport like pinocytosis. If the egg internalized by the ancestral poeciliid shared these properties it means that, from the inception of livebearing, developing embryos had the capacity to acquire resources from their mother. This capacity sets the stage for conflict between embryo acquisition and maternal allocation. As such, it may represent a "preadaptation" for the evolution of matrotrophy as a product of the escalation of this conflict over resource allocation. Since the eggs of other distantly related fish species have been found to have the same ability to acquire small organic molecules from their surroundings (Terner, 1968; Siebers

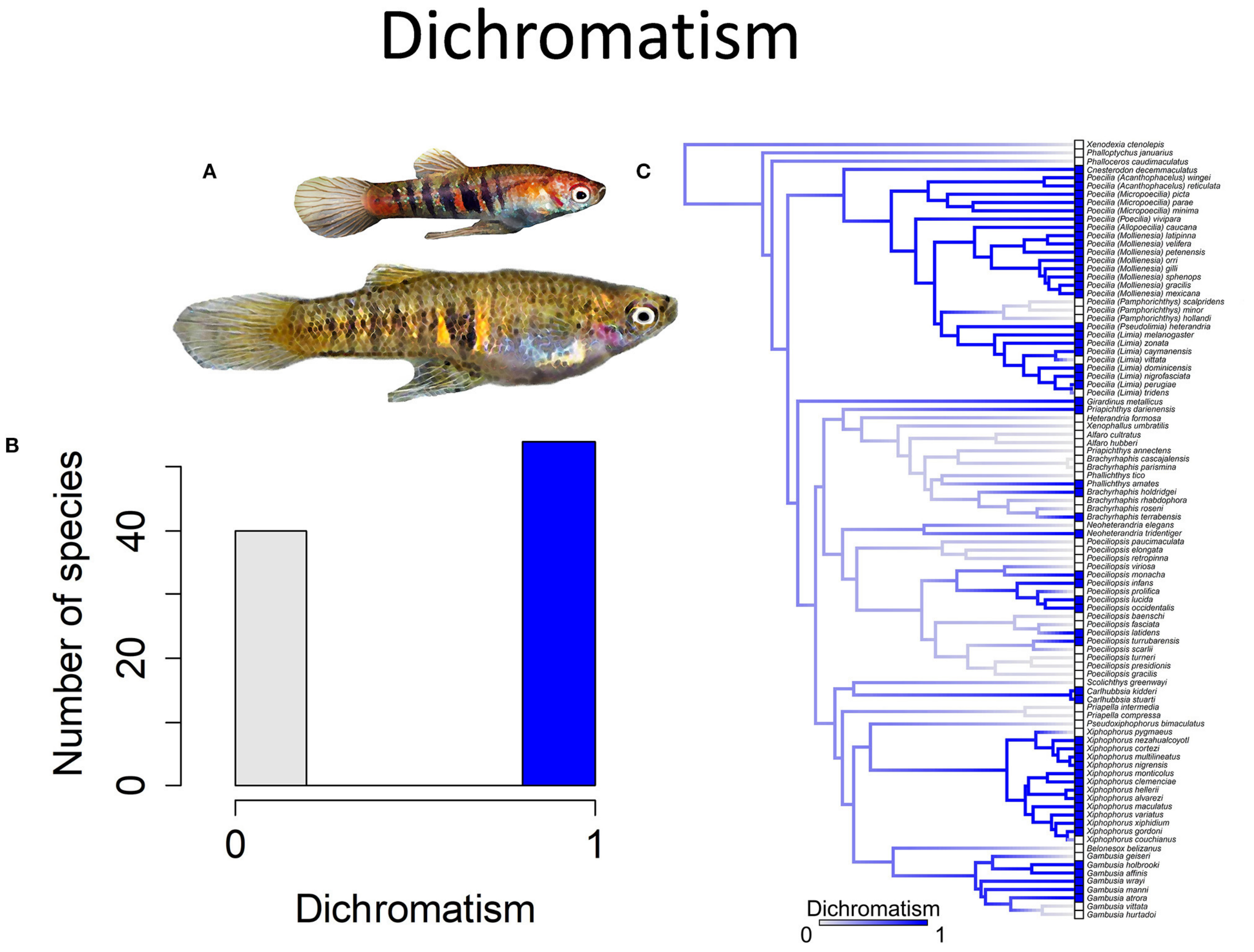

FIGURE 5 | Sexually dichromatic coloration in the family Poeciliidae is when males exhibit brighter and more colorful patterns than females; the alternative is sexually monochromatic coloration in which males and females exhibit similar coloration patterns and intensity. **(A)** *Neoheterandria elegans*, exhibits dichromatism with males more brightly colored than females (pictured). **(B)** Frequency histogram of presence or absence of dichromatism (*n* = 94). **(C)** Ancestral state reconstruction of dichromatism as a binary character was performed using stochastic character mapping in the R package phytools. Branch colors represent posterior densities of edge states based on 1000 stochastic character maps of each reconstruction. The phylogeny tips depict a binary categorization of dichromatism (i.e., absence vs. presence). Figure redrawn after Furness et al. (2019). The most likely ancestral state of the family is the absence of dichromatism (Furness et al., 2019).

and Rosenthal, 1977) this property may explain why matrotrophy has evolved much more readily in fish than in amniotes; 12 of the 14 lineages of bony fishes that have evolved livebearing also evolved matrotrophy, as compared to only 2–4 of the 115 lineages of squamates that evolved livebearing (Blackburn, 2015).

These results may also explain those of earlier investigators who injected radiolabeled amino acids into pregnant females of lecithotrophic species of Poeciliidae (Marsh-Matthews et al., 2001, 2005; Marsh-Matthews and Deaton, 2006; Riesch et al., 2010). These authors found that the label appeared in developing embryos within hours of the injection. They interpreted the result as evidence for the presence of matrotrophy. Morrison et al. (2017) found that the killifish eggs acquired nutrients from the surrounding environment at a comparable rate to lecithotrophic poeciliid embryos developing inside their mother. By our interpretation, rather than demonstrating matrotrophy, these investigators were instead characterizing a form of active transport retained from the egg-laying ancestors of the Poeciliidae.

Inferences about how mothers provision their young are derived from the patterns of change in the dry mass of the young during development. This pattern of weight change is characterized with the "matrotrophy index" (MI), or the ratio of the estimated mass of offspring at birth divided by the estimated mass of the egg at fertilization. Lecithotrophic species have embryos that lose dry mass over the course of development. The embryos of matrotrophic species instead gain dry mass during development. The modal value for MI in the family is ∼0.7 (Pollux et al., 2009), implying that the embryos lose 30% of their dry mass during the course of development. This is comparable to the mass difference between a newly laid bird egg and the mass of the hatching that emerges (Sotherland and Rahn, 1987), hence

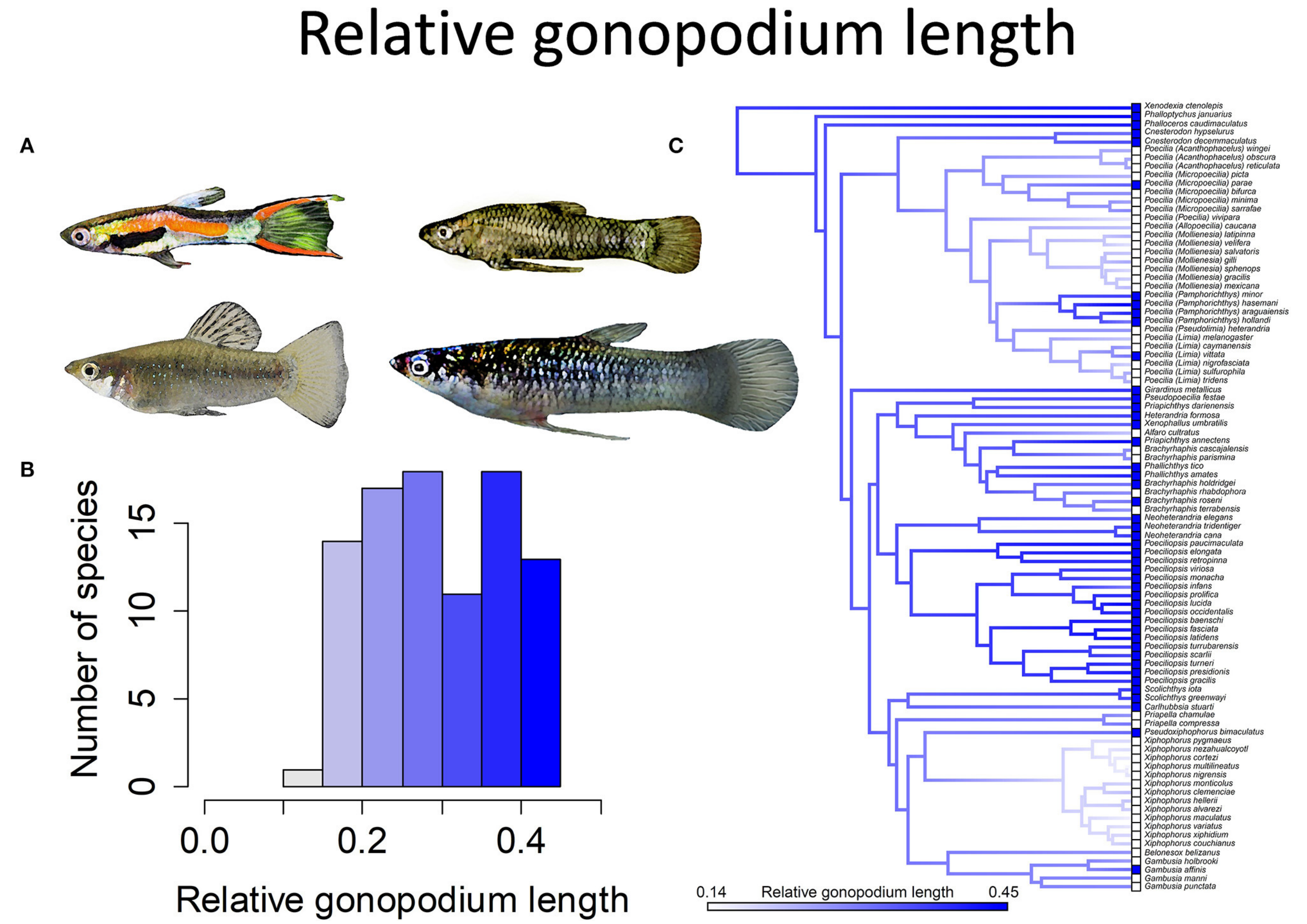

FIGURE 6 | Relative gonopodium length in the family Poeciliidae ranges from 14 to 45% of male standard length. **(A)** Males of *Poecilia wingei* and *Limia perugiae* (pictured left) have relatively short gonopodia while males of *Phalloptychus Januarius* and *Poeciliopsis elongata* (pictured right) have relatively long gonopodia. **(B)** Frequency histogram of male gonopodium length ($n = 92$). **(C)** Ancestral state reconstruction of relative gonopodium length as a continuous character using the contMap function in the R package phytools, which estimates the Maximum likelihood ancestral states at all internal nodes and interpolates the states along each edge. The phylogeny tips depict a binary categorization of relative gonopodium length (i.e., short vs. long). The most likely ancestral state of the family, when treated as a binary character, is that of a long gonopodium (Furness et al., 2019).

the inference that it represents eggs that were fully provisioned prior to fertilization. There is then a continuous distribution of greater MI values, with the tail of the distribution extending to values of >100, which means that the mass of the offspring at birth is more than 100 times the mass of the egg at fertilization (**Figure 8**).

A second type of variation is in the presence or absence of superfetation. Most species lack superfetation, which means that they produce a single batch of eggs that is fertilized, then embryos develop and are born together (**Figure 9**). The process then begins again, with the yolking and fertilization of a new batch of eggs. Females of such species usually give birth every 3–4 weeks. Species with superfetation have the capacity to carry multiple broods of developing young in different stages of development (Pollux et al., 2009). Such species initiate a litter of developing young, then yolk a new batch of eggs and initiate the next litter before the previous litter is born (Turner, 1937). Species and populations within species vary in how many litters they carry at one time, ranging from as few as two to up to 8 litters at a time (Turner, 1937). A consequence of superfetation is that females give birth more frequently but to fewer young at a time. Species that typically have two developing litters at a time will give birth every 12–14 days. Those that typically have seven or eight developing litters at a time will give birth every 2 or 3 days (Turner, 1937; Pollux and Reznick, 2011).

CHRONOLOGY OF EVOLUTION AND THE SIGNATURE OF CONFLICT

Trends in Male and Female Evolution

Our next step was to integrate our comparative data on males and females with a well-resolved DNA-based family tree (Pollux et al., 2014; Reznick et al., 2017), enabling us to make inferences about the evolution of male and female traits

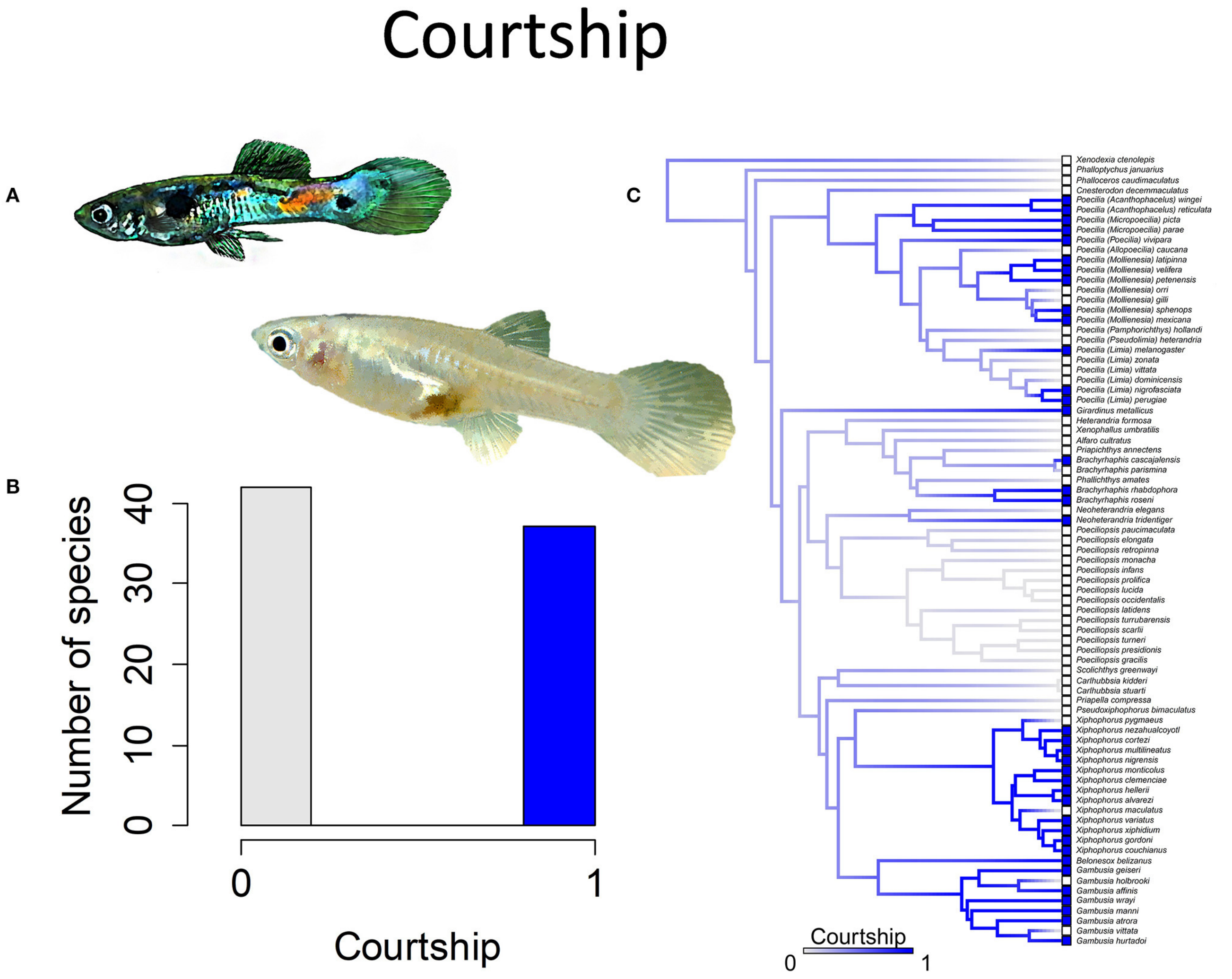

FIGURE 7 | Courtship in the family Poeciliidae involves males displaying for females by spreading their unpaired fins and is often accompanied by stereotypical movements such as a sigmoid posture; the alternative to courtship is coercive or sneak-chase mating. **(A)** A male guppy, *Poecilia reticulata*, displays for a female (pictured) to elicit cooperation during mating. **(B)** Frequency histogram of presence or absence of male courtship ($n = 79$). **(C)** Ancestral state reconstruction of courtship as a binary character was performed using stochastic character mapping in the R package phytools. Branch colors represent posterior densities of edge states based on 1000 stochastic character maps of each reconstruction. The phylogeny tips depict a binary categorization of courtship (i.e., absence vs. presence). Figure redrawn after Furness et al. (2019). The most likely ancestral state of the family is the absence of courtship (Furness et al., 2019).

(Furness et al., 2019). The most likely condition of males in the common ancestor of the family is to lack sexual dichromatism, ornamentation and courtship (**Figures 4**, **5**, **7**). The males were likely to have been much smaller than females (**Figure 1**) and the gonopodium was likely to have been relatively long (**Figure 6**). Courtship, dichromatism and male ornamentation proved to be evolutionarily labile; they were as likely to be lost as gained throughout the family tree (Furness et al., 2019). For example, phylogenetic analyses reveal that *Poecilia latipunctata* lost the enlarged dorsal fin that characterizes the rest of its clade, but retained courtship behavior (Ptacek and Breden, 1998; Ptacek et al., 2005). *Xiphophorus continens* has lost the sword, coloration, and courtship behavior typical of its clade (Morris et al., 2005). More generally, phylogenetic analyses reveal that the sword, typical of the genus *Xiphophorus*, was ancestral to the genus as a whole then repeatedly gained and lost throughout the diversification of the genus (Meyer et al., 1994).

The females of the common ancestor of the family likely had internal fertilization, bore live young, were lecithotrophic and lacked superfetation (Furness et al., 2019). Superfetation evolved four times and was lost once. The loss was in the common ancestor of a clade of Central American species, including the genera *Brachyraphis*, *Phallichthys*, and *Alfaro* (**Figure 9**). Placentotrophy evolved nine times and, once gained, was never lost (**Figure 8**). The independent origins of the placenta are *Xenodexia*, *Phalloptychus*, *Phalloceros*, *Heterandria formosa*, the *Micropoecilia* and *Pamphorichthys* subclades of *Poecilia*, and three times within the genus *Poeciliopsis*. There are

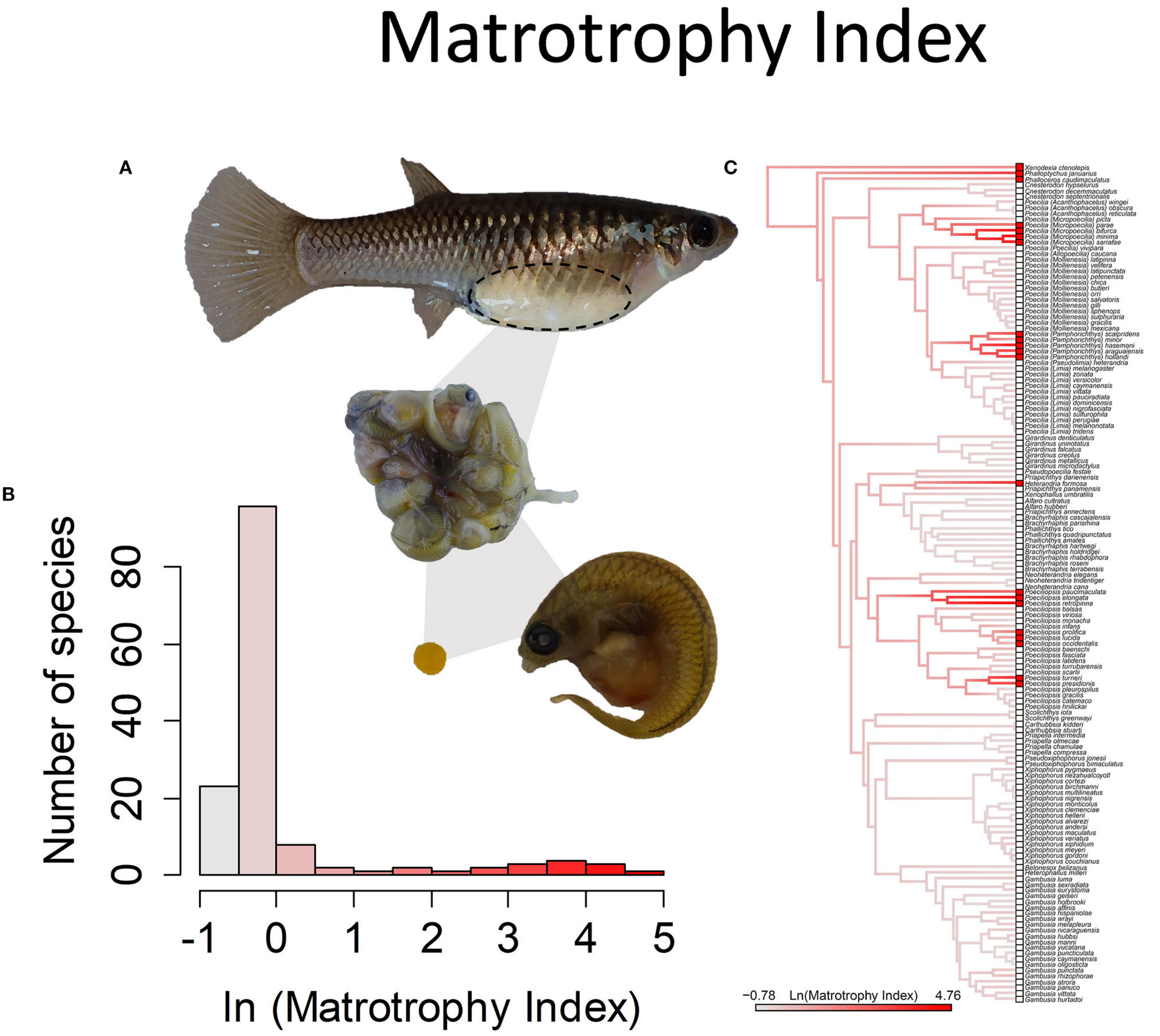

FIGURE 8 | The Matrotrophy Index, measured as the ratio of offspring dry mass at birth to that of a just fertilized embryo, is a unitless measure of the degree of post-fertilization maternal embryo provisioning. In the family Poeciliiidae, this value ranges from 0.46 to 117. Values <1 indicate embryos lose mass over the course of gestation and are considered lecithotrophic or "yolk feeding" (a value of 0.7 represents the loss of mass in egg-laying species), and larger values indicate matrotrophy or "mother feeding." **(A)** *Poeciliopsis retropinna* is a highly matrotrophic species (Reznick et al., 2002, Hagmayer et al., 2018, Hagmayer et al., 2020). Over the course of gestation in the ovary (pictured), embryos undergo an up to 117-fold increase in dry weight, due to nutrient provisioning by means of a follicular placenta. **(B)** Frequency histogram of the natural log transformed Matrotrophy Index ($n = 146$). **(C)** Ancestral state reconstruction of the Matrotrophy Index as a continuous character using the contMap function in the R package phytools, which estimates the Maximum likelihood ancestral states at all internal nodes and interpolates the states along each edge. The phylogeny tips depict a binary categorization of the placenta (i.e., absent vs. present). Please note, the reconstruction depicted here omits a basal lineage, *Tomeurus gracilis*, the sole non-livebearing member of the family, as well as non-livebearing outgroup taxa. A reconstruction of placentation as a binary presence or absence character, with the inclusion of these taxa, suggests the most likely ancestral state of the family is to lack placentation (Furness et al., 2019).

also hints of evolving matrotrophy elsewhere in the family. For example, we have observed matrotrophy indices of between 1 and 2 in *Gambusia rhizophorae* (unpublished obsvn.). Zuniga-Vega et al. (2011) similarly observed matrophy indices that ranged from 1 to 5.8 in different populations of Poecilia butleri. Both species are from clades in which all other species are lecithotrophic.

Superfetation is almost always associated with matrotrophy, so much so that it was once suggested that they were different facets of the same adaptation (Constanz, 1989).

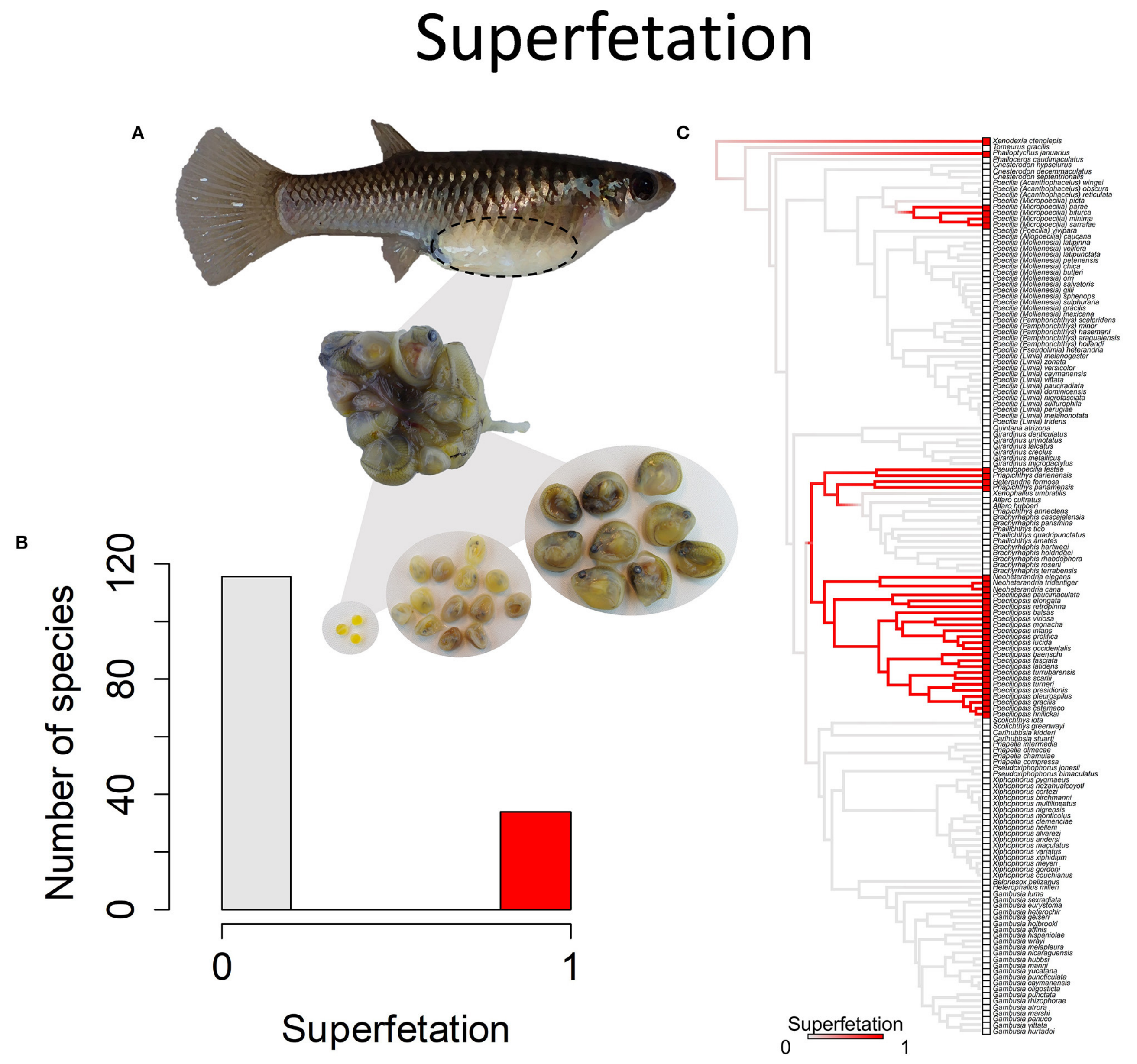

FIGURE 9 | Superfetation refers to the capacity of females to gestate more than one brood of offspring at different stages of development. **(A)** *Poeciliopsis retropinna* exhibits superfetation (Hagmayer et al., 2018, Hagmayer et al., 2020). Broods at different stages of development (pictured) are simultaneously gestated in the ovary. **(B)** Frequency histogram of presence or absence of superfetation (*n* = 150). **(C)** Ancestral state reconstruction of superfetation as a binary character was performed using stochastic character mapping in the R package phytools. Branch colors represent posterior densities of edge states based on 1000 stochastic character maps of each reconstruction. The phylogeny tips depict a binary categorization of courtship (i.e., absence vs. presence). Figure redrawn after Furness et al. (2019). The most likely ancestral state of the family is the absence of superfetation, a pattern which becomes clearer with the inclusion of additional outgroup taxa (Furness et al., 2019).

The phylogenetic reconstructions reveal all possible orders of origin of superfetation and placentotrophy (Furness et al., 2019). Superfetation evolved before placentotrophy four times, placentotrophy and superfetation appear together three times, and placentotrophy evolved twice before superfetation. Superfetation appears before matrotrophy in the common ancestor of *Neoheterandria* and *Poeciliopsis*. Matrotrophy evolves later, at least three different times, within *Poeciliopsis*, but all other species within these two genera have superfetation without matrotrophy. The common ancestors of *Phalloceros* and *Pamphorichthys* had placentotrophy without superfetation, but superfetation has recently been seen in one population each of *Phalloceros harpagos* (Gorini-Pacheco et al., 2018) and *Pamphorichthys hasemani* (Pires and Reznick, 2018). The inclusion of these species and new information would bring the number of origins of superfetation in the family to six, and likely

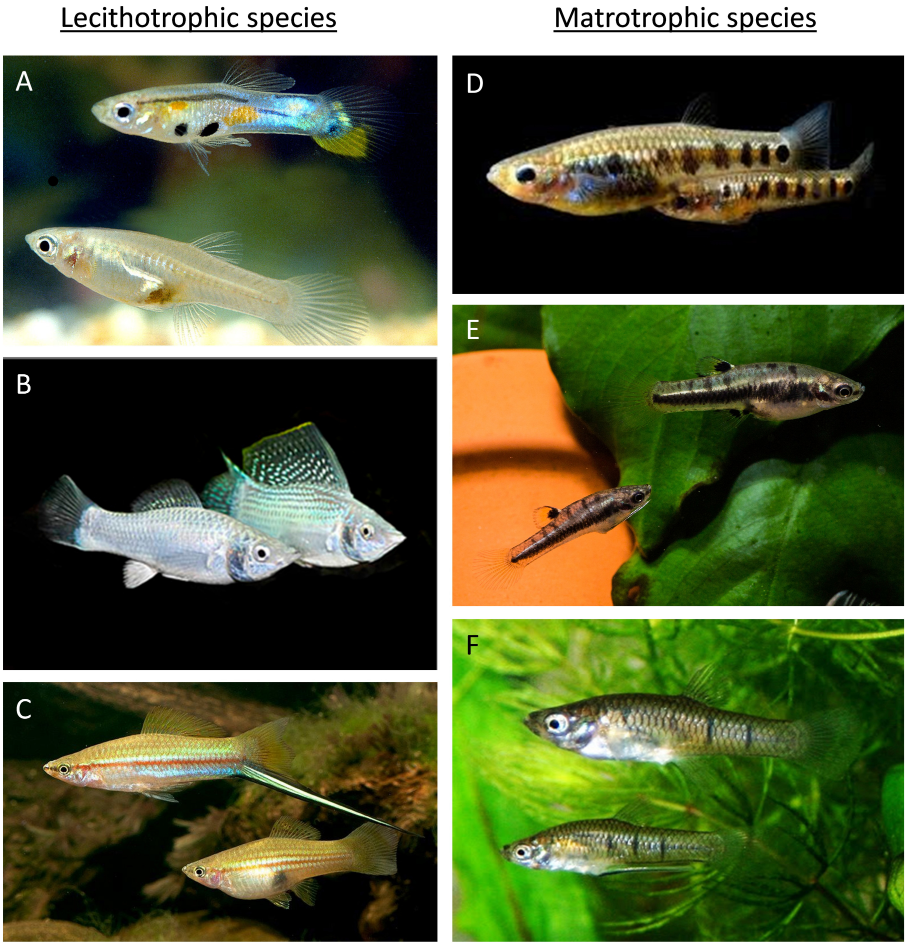

FIGURE 10 | Lecithotrophic species include many popular in aquariums such as the sexually dichromatic guppy, *Poecilia reticulata* **(A)**, the sailfin molly, *Poecilia latipinna* **(B)**, and the swordtail, *Xiphophorus helleri* **(C)**. Males of the latter two species also exhibit ornamental display traits in the form of a sail-like dorsal fin, and sword-like caudal fin. Males of all three species exhibit courtship and tend to have short gonopodia. In contrast, males of matrotrophic species such as **(D)** *Poeciliopsis turneri*, **(E)** *Heterandria formosa*, and **(F)** *Phalloptychus juanarius* tend to have sexually monochromatic coloration, lack ornamental display traits, and exhibit sneak-chase mating behavior rather than courtship. Furthermore, males in these species have long gonopodia and are considerably smaller than females. Photo credits: **(A)** Wikimedia Commons, **(B)** Johnny Jensen, JJPhoto.dk, **(C)** http://scontent-lhr3-1.xx.fbcdn.net, **(D)** aquafisher.org.ua, **(E)** Chiara Sciarone, **(F)** http://leo-aquarium.blogspot.com/.

strengthen the already significant association between these two traits.

Why do we see this strong association between superfetation and matrotrophy? It is possible that superfetation promotes polyandry. Superfetation is known to promote polyandry in some mammals because females can re-mate each time they ovulate, so sequential litters can be sired by different males (Shackelford, 1952; Yamaguchi et al., 2004). If the same is true of the poeciliids, meaning that if females were inclined to mate after each time they gave birth, then it follows that superfetation may become associated with plancentotrophy because it would contribute to Zeh and Zeh (2000) prediction that matrotrophy favors polyandry (Pollux et al., 2014).

In conclusion, the diversity of male attributes in the family represents multiple independent gains and losses of the traits associated with sexual selection. Likewise, the placenta and superfetation have evolved repeatedly throughout the family. The multiple, independent origins of male and female traits gives us the statistical power to test whether the evolution of male and female attributes are associated with each other. The availability of a well-resolved fossil-calibrated phylogenetic tree (Pollux et al., 2014) also enables us to overlay the evolution of male and female traits, then address the order of evolution and hence make inferences about the causal relationships between the evolution of male traits and female mode of reproduction.

Associations Between Male and Female Attributes

The VDCH predicts that females of species that lack placentas will be choosier about who they mate with, making it more likely that the males of these species will have bright coloration, ornamentation, and courtship (Pollux et al., 2014). We also expected that the evolution of elaborate male displays will be accompanied by an increase in male body size since size so often plays a role in female choice or in male-male combat for access to females (Rios-Cardenas and Morris, 2011). If mating is cooperative, then selection may also favor the evolution of shorter gonopodia. Conversely, if females are placental, then the VDCH predicts that females will tend to mate with multiple males then make a post-copulatory choice of who sires her offspring. The prediction is thus that males will not experience the sort of pre-copulatory mate choice that favors the evolution of traits like bright coloration, ornamentation or courtship. Selection should instead favor the enhancement of traits associated with sneaky, forced copulation, such as the evolution of longer gonopodia and smaller male body size.

Selection could also favor the evolution of sperm attributes, such as greater sperm longevity, higher sperm velocity, and perhaps attributes of semen that promote fertility, as has been described in *Drosophila* (e.g., Swanson et al., 2001). However, some of these characteristics could also be favored by selection in non-placental species for males to subvert the effects of female choice. There is evidence that sperm characteristics can be associated with other sexually selected features in non-placental species (Pilastro et al., 2004; Locatello et al., 2006). This does not falsify the prediction from the VDCH; the prediction is that the major focus of selection in non-placental species is pre-copulatory while the major focus in placental species is postcopulatory. There is, to date, only a single study in a single species (guppies–*Poecilia reticulata*) that has attempted to parse the opportunity for sexual selection into pre- and post-copulatory phases (Devigili et al., 2015). Thus, whether sperm or components of semen evolve to circumvent female choice or simply via male-male competition is an interesting question, as is the question of whether the answer differs between placental and non-placental species.

Our comparative phylogenetic analyses support all of the predictions of the VDCH. Species with placentas have males that are smaller relative to females, have longer gonopodia and are less likely to have bright coloration, ornamentation, and courtship behavior than species that lack placentas (Pollux et al., 2014) (**Figure 10**).

The Evolution of Maternal Provisioning Governs the Evolution of Male Attributes

We next considered the joint evolution and possible inter-dependence of the evolution of male and female attributes (Furness et al., 2019). If the absence of placentation favors the evolution of male traits associated with pre-copulatory mate choice, then these traits should be significantly more likely to evolve in lineages that lack placentas than in lineages that have them. We found significant interdependence in male and female evolution for three of the five male traits associated with sexual selection—the presence or absence of courtship, sexual dichromatism and sexual size dimorphism. In all three cases, these traits were more likely to evolve when placentas were absent than when they were present, as predicted. In contrast, we did not find support for the evolution of maternal provisioning conditioned on male traits (Furness et al., 2019).

Furthermore, male traits associated with sexual selection were also lost at higher rates in lineages that lack placentas (Furness et al., 2019). An inherent feature of conflict is that the fitness of one sex is gained at a cost to the opposite sex (Rice, 1996), so sustaining the trait requires a balance between the two. Male traits that evolve under such circumstances should be less stable than traits that evolve under an alternative form of sexual selection like the good genes hypothesis, under which both sexes benefit. This elevated rate of gain and loss of male traits associated with sexual selection in lineages that lack placentas is illustrated in Figure 4 of Furness et al. (2019).

Conflict between sexual and natural selection is a second source of the evolutionary lability (accelerated gains and losses) of male traits associated with sexual selection. Traits associated with sexual selection are often costly; e.g., the bright coloration of male guppies attracts females, but also attracts the unwanted attention of predators (Endler, 1984). The contribution of these traits to fitness thus represents a balance between the costs and benefits associated with them. A tilt in that balance in favor of natural selection could result in net selection against such traits.

To summarize, species with placental reproduction tend to retain the ancestral state of males for the family, meaning that the males tend to lack sexual dichromatism, ornamentation, and courtship, be small relative to females and to have long gonopodia (**Figure 10**). Male traits associated with sexual selection evolved within lineages that lacked placentas. **Figure 11** illustrates the complementary nature of male and female evolution in the form of mirrored phylogenetic trees of the evolution of male attributes with the evolution of placentation in females.

Macroevolutionary Consequences of Conflict—Rate of Speciation

Zeh and Zeh (2000) predict that post-zygotic reproduction isolation will evolve more rapidly in viviparous than oviparous species because development of the embryo within the mother creates a physiological arena for genomic conflict absent in species that lay eggs (Zeh and Zeh, 2000). Reproductive isolation may evolve among separate populations as a byproduct of differences between them in how conflict is resolved. Evidence for this hypothesis includes the demonstration that oviparous (spawning or egg laying) species pairs retain the capacity to produce viable hybrids tens of millions of years following divergence, an order of magnitude longer than for many viviparous (live-bearing) species (Zeh and Zeh, 2000). Vrana (2007) details a specific mechanism in crosses among closely related species of *Peromyscus* mice that differ in the intensity of conflict because one is monogamous and the other polygamous. Hybrids experience offspring growth dysregulation caused by disrupted placental function and genomic imprinting. This results in severe offspring under- or over growth depending on the direction of the reciprocal cross. There is thus a direct link between placental reproduction and post-zygotic reproductive isolation. Since reproductive isolation is the hallmark of the biological species concept, a natural extension is that

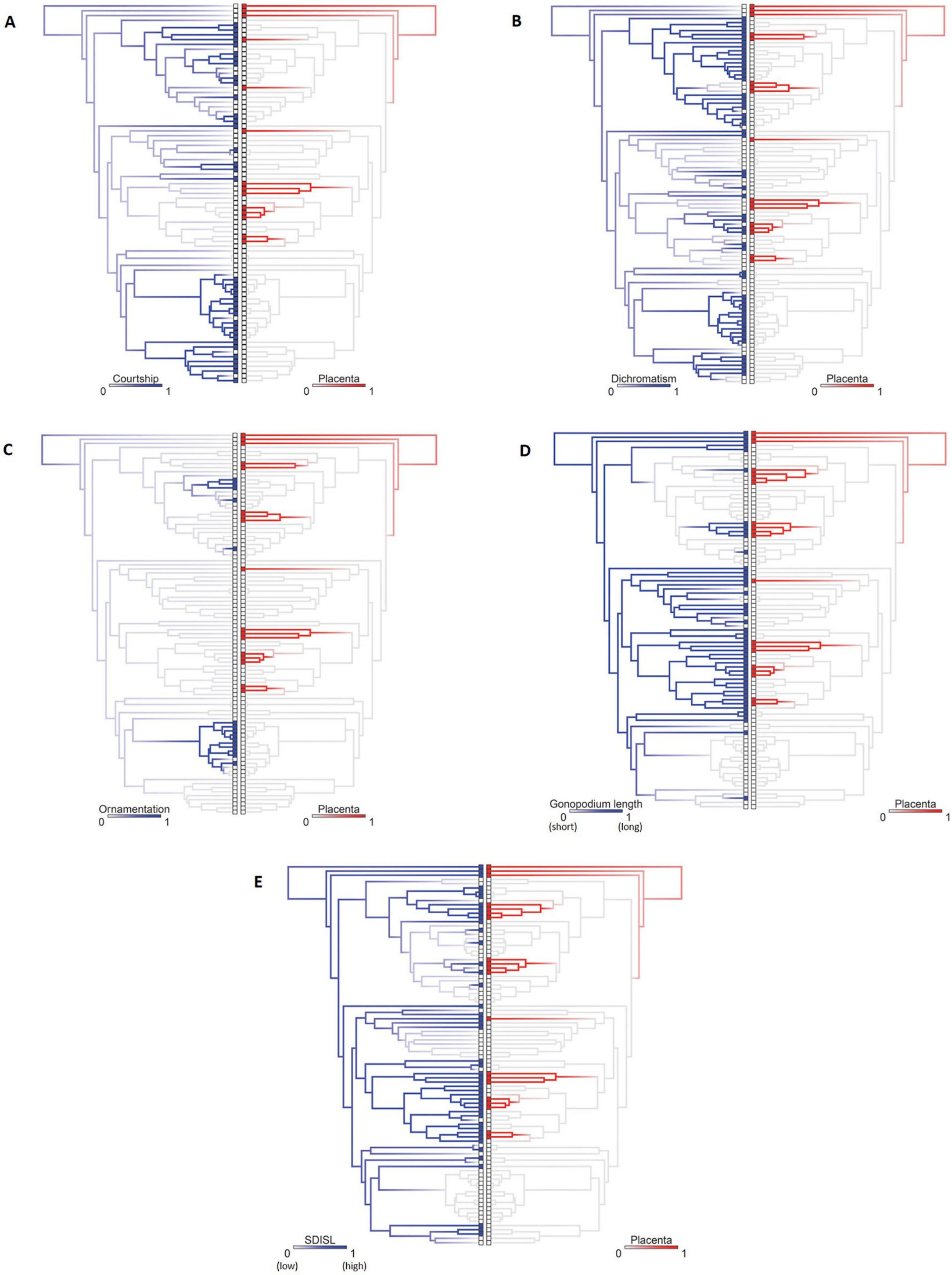

FIGURE 11 | Mirrored trees illustrating potential correlated evolution between **(A)** courtship, **(B)** dichromatism, **(C)** ornamentation, **(D)** relative gonopodium length, and **(E)** sexual dimorphism index (standard length) and the placenta. Ancestral state reconstructions of both binary traits by stochastic character mapping. Phylogeny restricted to species with both traits. Branch colors represent posterior probability densities of edge states based on 1000 stochastic character maps of each econstruction. BayesTraits Discrete analyses support dependent (i.e., correlated) evolution between placentation and the absence of courtship, the absence of sexual dichromatism and increased sexual size dimorphism (males much smaller than females) but not with absence of ornamentation or long relative gonopodium length (Furness et al., 2019). The lack of association between placentation and absence of ornamentation is almost certainly due to the limited sample size of species with ornamentation. However, even though not statistically supported, all origins of ornamentation are in non-placental lineages (*Xiphophorus and Poecilia*). Likewise, the placenta tends to evolve in lineages with long gonopodia. In sum, species with placentas tend to lack male courtship, dichromatism, and ornamentation, have long gonopdia, and have a high degree of sexual size dimorphism (males much smaller than females). Figures redrawn from Furness et al. (2019).

reproductive mode might affect the rate of speciation. If true, then this hypothesis yields the prediction that placental species will have a higher rate of speciation than lecithotrophic species.

An alternative hypothesis is that lineages in which males are enhanced by sexual selection will have higher rates of speciation because sexual selection can cause the accelerated evolution of pre-copulatory reproductive isolation (Rice and Holland, 1997; Coyne and Orr, 2004). There is, in fact, a strong association between enhanced sexual selection and the rate of speciation in cichlid and centrarchid fishes, insects and organisms that use bioluminescence as part of sexual displays (Arnqvist et al., 2000; Wagner et al., 2012; Seehausen, 2015; Smith et al., 2015; Ellis and Oakley, 2016). More generally, it is of interest to illuminate the relative importance of pre- vs. post-copulatory reproductive isolation in causing speciation to occur (Coyne and Orr, 1989, 2004). The Poeciliidae provide a rare opportunity to compare the two side by side because of the tendency for a hypothesized driver of post-copulatory reproductive isolation (placentation) to be disassociated from a hypothesized driver of pre-copulatory reproductive isolation (sexual selection).

Contrary to the prediction of Zeh and Zeh, we found that non-placental species exhibited a higher rate of speciation than placental species. Furthermore, we found that the speciation rate is higher in lineages that exhibit courtship, dichromatism, and ornamentation, and especially the combination of all three traits (Furness et al., 2019). We can separate the role of male traits from mode of maternal provisioning because males that lack these traits represent the ancestral state of the family and there remain many non-placental lineages that retain this ancestral state. It thus appears that sexual selection and the associated elaboration of male traits may be a stronger driver of speciation rate than female reproductive mode (i.e., placentation) in this family of fishes. In this view the low rate of speciation in placental lineages could be a byproduct of the shift away from pre-copulatory and toward post-copulatory mate choice, causing placental lineages to lack courtship, dichromatism, and ornamentation. These patterns of association suggest that pre-copulatory reproductive isolation dominates post-copulatory reproductive isolation in governing the rate of speciation in the poeciliid fishes.

DISCUSSION

What Do We Know About Multiple Paternity in the Poeciliidae?

High levels of concurrent multiple paternity have been reported in 10 species of Poeciliidae (Coleman and Jones, 2011; Evans and Pilastro, 2011; Girndt et al., 2012; Dekker et al., 2020)– four species of *Xiphophorus*, two of *Gambusia*, three of *Poecilia*, *Poeciliopsis monacha*, and *Heterandria formosa*). Only one of these species (*H. formosa*) is placental.

The existing analyses of concurrent multiple paternity harbor diverse sources of bias that preclude any attempt at generalization. First, while most of these studies have considerable statistical power to test the hypothesis that more than one sire is necessary to account for the genetic diversity of offspring, the actual estimates of the numbers of sires represented in a litter are based on different underlying algorithms and statistical approaches (Jones et al., 2010) and have large sampling errors. Second, not all of these studies were able to delimit, accurately, the distribution of reproductive success among males siring offspring. It is important to do so because different distributions of paternity among embryos will produce differences in the effective level of polyandry within a brood and the nature and strength of prenatal conflicts (Zeh and Zeh, 2000; Schrader et al., 2011). A brood of 15 embryos where one male sired 13 and two males sired one embryo each presents a different scenario for conflict than a brood of 15 in which three males sire five embryos apiece. Third, there is considerable variation among studies in the number of genetic loci assayed and the number of alleles per locus. The probability of detecting concurrent multiple paternity is directly proportional to the genetic variation present in the loci used to detect it (Neff et al., 2002; Jones et al., 2010; Schrader et al., 2011) and not all of these studies have corrected their estimates for the levels of segregating genetic variation. Fourth, some studies show substantial variation among populations in the rate of concurrent multiple paternity (Trexler et al., 1997; Gao et al., 2019). This suggests that studies of single populations may not represent patterns in the species as a whole. In addition, if populations differ in levels of neutral genetic variation, conclusions about population variation in the rates of multiple paternity depend crucially on whether the estimates have been corrected for those different levels of segregating genetic variation (Schrader et al., 2011). Finally, the existing data represent an *ad hoc* collection of distantly related species rather than a pairing of closely related species that differ in mode of maternal provisioning.

We are currently trying to improve on the situation by comparing levels of concurrent multiple paternity in three pairs of closely related species that represent three independent origins of placentation: *Poeciliopsis prolifica* (matrotrophic, denoted M) and *P. infans* (lecithotrophic, denoted L); *Poeciliopsis presidionis* (M) and *P. gracillis* (L); and *Poecilia bifurca* (M) and *Poecilia reticulata* (L) (Meredith et al., 2010). We are doing so in a way that addresses what we feel were weak points in prior analyses of multiple paternity. We hope, provide a better assessment of whether Zeh and Zeh (2000) prediction of higher rates of multiple paternity in placental species is upheld.

Alternative Explanations for the Associations Between Male and Female Attributes

Our presentation implicitly assumes that the associations among male and female traits are solely attributable to conflict. There are adaptive hypotheses for the evolution of placentas that may offer alternatives. One such hypothesis is derived from the observation that species with placentas tend to have a lower reproductive burden (Reznick et al., 2007b; Bassar et al., 2014) and to be more streamlined (Fleuren et al., 2018, 2019), which suggests that the evolution of placentation may be favored in performance demanding habitats, such as those with high risk of predation

(Hagmayer et al., 2020). High risk of predation also favors the evolution of less colorful guppies (Endler, 1978) so it is possible to imagine predation independently shaping male and female attributes. Alternatively, Trexler and DeAngelis (2003) propose that placentation is most likely to evolve in stable, high resource availability environments because it can yield a fecundity advantage. If such high resource environments are associated with high population densities and a reduced opportunity for males to monopolize females, then it could simultaneously favor a loss of male traits associated with sexual selection. Adaptive and conflict hypotheses are not necessarily exclusive of one another. For example, superfetation and placentation may have initially conveyed an adaptive advantage, but once adopted they would also have shifted the venue of sexual conflict (Furness et al., 2015). We have recently performed comparative analyses on the same data set used here and failed to find consistent family-wide associations between placentation, superfetation and habitat, and failed to find consistent support for either of these adaptive hypotheses (Furness et al., 2021), so these alternatives do not seem viable at this time.

CONCLUSIONS

We can now answer the seven questions posed at the opening of this paper.

- How often have male attributes associated with sexual selection evolved? The reversibility of the evolution of these traits makes it difficult to arrive at a precise number of events, but we can at least say that these attributes have been gained, and lost, repeatedly in the family. We can also identify “hot spots” of sexual selection in the genera *Poecilia* and *Xiphophorus*, where we see species with a concentration of those attributes associated with sexual selection.
- How often has the equivalent of a mammalian placenta evolved? Placentation evolved at least nine times. There is evidence of other origins in individual species nested within clades that are otherwise non-placental.
- How often has superfetation, a different variation on the theme of maternal provisioning, evolved? Superfetation was gained at least four times and lost at least once in the family. We also have discovered superfetation emerging two additional times in lineages that had previously evolved placentation (*Pamphorichthys* and *Phalloceros*).
- How often are these traits lost? Specifically, are there differences in the evolutionary lability of these male and female attributes? There are indeed strong differences in the reversibility of male traits associated with sexual selection and mode of reproduction in females. Male traits are much more labile. One obvious explanation is they reflect a continuous conflict of interest between sexual and natural selection to a greater degree than female mode of reproduction, which perhaps evolves as an adaptive trait, but one that shifts the venue of sexual conflict. There may be deeper differences between male and female traits in terms of the underlying genetic mechanisms and how reversible they are. For example, the genetic basis of the evolution of male traits may be relatively simple while those underlying the evolution of mode of reproduction may be the consequence of multiple, sequential steps that are more difficult to reverse (Lee and Shine, 1998; Furness et al., 2015; Furness and Capellini, 2019).
- Are there associations between the presence of male attributes associated with sexual selection and the mode of maternal provisioning? Yes. As predicted by the VDCH hypothesis, male traits associated with sexual selection are much more likely to be seen in species that lack placentas.
- Are there patterns in the order of evolution of male traits associated with sexual selection and the mode of maternal provisioning that suggest a cause-and-effect relationship? Yes. The evolution of male traits associated with sexual selection is conditioned on the mode of maternal provisioning. Species that lack placentas are much more likely to gain, and lose, male traits associated with sexual selection.
- Finally, is the evolution of a placenta associated with the accelerated evolution of post-copulatory reproductive isolation and speciation, as predicted by the VDCH? No. Lineages with placentas have slower rates of speciation than those without placentas. The reason those without placentas have higher average rates of speciation is because a subset of the non-placental lineages are the ones in which we see the elaboration of male traits associated with sexual selection. The accelerated rates of speciation are concentrated in these lineages.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://doi.org/10.5061/dryad.7g5b162.

AUTHOR CONTRIBUTIONS

DR conceived of the paper, wrote the first draft, and edited the final product. AF revised the first draft and made all of the figures. JT revised the first draft and contributed some new material to the manuscript. BP contributed the male data and revised the final draft of the manuscript. All authors approved the submitted version.

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Examination of the Trexler-DeAngelis Model of Maternal Provisioning Reveals That Matrotrophy Is Costly

Nabila Saleh-Subaie[1], Gonzalo A. Ramírez-Cruz[2] and J. Jaime Zúñiga-Vega[2]*

[1] Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, Mexico, [2] Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad de México, Mexico

The evolution of matrotrophy (post-fertilization maternal provisioning to developing embryos) has been explained through several hypotheses. Trexler and DeAngelis proposed in 2003 a theoretical model that defines the ecological conditions under which matrotrophy would be favored over lecithotrophy (pre-fertilization maternal provisioning). According to this model, matrotrophy offers a selective advantage in environments with abundant and constantly available food, whereas environments with limited and fluctuating food resources should instead promote a lecithotrophic mode of maternal provisioning. This model also proposes that matrotrophy entails the consequence of leaner reproductive females and in turn shorter lifespans. In this study, we examined the Trexler-DeAngelis model using data from 45 populations of five viviparous species from the fish genus *Poeciliopsis* (family Poeciliidae). We used the matrotrophy index (MI) as a measure of post-fertilization maternal provisioning, and the index of stomach fullness and individual body condition (BC) as proxies for food availability. We also estimated the magnitude of fluctuations in food availability by calculating the temporal variances of these two proxies. Neither abundant nor constantly available food were associated with greater degrees of matrotrophy, which fails to support the predictions of the Trexler-DeAngelis model with respect to the ecological drivers of increased post-fertilization provisioning to embryos. Nonetheless, in all five species we observed that females with greater degrees of matrotrophy had poorer BC compared to females that provided less nutrients to embryos after fertilization. This finding is consistent with one of the expected consequences of advanced matrotrophy according to the Trexler-DeAngelis model, namely, a detriment to the nutritional status of females. Our study provides compelling evidence that gestating females experience a trade-off between post-fertilization provisioning to embryos and self-maintenance, revealing in turn that matrotrophy is a costly reproductive strategy.

Keywords: lecithotrophy, matrotrophy, maternal provisioning, Poeciliidae, *Poeciliopsis*, reproductive modes, viviparous fishes

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Michael Tobler,
Kansas State University, United States

Reviewed by:
Zachary Culumber,
University of Alabama in Huntsville,
United States
Rudiger Riesch,
University of London, United Kingdom
Daniel Blackburn,
Trinity College, United States

***Correspondence:**
J. Jaime Zúñiga-Vega
jzuniga@ciencias.unam.mx

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INTRODUCTION

Viviparity is a reproductive strategy in which the embryo develops inside a specialized structure or cavity within the female after fertilization (Blackburn, 1999). This reproductive strategy can be found in mammals, sharks, anurans, salamanders, snakes, lizards, and fishes (Shine and Bull, 1979; Guillette and Jones, 1985; Greven and Guex, 1994; Castro, 2009; Renfree et al., 2013; Wake, 2015). In addition, viviparity also occurs in numerous invertebrate taxa, including several species from the following classes: Arachnida, Chromadorea, Digenea, Demospongiae, Insecta, and Stenolaemata, among others (Hagan, 1931; Meier et al., 1999; Ostrovsky et al., 2009, 2016). Females of viviparous species provide nutrients to their developing embryos by means of two different mechanisms. The first mechanism is lecithotrophy, in which the mother transfers all nutrients to her embryos before fertilization in the form of yolk and does not transfer additional resources during their development (Blackburn, 1992). The second mechanism is matrotrophy, which unlike lecithotrophy, involves the continuous provisioning of nutrients from the mother to the developing embryos after fertilization, usually through specialized structures (e.g., placentas; Wourms, 1981; Lombardi and Wourms, 1985; Blackburn, 1992; Marsh-Matthews, 2011; Kwan et al., 2015; Olivera-Tlahuel et al., 2019).

Theoretical models about the evolution of matrotrophy from a lecithotrophic strategy suggest that food availability is an ecological factor that could promote changes in the way that mothers provide nutrients to their developing offspring. Trexler and DeAngelis (2003, 2010) proposed a model that suggests that the amount of food available in the environment may favor the evolution of maternal adaptations such as matrotrophy. They suggested that one of the benefits of matrotrophy is that females may be able to produce a greater number of embryos than lecithotrophic females. The reason for this expected difference in fecundity is that lecithotrophic eggs are larger and more energetically costly than matrotrophic eggs because they contain all the necessary nutrients to complete embryo development. In contrast, matrotrophic eggs are notably smaller and imply a lower energy demand at the onset of reproduction, which should allow females to initiate gestation with a larger brood. However, this benefit of higher fecundity for matrotrophic females would only be possible if pregnant females have constant access to enough food to simultaneously meet their own energetic demands (i.e., self-maintenance) and to provide nutrients to their embryos all throughout development. Therefore, matrotrophy should be favored in environments with constant and high food availability. Lecithotrophy, on the other hand, should be favored in environments where resource availability is low or highly variable, because lecithotrophic females do not need a constant surplus of nutrients to support embryo development, but instead can store nutrients in the form of yolk during periods of relatively high food availability (Trexler and DeAngelis, 2003, 2010).

Viviparous fishes of the family Poeciliidae exhibit wide variation in the amount of nutrients that females can transfer to their developing embryos before and after fertilization. Some species are strictly lecithotrophic (e.g., *Gambusia hubbsi* and *Gambusia. alvarezi*), whereas other species exhibit extensive matrotrophy (e.g., *Poecilia parae* and *Poeciliopsis prolifica*), with several species showing varying degrees of the relative amounts of pre- and post-fertilization provisioning (Reznick et al., 2002; Pires et al., 2007, 2010; Pollux et al., 2009; Pollux and Reznick, 2011; Torres-Mejia, 2011; Olivera-Tlahuel et al., 2015). In addition, some studies have demonstrated that the degree of matrotrophy (i.e., the amount of post-fertilization maternal provisioning) may also vary among different populations of the same species (Reznick et al., 2007; Turcotte et al., 2008; Pires et al., 2010; Molina-Moctezuma et al., 2020). Due to the large variation in the way poeciliid females provide resources to their embryos, some studies have attempted to understand the potential benefits of matrotrophy over lecithotrophy (Trexler and DeAngelis, 2003, 2010; Pollux et al., 2009, 2014; Saleh-Subaie and Zúñiga-Vega, 2019).

Several studies have previously tested the Trexler-DeAngelis model under laboratory (Marsh-Matthews and Deaton, 2006; Pires et al., 2007; Banet and Reznick, 2008; Banet et al., 2010; Pollux and Reznick, 2011; Itonaga et al., 2012; Van Dyke et al., 2014; Molina-Moctezuma et al., 2020) and natural conditions (Schrader and Travis, 2008, 2012; Riesch et al., 2013; Molina-Moctezuma et al., 2020) obtaining mixed results. Interestingly, most of these studies have been conducted on poeciliid fishes and only three studies have evaluated this model with non-fish taxa, specifically using lizards as model systems (Cadby et al., 2011; Itonaga et al., 2012; Van Dyke et al., 2014). Some authors suggest that the Trexler-DeAngelis model is at least partially supported according to the relatively high levels of matrotrophy and the tendency to decrease the size of oocytes observed in sites with high food availability in both natural and experimental conditions (Pires et al., 2007; Schrader and Travis, 2008, 2012; Pollux and Reznick, 2011; Itonaga et al., 2012; Riesch et al., 2013; Van Dyke et al., 2014; Molina-Moctezuma et al., 2020). Nonetheless, other authors suggest that the model is not supported because they did not observe a significant decrease in oocyte size in matrotrophic females, nor did they find differences in the level of matrotrophy in experiments where the amount of food was constant or variable (Marsh-Matthews and Deaton, 2006; Banet et al., 2010; Pollux and Reznick, 2011; Riesch et al., 2013).

Since no conclusive results have been found, neither under experimental or natural conditions, it is important to study the generality of this phenomenon to determine if a higher degree of matrotrophy (i.e., greater amount of post-fertilization provisioning) indeed occurs with greater quantity and stability of food. Therefore, the main objective of this study is to evaluate the Trexler-DeAngelis model (Trexler and DeAngelis, 2003, 2010). In particular, we address the specific conditions that would lead to increased matrotrophy by comparing natural populations of five species of the genus *Poeciliopsis*. We predict that females from populations where the amount of available food resources is relatively high and constant, will show higher levels of matrotrophy. In contrast, we expect less post-fertilization maternal provisioning in populations that inhabit sites where there is evidence that the amount of food is lower or fluctuating. We used two proxies for food availability: an index of stomach fullness (IF) and an estimate of body condition (BC). High IF and/or BC values indicate that females inhabit an environment with high food availability, whereas low IF and/or BC values indicate that females inhabit an environment with low food availability. In addition, we estimated the variance among months of both IF and BC to quantify the degree of temporal variability in food availability. In environments where food resources are constantly available (i.e., populations with small temporal variances in IF and/or BC), we expect to find higher degrees of matrotrophy, whereas in environments where food availability fluctuates (i.e., populations with large temporal variances in IF and/or BC), we expect to find females exhibiting less post-fertilization provisioning. Our study represents an empirical test of this important theoretical model that attempts to explain how different strategies for embryo nourishment have evolved in viviparous organisms.

MATERIALS AND METHODS

Study Species and Datasets

We focused on five species of the genus *Poeciliopsis* (family Poeciliidae) with relatively wide geographic distributions, which allowed us to examine interpopulation differences in food availability and how such differences affect the patterns of embryo nourishment. All five species exhibit superfetation, which is the ability of females to simultaneously bear two or more groups of embryos at different developmental stages (Scrimshaw, 1944), but differ in the degree of matrotrophy (i.e., lecithotrophy, matrotrophy, and incipient matrotrophy) (**Table 1**). The number of populations differed among species, from 6 to 11, for a total of 45 populations. The number of females per population varied from 14 to 141 (**Table 1**). Geographic coordinates of all populations can be found in **Supplementary Table 1**. For *Poeciliopsis gracilis*, *Poeciliopsis infans*, and *Poeciliopsis turrubarensis* we combined data from published studies (Zúñiga-Vega et al., 2007; Frías-Alvarez et al., 2014; Frías-Álvarez and Zúñiga-Vega, 2016), and from our own dissections of pregnant females, which we obtained from the National Collection of Fishes (Instituto de Biología, Universidad Nacional Autónoma de México). For the remaining two species (*Poeciliopsis baenschi* and *P. prolifica*), all data was obtained by dissecting preserved females, also from the National Collection of Fishes (**Table 1**). All specimens were preserved in ethanol (70%) until they were dissected.

Laboratory Measurements

To test our hypothesis, we calculated the matrotrophy index (MI), and two proxies for food availability: the index of fullness (IF) and individual BC. To quantify these variables we measured the standard length (SL) of females before dissection with a digital caliper (±0.1 mm), from the tip of the mouth to the base of the caudal fin at the level of the lateral line. We then dissected females to quantify brood size (number of embryos in the same developmental stage) and superfetation (number of simultaneous broods). We used the classification of embryonic stages proposed by Haynes (1995) to identify the developmental stage of each brood. Individual embryo mass was quantified by drying an entire brood for 48 h at 55°C, weighing it with a precision scale (±0.05 mg), and dividing brood dry mass by the number of embryos in the brood. Upon dissection, the entire digestive tract (stomach and intestines) was removed from each female. We quantified the somatic dry mass of each female (excluding digestive and reproductive tracts) as well as the dry mass of the digestive tract (including all its content) following the same drying and weighing protocol that we implemented for embryos.

Matrotrophy Index

The MI is a standard measure of post-fertilization maternal provisioning, and is calculated as the dry mass of the offspring at birth divided by the dry mass of the egg at fertilization (Reznick et al., 2002; Marsh-Matthews, 2011). MI values <0.75 indicate that females provide all nutrients before fertilization in the form of yolk. MI values between 0.75 and 1 indicate that females provide most nutrients before fertilization and small amounts of nutrients during embryo development (i.e., incipient matrotrophy). MI values >1 indicate that a substantial amount of nutrients are provided after fertilization. MI values >5 indicate extensive matrotrophy (Marsh-Matthews, 2011).

We calculated the MI in two different ways. First, we calculated an MI separately for each population by using data from all females to estimate the dry mass at birth and the dry mass of

TABLE 1 | List of the five study species from the fish genus *Poeciliopsis* with information on the type of maternal provisioning to developing embryos (matrotrophy, incipient matrotrophy, or lecithotrophy), matrotrophy index (MI), number of populations per species, and sample size (n = number of reproductive females) per population.

Species	Type of maternal provisioning	MI	Number of populations	*n* per population	References
Poeciliopsis baenschi	Matrotrophy	1.58	6	48, 38, 32, 29, 45, 34	Pollux et al., 2014; Olivera-Tlahuel et al., 2015; Zúñiga-Vega et al., 2017; this study
Poeciliopsis gracilis	Lecithotrophy	0.72	11	15, 58, 78, 116, 52, 64, 74, 23, 90, 85, 91	Bassar et al., 2014; Pollux et al., 2014; Frías-Álvarez and Zúñiga-Vega, 2016; this study
Poeciliopsis infans	Incipient matrotrophy	0.86	11	50, 74, 131, 55, 59, 56, 38, 52, 50, 65, 63	Frías-Alvarez et al., 2014; Pollux et al., 2014; Frías-Álvarez and Zúñiga-Vega, 2016; this study
Poeciliopsis prolifica	Matrotrophy	5.40	8	19, 32, 145, 18, 93, 49, 78, 44	Pires et al., 2007; Banet and Reznick, 2008; Pollux et al., 2014; this study
Poeciliopsis turrubarensis	Incipient matrotrophy	0.95	9	37, 71, 49, 40, 38, 77, 21, 40, 14	Zúñiga-Vega et al., 2007; Bassar et al., 2014; Pollux et al., 2014.

the egg at fertilization (Reznick et al., 2002; Marsh-Matthews, 2011; **Supplementary Table 2**). For this purpose, we fitted a linear regression to our data on individual embryo mass (response variable, log-transformed) and developmental stage (predictor variable), separately for each population. From this regression, we used the predicted dry mass at stage 4 [recently fertilized eggs according to Haynes (1995)] as well as the predicted dry mass at stage 11 (last stage of development and, thus, an estimate of mass at birth) to calculate the MI. In this regression, each female was represented by a single data point (i.e., the average dry mass of individual embryos in a particular developmental stage). Because all species in this study exhibit superfetation, we randomly chose one brood from each female to quantify individual embryo mass (as per Frías-Alvarez et al., 2014; Gorini-Pacheco et al., 2018). In this way, we ensured that each female was represented only once in our linear regressions to avoid the problem of non-independence in the data.

Second, we calculated an matrotrophy index per individual female (MI_{ind}) by selecting females that had simultaneous broods in developmental stages 4 (recently fertilized) and 11 (close to be born). We calculated an MI_{ind} for each of these females by dividing the average dry mass of her embryos at stage 11 by the average dry mass of her embryos at stage 4. In total, 51 females across all species had broods in stages 4 and 11 simultaneously. Our MI_{ind} values represent how embryo mass changes throughout development within individual females and, hence, are individual estimates of the amount of post-fertilization provisioning. In contrast, our MI values per population are an average representation of the degree of matrotrophy across all the females that inhabit a population.

Index of Fullness and Body Condition as Proxies for Food Availability

To estimate food availability, we used the index of stomach fullness (IF), which was calculated for each female as the dry mass of her digestive tract (including all its content) divided by her somatic dry mass, and multiplying the result by 100 (Hyslop, 1980). The IF is an indirect measure of the amount of available food and, more specifically, it is a measure of how much females ate at a certain time. It gives us a proxy for food availability at the time when we collected the females, and maybe for a few days before sampling. High IF values indicate that females were in an environment with high food availability, whereas low IF values indicate that females were in an environment with low food availability. Once we obtained the IF for each female, we calculated an average IF (IF_A) for each population (**Supplementary Table 2**). Given our hypothesis, we expected that in environments where food is abundant (i.e., populations with high IF_A), females will have higher degrees of matrotrophy, whereas in environments where food is less abundant (i.e., populations with low IF_A), we expected to find less matrotrophic females.

In addition, to quantify the magnitude of fluctuations in food availability, we calculated for each population the variance of the IF among months ($\text{VarIF}_{\text{months}}$; **Supplementary Table 2**). Given that the amount of available food can vary over time, in those cases in which we had several collection dates (i.e., conducted in different months) from the same population (**Supplementary Table 3**), we calculated an IF_A per month and then the variance among these monthly IF_A values. Populations with large values of $\text{VarIF}_{\text{months}}$ are those with pronounced temporal fluctuations (among months or between seasons) in food availability. According to the Trexler-DeAngelis model (Trexler and DeAngelis, 2003, 2010), we expected a negative relationship between MI and $\text{VarIF}_{\text{months}}$. That is, matrotrophy should be favored in populations where food availability is relatively constant, whereas lecithotrophy should be favored in environments with substantial variability in the access to food resources. Our data from populations with several collection dates revealed that, in some of these populations, both the MI and the index of stomach fullness varied substantially among months (**Supplementary Figures 1, 2**).

In most of the populations that were sampled in different occasions, the collection dates occurred within a period of 2 years (**Supplementary Table 3**). Given that the lifespan of poeciliid fishes varies between 2 and 4 years (Coad, 2017; Young et al., 2017), our multiple samples provide a reliable representation of the temporal fluctuations in food availability that a single generation of females experienced. Therefore, this is the appropriate temporal scheme to test the prediction that females facing changing conditions, in terms of food supply, over the course of their lifespan would increase their pre-fertilization investment (a less matrotrophic strategy).

We also calculated BC as another proxy for food availability. We used the scaled mass index as an estimate of individual BC (Peig and Green, 2009). This index is a more reliable indicator of the relative size of energy reserves compared to other indices of BC (Peig and Green, 2010). We calculated the scaled mass index, which we denote here as BC because it is our measure of BC, for each female as follows:

$$\text{BC}_i = \text{M}_i \left[\frac{\text{SL}_0}{\text{SL}_i} \right]^{b/r}$$

where M_i and SL_i are the somatic dry mass and SL of female i, respectively, SL_0 is the average SL of the corresponding species, b is the slope from a linear regression of female dry mass on SL (both variables log-transformed), and r is the correlation coefficient between log-transformed female dry mass and SL. Given that female mass and SL differed substantially among species, we calculated the scaled mass index separately for each species, but pooling data from different populations. This index (BC_i) is an estimate of the dry mass that each female i would have at a fixed body length, which in our case is the average SL of each species (SL_0). Thus, smaller values of BC indicate poorer nutritional status and less energetic reserves, whereas larger values of BC indicate better nutritional status and greater energetic reserves (Peig and Green, 2009, 2010).

We then calculated an average of this scaled mass index separately for each population [average body condition (BC_A)] (**Supplementary Table 2**). BC is an estimate of food availability on a long time scale (weeks before capturing the females) because it reflects if females were able to gain somatic mass as a result of abundant food intake during the past weeks or, alternatively, if they lost mass as a consequence of food scarcity. According to our hypothesis, females will have good BC in environments with plenty of available food and, therefore, high degrees of matrotrophy. In contrast, poor BC indicates that females have experienced reduced food availability and, thus, they should exhibit lower degrees of matrotrophy.

Estimates of BC may be affected by morphological differences among females. For instance, heavier females may also have deep bodies and extended abdomens whereas leaner females may have thinner and elongated bodies. Given that body shape may be affected by ecological factors other than food availability, such as predation risk and water velocity (Zúñiga-Vega et al., 2007; Langerhans, 2009; Langerhans and Makowicz, 2009), an association between morphology and BC could imply that the observed differences among populations in average BC may have been driven by these other factors. This in turn would imply that our estimates of BC do not reflect food availability but indicate instead interpopulation differences in predation intensity or water velocity. We discarded a confounding effect of body shape on our estimates of BC by means of a morphological analysis based on geometric morphometric techniques (**Supplementary Material: Methods**). This analysis was conducted on four of our five study species because no digital photographs were available for one species (*P. turrubarensis*). The correlations between the scaled mass index and the first two axes of shape variation were rather weak in all four species (all correlation coefficients ranged between −0.20 and 0.31; **Supplementary Table 4**). Thus, in these *Poeciliopsis* species, the observed intraspecific variation in BC cannot be explained by morphological differences among females.

Similar to what we did for the IF, for each population we quantified temporal fluctuations in food availability by means of the variance of body condition among months ($\mathrm{VarBC_{months}}$; **Supplementary Table 2**). This temporal variance was calculated only for those populations that were sampled in different months (**Supplementary Table 3**). First, we calculated an average BC ($\mathrm{BC_A}$) per month and then the variance among these monthly $\mathrm{BC_A}$ values. We expected a negative relationship between MI and $\mathrm{VarBC_{months}}$. Consistent with the observed temporal variation in both the MI and the IF, BC also varied substantially among months within some of the populations that were sampled in different occasions (**Supplementary Figure 3**).

Quantifications of stomach contents and BC indices have been used previously as indicators of food availability in the surrounding environment. In particular, studies on other fish species have demonstrated that both the index of stomach fullness and individual BC correlate positively with the abundance of important food sources, such as nutrient-rich prey (Simpkins and Hubert, 2000; Pothoven et al., 2001; Rikardsen et al., 2006). These previous observations give support to the use of IF and BC as reliable proxies for food availability.

Statistical Analyses

We implemented different sets of linear models using the R statistical software v. 3.4.2 (R Core Team, 2019). We used the Akaike information criterion adjusted for small sample sizes (AICc; Akaike, 1973) to select the best model (i.e., the model with smallest AICc). However, models that differ by less than seven units of the AICc with respect to the best model ($\Delta\mathrm{AICc} < 7$) also have some support in the data (Burnham and Anderson, 2002; Burnham et al., 2011). To deal with model uncertainty, we calculated Akaike weights (w_i), which are measures of the relative support for each model i in the data (Burnham and Anderson, 2002). Then, we compared the support of two models relative to each other using evidence ratios, which are calculated as the quotient of their respective Akaike weights (w_1/w_2). These evidence ratios indicate how much greater the evidence is in favor of model 1 compared to that of model 2. We considered that strong evidence in support of the Trexler-DeAngelis model would come from two complementary lines of evidence. First, the best-fitting model must include one of our predictors of interest (IF, $\mathrm{IF_A}$, $\mathrm{VarIF_{months}}$, BC, $\mathrm{BC_A}$, or $\mathrm{VarBC_{months}}$). Second, this best-fitting model must have at least four times stronger support than models that do not include this predictor (i.e., an evidence ratio ≥ 4). In those cases where two or more supported models (models within $\Delta\mathrm{AICc} < 7$) had smaller evidence ratios (because such supported models had relatively large and similar Akaike weights), we chose the model that contained fewer parameters (the simplest model), since adding the additional parameters did not substantially improve model fit.

In addition, based on the Akaike weights, we calculated model-averaged regression coefficients, as instructed by Burnham and Anderson (2002), for those predictor variables with evident statistical effects. These model-averaged slopes account for model uncertainty and are more robust than those derived from any single model (Johnson and Omland, 2004). We used the R package "MuMIn" to implement all these procedures for model selection and multi-model inference (Bartoń, 2019).

For the first model set we used the MI per population (log-transformed) as response variable and $\mathrm{IF_A}$ as the main explanatory variable. We also considered the factor "species" to take into account differences among species in the degree of matrotrophy. We included additive and interactive effects of $\mathrm{IF_A}$ and species, as well as an intercept-only model, for a total of five competing models (**Table 2**). In this first model set, we had 45 data points, one for each of our study populations.

In our second model set we also used MI per population (log-transformed) as response variable, but in this case, we analyzed the effects of temporal fluctuations in the IF by using $\mathrm{VarIF_{months}}$ as explanatory variable. We also considered differences among species, additive and interactive effects of $\mathrm{VarIF_{months}}$ and species, and an intercept-only model, for a total of five competing models (**Table 2**). Given that not all our study populations were visited in different months (**Supplementary Table 3**), we had a reduced number of data points (28) for this model set. In fact, all populations of *P. baenschi* and *P. turrubarensis* lacked data from different months and, thus, these two species were excluded from this model set.

The third model set was implemented using the $\mathrm{MI_{ind}}$ female (log-transformed) as response variable. The main explanatory variable was the IF calculated for each female. In addition, we considered differences among species, additive and interactive effects of IF and species, and an intercept-only model, for a total

TABLE 2 | Values of the Akaike information criterion adjusted for small sample sizes (AICc), differences in AICc with respect to the best-fitting model (ΔAICc), and Akaike weights (*w*) for competing models that examined variation in the MI per population.

	Models	AICc	ΔAICc	*w*
First model set	Species	47.52	0	0.70
	Species+IF_A	50.27	2.75	0.18
	Species×IF_A	51.00	3.48	0.12
	IF_A	65.48	17.96	0
	Intercept only	67.47	19.95	0
Second model set	Species	22.39	0	0.75
	Species+$VarIF_{months}$	24.70	2.31	0.24
	Species×$VarIF_{months}$	30.33	7.94	0.01
	Intercept only	52.31	29.92	0
	$VarIF_{months}$	54.38	31.99	0

In the first model set, we examined the effects of the average index of fullness (IF_A) while accounting for differences among species. In the second model set, we examined the effect of the variance of the index of fullness among months ($VarIF_{months}$) also accounting for differences among species. The best-fitting models are indicated by ΔAICc = 0. Models are listed according to their fit to the data, from best to worst.

TABLE 3 | Values of the Akaike information criterion adjusted for small sample sizes (AICc), differences in AICc with respect to the best-fitting model (ΔAICc), and Akaike weights (*w*) for competing models that examined variation in the matrotrophy index per individual female (MI_{ind}).

Models	AICc	ΔAICc	*w*
Species	50.02	0	0.65
IF+Species	51.28	1.26	0.35
IF×Species	60.63	10.61	0.003
Intercept only	85.18	35.16	0
IF	85.18	35.16	0

In this third model set, we examined the effect of the index of fullness per individual (IF) while accounting for differences among species. The best-fitting model is indicated by ΔAICc = 0. Models are listed according to their fit to the data, from best to worst.

TABLE 4 | Values of the Akaike information criterion adjusted for small sample sizes (AICc), differences in AICc with respect to the best-fitting model (ΔAICc), and Akaike weights (*w*) for competing models that examined variation in the MI per population.

	Models	AICc	ΔAICc	*w*
First model set	Species	47.52	0	0.80
	Species+BC_A	50.34	2.82	0.20
	Species×BC_A	57.62	10.10	0.01
	BC_A	65.59	18.07	0
	Intercept only	67.47	19.95	0
Second model set	Species	22.39	0	0.46
	Species×$VarBC_{months}$	22.82	0.43	0.38
	Species+$VarBC_{months}$	24.50	2.11	0.16
	$VarBC_{months}$	46.53	24.14	0
	Intercept only	52.31	29.92	0

In the first model set, we examined the effects of the average body condition (BC_A) while accounting for differences among species. In the second model set, we examined the effect of the variance of body condition among months ($VarBC_{months}$) also accounting for differences among species. The best-fitting models are indicated by ΔAICc = 0. Models are listed according to their fit to the data, from best to worst.

of five competing models (**Table 3**). In this case, we expected a positive relationship between MI_{ind} and the IF. Unlike the previous two model sets, which were conducted at the population level, this third model set was conducted at the individual level with 51 data points, one for each female with an estimate of MI_{ind}.

Additionally, we implemented all three model sets, but using BC as proxy for food availability, instead of the IF. This means that the response variable was the same (MI per population in the first and second model sets, and MI_{ind} in the third model set), but we used BC_A as the main predictor of interest in the first model set (**Table 4**), $VarBC_{months}$ in the second (**Table 4**), and BC per individual female in the third (**Table 5**). We also implemented all other models that we described above (i.e., differences among species, additive and interactive effects, and an intercept-only model).

RESULTS

Matrotrophy and the Index of Fullness

In the first model set, using IF as proxy for food availability, we found no evident effect of IF_A on the MI per population (**Figure 1A**). The top model indicated that MI per population only differed among species (**Table 2**). Two other models were within ΔAICc < 7 and both included the effect of IF_A in addition to differences among species (additive effect in the second model and interactive effect in the third model). However, these additional effects of IF_A did not improve model fit compared to the simpler model that only included differences among species (**Table 2**). Similarly, in the second model set, we found no evidence of an effect of $VarIF_{months}$ on the MI per population (**Figure 1B**). Also in this case, the top model indicated differences among species and adding the effect of $VarIF_{months}$ did not substantially improve model fit (**Table 2**). The highest degrees of matrotrophy occurred in four populations of *P. prolifica*, and the lowest (indicating a lecithotrophic mode of maternal provisioning) in one population of *P. gracilis* (**Figure 1**).

We obtained a similar result when we analyzed the potential effect of the individual IF on MI_{ind} in our third model set. The model with the best fit only included differences among species in the MI_{ind} (**Table 3**). The second model was within seven AICc units from the best-fitting model and included an additive effect of IF and species. Hence, evidence of an effect of IF on MI_{ind} was weak because adding IF did not substantially improve model fit with respect to the simpler model that only included differences among species (**Table 3**). Most females of *P. prolifica* had higher degrees of matrotrophy compared to females from all other species, whereas three females of *P. gracilis* had the lowest values of the MI_{ind} (**Figure 2**).

Matrotrophy and Body Condition

In our first model set, using BC as proxy for food availability, BC_A did not affect the MI per population (**Figure 3**). Again in this case, the best-fitting model indicated differences among

TABLE 5 | Values of the Akaike information criterion adjusted for small sample sizes (AICc), differences in AICc with respect to the best-fitting model (ΔAICc), and Akaike weights (*w*) for competing models that examined variation in the MI$_{ind}$ female.

Models	AICc	ΔAICc	*w*
BC$\times$Species	46.90	0	0.63
BC+Species	48.78	1.88	0.24
Species	50.02	3.12	0.13
Intercept only	85.18	38.28	0
BC	86.57	39.67	0

In this third model set, we examined the effect of individual body condition (BC) while accounting for differences among species. The best-fitting model is indicated by ΔAICc = 0. Models are listed according to their fit to the data, from best to worst.

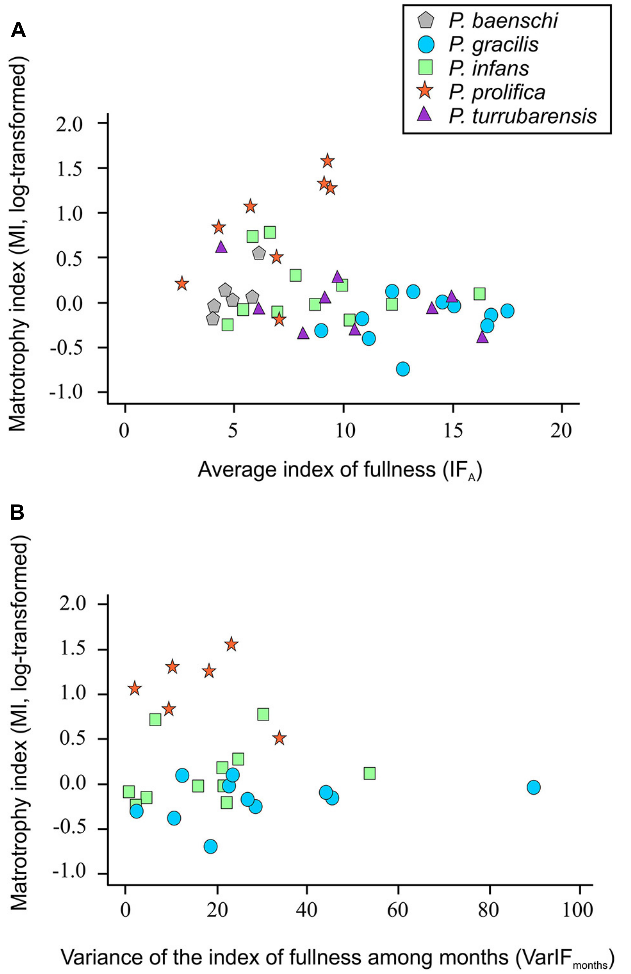

FIGURE 1 | (A) Lack of association between the matrotrophy index per population (MI) and average index of fullness (IF$_A$) for 45 populations of five fish species from the genus *Poeciliopsis*. **(B)** Lack of association between the MI per population and the variance of the index of fullness among months (VarIF$_{months}$) for 28 populations of three fish species from the genus *Poeciliopsis*.

species in the MI (**Table 4**). The model that ranked second was within ΔAICc < 7 and included the additive effect of species and BC$_A$. However, adding BC$_A$ did not improve model fit compared to the model that only included differences among species. Similarly, in our second model set, the top model indicated that MI only differs among species (**Table 4**). Two other models had support (i.e., ΔAICc < 7) and both included the effect of VarBC$_{months}$ (interacting with species in the second model and as an additive effect in the third model). Hence, adding VarBC$_{months}$ did not substantially improve model fit, indicating a weak effect of this predictor on the MI per population (**Figure 4**). In *P. prolifica*, MI apparently decreased as VarBC$_{months}$ increased (**Figure 4C**). However, this negative association was caused by a single population with large temporal variance and quite small MI. For this reason, the model including the interaction between VarBC$_{months}$ and species did not outperform the simpler model that only included differences among species (**Table 4**).

Finally, in the third model set in which we used BC as proxy for food availability, the model that included an interactive effect of individual BC and species on MI$_{ind}$ ranked first (**Table 5**). The model that included an additive effect of these two predictors ranked second. The top model had 2.6 times stronger support than the second model (evidence ratio: 0.63/0.24 = 2.63). The third model only included differences among species with no effect of BC. However, the top model had almost five times stronger support than this third model (evidence ratio: 0.63/0.13 = 4.85) (**Table 5**), which in this case represents compelling evidence of a combined effect of both BC and species on MI$_{ind}$. Contrary to our prediction, model-averaged regression coefficients revealed a negative relationship between MI$_{ind}$ and individual BC for females of all five species (**Figure 5**). This negative relationship was weaker (i.e., a less steep negative slope) for females of *P. prolifica* (**Figure 5D**).

DISCUSSION

Matrotrophy Negatively Affects Body Condition and Is Apparently Not Influenced by Food Availability

In this study, we examined the Trexler-DeAngelis model of maternal provisioning to developing embryos (Trexler and DeAngelis, 2003, 2010). According to this model, higher levels of matrotrophy are only possible if food is relatively abundant and constantly available. To test this prediction, we used the index of stomach fullness and BC as proxies for the amount of available food in the environment. Based on these two variables, we also quantified the degree of temporal variability in food availability. We found that females with higher levels of matrotrophy had poorer BC compared to females that provided less nutrients to embryos after fertilization. This negative association was evident in females of all five species and is opposite to our prediction of greater matrotrophy in females that exhibit better BC, presumably as a result of constant access to abundant food resources. In contrast, our main finding implies that providing relatively large amounts of nutrients to embryos after fertilization has a negative consequence on the nutritional status of the females. Possibly, females experience a trade-off

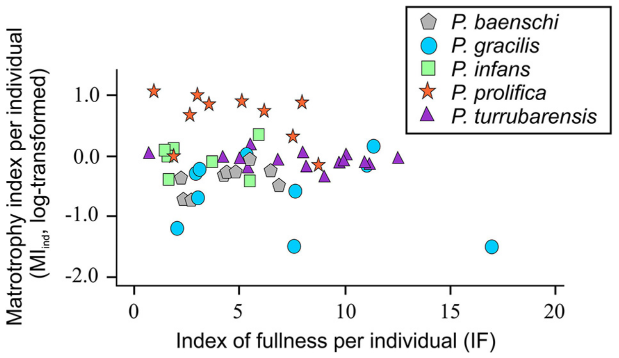

FIGURE 2 | Lack of association between the matrotrophy index per individual (MI$_{ind}$) and the individual index of fullness (IF) for 51 females of five fish species from the genus *Poeciliopsis*.

between active provisioning of embryos during gestation and allocation of energy and nutrients to somatic tissues, suggesting that matrotrophy is a costly physiological process. Severe demands for energy and nutrients are well-known consequences of large investments in reproduction (Koskela et al., 1998; Jonsson et al., 2002; Velando and Alonso-Alvarez, 2003).

According to the Trexler-DeAngelis model, one of the main benefits of matrotrophy is a potentially higher fecundity, because lecithotrophy implies that eggs must be full of yolk before fertilization and thus represent a large energetic investment since the onset of gestation. In contrast, matrotrophic eggs are considerably smaller and less energetically costly, which could allow females to initiate gestation with a higher number of these smaller eggs. If resources are abundant enough to provide embryos with the necessary nutrients all throughout gestation, then matrotrophy would be favored over lecithotrophy (Trexler and DeAngelis, 2003, 2010). Here, we have shown that neither the amount of food that is present in the digestive tract of females nor their nutritional status reflected in their BC are positively associated with the degree of matrotrophy, which in turn suggests that high food availability is not the main driver of advanced degrees of matrotrophy. In contrast, we detected in all five species that females that provided more nutrients to embryos after fertilization were in poorer BC compared to females that relied more on pre-fertilization provisioning. This observed pattern represents compelling evidence that, if advanced matrotrophy allows females to gestate more embryos, such increased fecundity comes at the cost of a reduced investment in self-maintenance.

Our work with 45 populations of five species of poeciliid fishes did not support the expectation of increased matrotrophy in sites with abundant food. Furthermore, sites where food availability fluctuates over time (i.e., sites with large temporal variances in the index of stomach fullness and in BC) did not promote reduced levels of matrotrophy. According to the Trexler-DeAngelis model, if food-limiting conditions are likely to arise, then females must rely more on pre-fertilization provisioning because they can store as much surplus energy as possible in the form of yolk whenever food becomes available (during periods when food intake exceeds metabolic demands) (Trexler and DeAngelis, 2003, 2010). Our data did not support this prediction either. Interestingly, however, the original model specifies that the total cost per brood may be greater for matrotrophic females compared to lecithotrophic females, because the former initially invest less per egg (because eggs are smaller) which allows them to initiate gestation with larger broods. If the size of offspring at birth is the same between matrotrophic and lecithotrophic females, then producing a larger number of similar-sized young will result in an overall higher reproductive cost for females with advanced matrotrophy. As a consequence, Trexler and DeAngelis (2003, 2010) predicted leaner bodies and shorter lifespans in females that allocate more resources to developing embryos after fertilization. Our findings revealed poor BC in females with advanced matrotrophy and are thus consistent with this particular prediction of the model that is related to the consequences of matrotrophy. In conclusion, we failed to provide evidence in support of the expected ecological causes of this reproductive mode (high and constant food availability), but we have demonstrated a critical consequence of advanced matrotrophy that appears to be pervasive because it occurred in five different species.

The Costs of Matrotrophy Decrease as Yolk Dependency Decreases

Our findings, along with previous evidence, suggest that the severity of the physiological costs imposed by greater post-fertilization provisioning depends on the degree to which females rely on yolk reserves for embryo nutrition. A previous study in the congeneric *Poeciliopsis retropinna* found that the amount of post-fertilization provisioning was positively associated with maternal body fat and unrelated to maternal lean mass (Hagmayer et al., 2018). This finding is somehow contrary to what we observed in this study, because our estimates of BC included both lipids and lean tissue (i.e., we did not extract lipids from the females). Thus, our observed reduction in BC associated with higher degrees of matrotrophy likely entailed reductions in both body fat and lean mass. The reasons why in *P. retropinna* a higher investment in embryo development after fertilization does not entail a reduction in maternal body fat or in lean mass, whereas in the five congeneric species that we studied advanced matrotrophy clearly resulted in poorer BC, remain unknown. A tentative explanation may involve the remarkably high degree of matrotrophy of *P. retropinna*, in which embryos increase in mass more than 100-fold during development (MI = 117; Reznick et al., 2002). Such an extensive degree of matrotrophy could substantially reduce the costs derived from reproduction because mature ova are notably small with no nutrients before fertilization and, hence, overall investment in reproduction is low during a significant proportion of pregnancy. All our study species exhibit considerably lower degrees of matrotrophy, from an MI = 0.72 in *P. gracilis* (which in fact indicates strict lecithotrophy) to an MI = 5.40 in *P. prolifica* (**Table 1**). Therefore, energetic demands derived from a higher investment in embryos since the onset of gestation (in our study species mature ova already contain some nutrient-rich yolk before fertilization) are likely higher in our five study species than in *P. retropinna*.

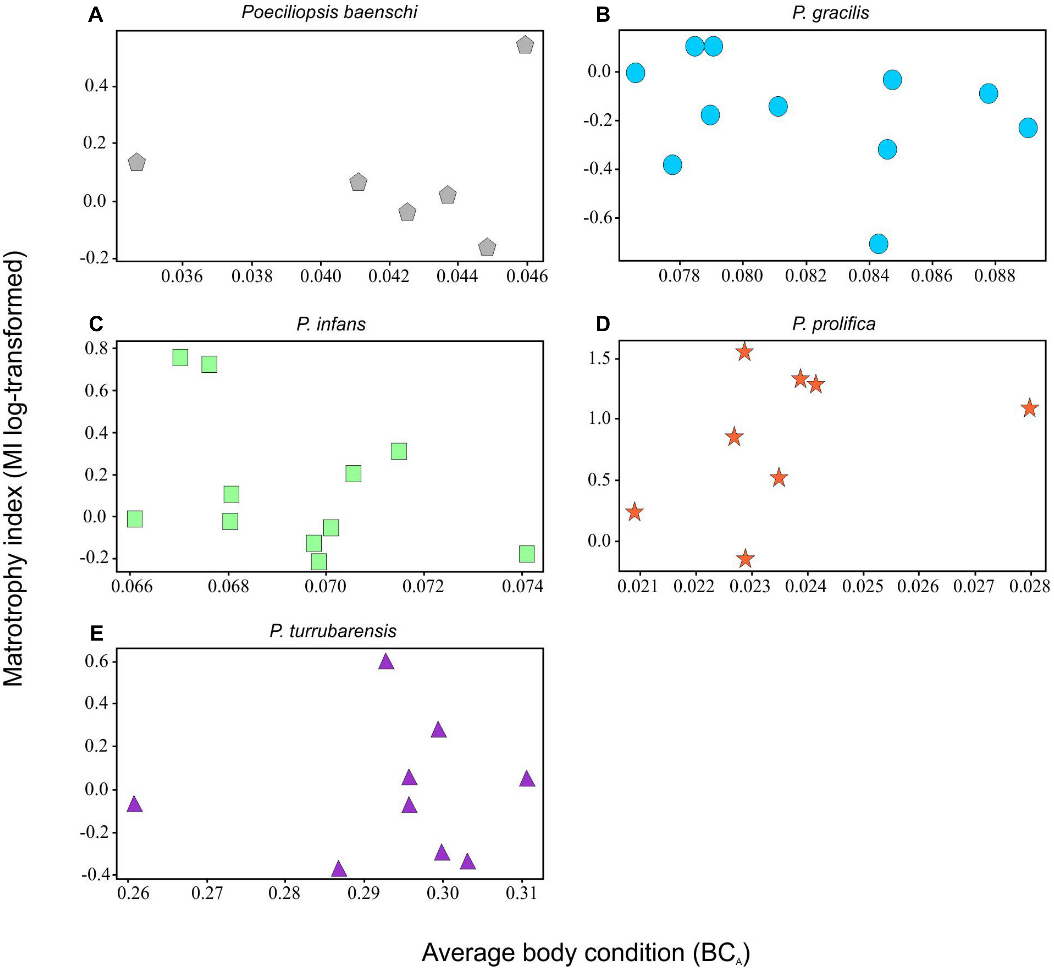

FIGURE 3 | Lack of association between the MI per population and average body condition (BC$_A$) for 45 populations of five fish species from the genus *Poeciliopsis*. Each species is shown in a different panel because of substantial differences among species in the values of body condition (BC). Each symbol represents a different species as per (**Figures 1**, **2**).

The weaker negative relationship between degree of matrotrophy and BC that we observed in *P. prolifica* confirms a lower physiological cost of actively providing embryos with nutrients during gestation for species that depend less on yolk reserves. Unlike *P. prolifica*, which exhibits a substantial amount of post-fertilization provisioning (MI = 5.40) and, hence, smaller amounts of yolk in mature ova, the other four species that we studied have a greater dependency on the yolk that is deposited into the eggs before fertilization for embryo nourishment (their MI values range between 0.72 and 1.58; **Table 1**; Reznick et al., 2002; Olivera-Tlahuel et al., 2015). Females of these four species lack specialized placentas that facilitate the transfer of nutrients from the mother to developing embryos and, instead, they have relatively simple maternal follicles that have the primary functions of gas exchange and osmoregulation (Kwan et al., 2015; Olivera-Tlahuel et al., 2019). Certainly, the observed variation in the degree of matrotrophy among individual females (**Figure 2**) as well as among populations (**Figure 1**) of these four species indicates that females are able to increase the amount of nutrients that are actively transferred to developing embryos after fertilization. Apparently, doing so without the specialized anatomical structures that are present in placental species such as *Poeciliopsis prolifica* and *Poeciliopsis turneri* (i.e., abundant microvilli and capillaries, thicker maternal follicles, numerous enlarged vesicles; Kwan et al., 2015; Olivera-Tlahuel et al., 2019) entails a greater energetic expenditure for gestating females of non-placental and yolk-dependent species that causes a detrimental effect on their somatic tissues and lipid reserves.

Caveats and Limitations

Even though previous studies have demonstrated that the index of stomach fullness and estimates of BC are accurate indicators of the amount and quality of food that is available in the surrounding environment (Simpkins and Hubert, 2000; Pothoven et al., 2001; Rikardsen et al., 2006), we recognize the potential limitations of using these proxies for food availability. These limitations may explain, at least partially, the lack of association between food availability and degree of matrotrophy

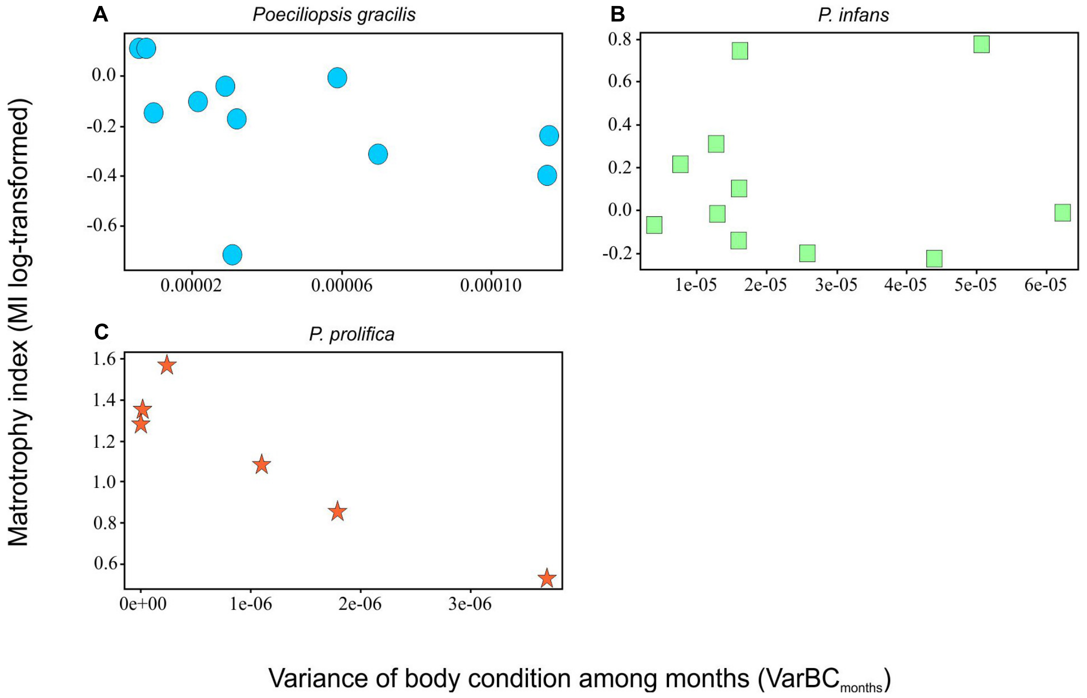

FIGURE 4 | Lack of association between the MI per population and the variance of body condition among months ($\text{VarBC}_{\text{months}}$) for 28 populations of three fish species from the genus *Poeciliopsis*. Each species is shown in a different panel because of substantial differences among species in the values of BC. Each symbol represents a different species as per (**Figures 1**, **2**).

that we observed in all five species. First, stomachs of females could be quite full, but most of their content may be of low nutritional value. For instance, females may have consumed abundant sediment or shells of mollusks that provide little to no energy (Langerhans et al., 2021). If this were the case, stomach fullness may not be correlated with the actual availability of nutritious food items.

Second, food availability, as well as stomach fullness and BC, may vary substantially over relatively short timescales (e.g., between weeks) and, hence, our collections of females may have occurred during an uncommon episode of low or high food abundance that does not reflect the general pattern of food availability in our study sites. Therefore, the lack of association between food availability and matrotrophy that we observed in all our focal species may have been caused by a mismatch between food availability at the time when our collections took place (e.g., low food availability during the past couple of days) and the observed degree of matrotrophy that was likely driven by the general long-term pattern of food availability of each population (e.g., high food availability during most of the past months).

Third, both stomach fullness and BC may be affected by factors other than food availability, such as temperature (which has a direct influence on digestive efficiency in ectotherms; Harlow et al., 1976), prevalence of pathogens (sick females may have poor BC even if food is abundant in the surrounding environment; Ageze and Menzir, 2018), and rates of activity (e.g., fish may maintain low foraging rates in the presence of abundant predators; Botham et al., 2006). Hence, females from populations where food is abundant may have less full stomachs or poor BC if these ecological conditions are suboptimal (low temperatures or abundant pathogens and predators). In these cases, neither stomach fullness nor BC would be reliable indicators of food availability. Therefore, the lack of associations between degree of matrotrophy and our proxies for food availability must be interpreted with caution, since future examination of additional measures of food availability, such as *in situ* abundances of algae and aquatic invertebrates (Quintans et al., 2009; Carbajal-Becerra et al., 2020), may provide supporting evidence for this prediction of the Trexler-DeAngelis model.

Finally, we must notice that our study is based on two important assumptions. (1) Populations differ in the average amount of food availability, which means that over the course of a relatively long period, a year for example, food availability is overall higher in a particular population whereas it is constantly lower in another. This assumption implies that our two proxies for food availability should reflect this same situation, namely, in the former population females must have fuller stomachs and better BC, whereas in the latter, females must have less full stomachs and poorer BC, regardless of the particular time of collection. If this assumption is true, then females experience the same regime of food availability during most of their reproductive lives (i.e., during most of their pregnancies), which in turn must promote the evolution of either reduced or advanced matrotrophy. (2) Fluctuations in food availability occur on relatively long timescales, such as among months or seasons. This means that a female

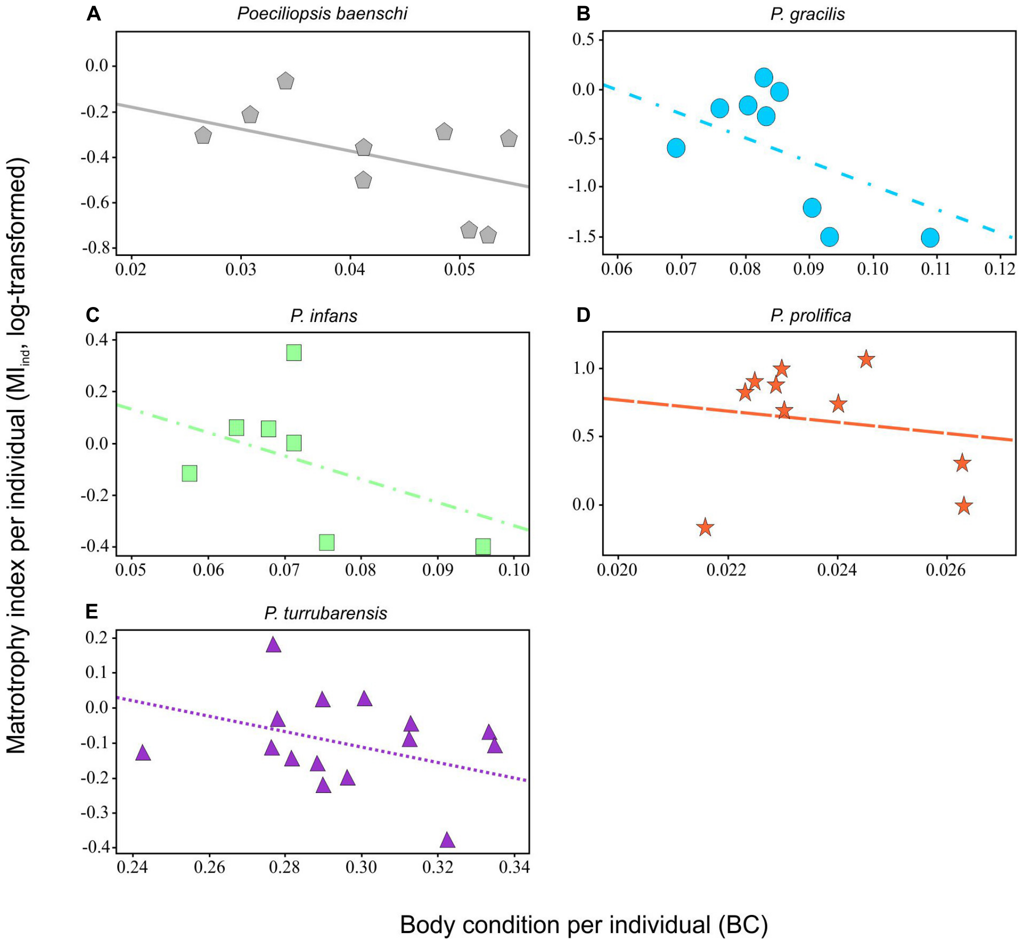

FIGURE 5 | Negative association between the $\mathrm{MI}_{\mathrm{ind}}$ and individual BC for 51 females of five fish species from the genus *Poeciliopsis*. Each species is shown in a different panel because of substantial differences among species in the values of BC. Fitted lines represent model-averaged regression coefficients. Each symbol represents a different species as per (**Figures 1**, **2**).

inhabiting a population where food supply fluctuates over time, experiences some months of food restriction and other months of abundant food. Given that gestation lasts approximately 30 days in poeciliid fishes (Veggetti et al., 1993; Bisazza and Marin, 1995), some broods are produced under limiting conditions and others under abundant food, which in the long term must promote the evolution of a predominantly lecithotrophic strategy. If these two assumptions are incorrect and food availability fluctuates drastically on shorter timescales (among weeks or days), then our statistical approach to estimate the degree of post-fertilization provisioning (a linear regression that predicts how embryo mass changes from fertilization to birth) may be invalid because the mass of embryos at different developmental stages, which depends to a large extent on the amount of food that females consume, would also change drastically within months or even weeks (during a single pregnancy). This, in turn, would promote short-term temporal changes in the overall degree of matrotrophy (i.e., in the MI). Thus, in summary, the temporal scale at which food availability fluctuates may be a critical aspect that could determine whether evidence in support of the Trexler-DeAngelis model is found or not.

Future Directions

The studies that have tested the Trexler-DeAngelis model of maternal provisioning have yielded mixed results, which may be attributed to different methodologies. Some studies were based on laboratory experiments (Marsh-Matthews and Deaton, 2006; Pires et al., 2007; Banet and Reznick, 2008; Banet et al., 2010; Pollux and Reznick, 2011; Itonaga et al., 2012; Van Dyke et al., 2014; Molina-Moctezuma et al., 2020), whereas others were conducted under natural conditions (Schrader and Travis, 2008, 2012; Riesch et al., 2013; Molina-Moctezuma et al., 2020). In controlled experiments, the amount of food and fluctuations in its availability are the only variables at play. Such experimental studies have provided evidence that abundant and constantly available food are positively associated with the amount of post-fertilization maternal provisioning, which supports the Trexler-DeAngelis model (Van Dyke et al., 2014; Molina-Moctezuma et al., 2020). In contrast, when data are obtained under natural conditions, several ecological factors interact and may mask the potential effects of food availability. Some of these studies have not found evidence in support of the Trexler-DeAngelis model (Schrader and Travis, 2008; Riesch et al., 2013). In addition to high and constant food availability, other ecological conditions may select for increased matrotrophy, such as a high predation risk. Given that matrotrophic embryos are substantially smaller during a large proportion of pregnancy, the ovarian mass and volume remain relatively small in gestating females, which provides them with an advantage in terms of swimming performance and escape velocity. Consistent with this hypothesis, in two poeciliid species, *P. retropinna* and *Phalloceros harpagos*, females from high-predation environments exhibit greater degrees of matrotrophy compared to females from low-predation environments (Gorini-Pacheco et al., 2018; Hagmayer et al., 2020). The combined effects of predation intensity and food availability on the relative amounts of pre- and post-fertilization provisioning to developing embryos have not been investigated yet and deserve further examination.

The Trexler-DeAngelis model was originally proposed using poeciliid fishes as model systems (Trexler and DeAngelis, 2003, 2010). To date, most empirical tests of this model have been conducted also on species of this group of viviparous fishes (Pires et al., 2007; Schrader and Travis, 2008, 2012; Pollux and Reznick, 2011). The few notable exceptions are three studies conducted on lizards (Cadby et al., 2011; Itonaga et al., 2012; Van Dyke et al., 2014). This means that we are still far from understanding if the causes and consequences of advanced degrees of matrotrophy are the same across diverse phylogenetic groups. The search for generalizations about the influence of food availability on post-fertilization maternal provisioning requires additional studies in other viviparous taxa such as some cartilaginous fishes and numerous invertebrates, which also exhibit wide variation in the relative amounts of pre- and post-fertilization maternal investment (Hamlett et al., 2005; Ostrovsky et al., 2016; Carter and Soma, 2020). These other taxa would be excellent model systems to test the predictions of the Trexler-DeAngelis model.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because we used only museum specimens.

AUTHOR CONTRIBUTIONS

JZ-V conceived the idea for the study and designed the statistical analyses. NS-S dissected the museum specimens, compiled the data, conducted the analyses, prepared the figures, and wrote the first draft of the manuscript. GR-C compiled part of the data and provided the ideas in all stages. All authors have contributed to the preparation, research, writing of the manuscript, reviewed and edited the manuscript, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.690430/full#supplementary-material

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