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RESEARCH ARTICLE

Multiple paternity in superfetatious live-bearing fishes

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Abstract

Superfetation, the ability to carry several overlapping broods at different developmental stages, has evolved independently multiple times within the live-bearing fish family Poeciliidae. Even though superfetation is widespread among poeciliids, its evolutionary advantages remain unclear. Theory predicts that superfetation should increase polyandry by increasing the probability that temporally overlapping broods are fertilized by different fathers. Here, we test this key prediction in two poeciliid species that each carry two temporally overlapping broods: *Poeciliopsis retropinna* and *P. turrubarensis*. We collected 25 females per species from freshwater streams in South-Eastern Costa Rica and assessed multiple paternity by genotyping all their embryos (420 embryos for *P. retropinna*; 788 embryos for *P. turrubarensis*) using existing and newly developed microsatellite markers. We observed a high frequency of unique sires in the simultaneous, temporally overlapping broods in *P. retropinna* (in 56% of the pregnant females) and *P. turrubarensis* (79%). We found that the mean number of sires within females was higher than the number of sires within the separate broods (2.92 sires within mothers vs. 2.36 within separate broods in *P. retropinna*; and 3.40 vs 2.56 in *P. turrubarensis*). We further observed that there were significant differences in the proportion of offspring sired by each male in 42% of pregnant female *P. retropinna* and 65% of female *P. turrubarensis*; however, this significance applied to only 9% and 46% of the individual broods in *P. retropinna* and *P. turrubarensis*, respectively, suggesting that the unequal reproductive success of sires (i.e. reproductive skew) mostly originated from differences in paternal contribution between, rather than within broods. Together, these findings tentatively suggest that superfetation may promote polyandry and reproductive skew in live-bearing fishes.

KEYWORDS
Poeciliidae, *Poeciliopsis*, polyandry, pregnancy, reproductive skew, reproductive strategy, superfetation, viviparity

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1 | INTRODUCTION

In some live-bearing animals, pregnant females can carry several overlapping broods that are in different developmental stages, a remarkable reproductive strategy referred to as superfetation (Gunn & Thresher, 1991; Reznick et al., 2007; Reznick & Miles, 1989; Roellig et al., 2011; Scrimshaw, 1944; Turner, 1937). Although superfetation purportedly occurs in a few mammal species, for example the European badger (*Meles meles*), the American mink (*Neovisonvison*) and the European brown hare (*Lepus europaeus*), these species do not appear to have proper superfetation (Roellig et al., 2011). In the American mink and European badger, fertilization of the second brood follows shortly after fertilization of the first, and implantation of these broods is simultaneous so that both broods are essentially in the same stage of development (a phenomenon that is referred to as superfertilization), and the European brown hare fertilizes her second brood shortly before parturition of the first, which means that there is little temporal overlap between the different broods (referred to as superconception; Roellig et al., 2011).

Among live-bearing fishes, superfetation is a more common reproductive strategy, being found in at least three different families from three distantly related orders: Clinidae (order Blenniiformes), Poeciliidae (order Cyprinodontiformes), and Zenarchopteridae (order Beloniformes) (Gunn & Thresher, 1991; Pollux et al., 2009, 2014; Reznick et al., 2007; Reznick & Miles, 1989; Scrimshaw, 1944; Turner, 1937; Wourms, 1981; Wourms et al., 1988). Superfetation is especially well-studied within the family Poeciliidae. In this family alone, superfetation evolved at least four times independently (Furness et al., 2019; Pollux et al., 2009, 2014), and variation in the degree of superfetation (defined as the number of simultaneous overlapping broods within the ovary) is observed among species, with the number of overlapping broods ranging from 2 to 14 (Pires, Banet et al., 2011; Pires, Bassar et al., 2011; Pollux & Reznick, 2011; Reznick & Miles, 1989; Scrimshaw, 1944; Turner, 1937). Even though superfetation is widespread in some live-bearing families, it remains unclear what its evolutionary advantages are (Zúñiga-Vega et al., 2010). The main hypotheses regarding its evolutionary advantages fall broadly into two categories: (a) superfetation may convey an adaptive advantage to females by reducing the peak cost of reproduction or (b) it may facilitate polyandry (i.e. female multiple mating).

The first category includes hypotheses that argue that superfetation conveys a benefit to females during pregnancy by reducing the peak cost associated with a live-bearing mode of reproduction. The argument is that superfetation is characterized by more frequent production of broods, typically in association with smaller brood sizes (Reznick & Miles, 1989; Thibault & Schultz, 1978). Theory predicts that this, all else being equal, should lead to lower peak reproductive allocation (the proportion of female mass allocated to reproduction) for species with superfetation compared with non-superfetatious species, notably without affecting their total reproductive output (Downhower & Brown, 1975; Pollux et al., 2009; Thibault & Schultz, 1978). There are in principle two ways in which a lower peak reproductive allocation and more frequent brood production may be beneficial for females. (a) Superfetation may be a favourable strategy in unstable environments by spreading reproduction across time. If the quality of the environment that a female's offspring will experience after birth influences their probability of survival and unpredictably fluctuates through time, then selection may favour a more frequent production of smaller broods over less frequent production of a single large brood (non-superfetation). Superfetation may thus act as a bet-hedging strategy to reduce the variance in fitness (Burley, 1980; Travis et al., 1987). To date, this theory has not been supported by empirical data. (b) Another way in which superfetation can potentially convey an adaptive advantage to females is by reducing their peak reproductive burden during pregnancy, which may lead to improved locomotor performance (Pires, Banet et al., 2011; Pires, Bassar et al., 2011; Pollux et al., 2009; Thibault & Schultz, 1978). In live-bearing fish, a lower reproductive allocation is associated with a more streamlined body shape (Fleuren et al., 2018, 2019), lower body drag (Quicazan-Rubio et al., 2019), higher sustained swimming performance (Plaut, 2002), improved fast-start escape response (Fleuren et al., 2019; Ghalambor et al., 2004) and enhanced probability of surviving a predator attack (Laidlaw et al., 2014; Plath et al., 2011; Walker et al., 2005). This suggests that superfetation may be beneficial when living in performance-demanding environments, such as environments that are predator rich or fast-flowing. While some studies provide tentative evidence for an adaptive advantage of superfetation in natural populations of *Poeciliopsis turrubarensis*, *P. retropinna* and *P. paucimaculata* (family Poeciliidae) that inhabit fast-flowing (micro)habitats in Costa Rican rivers (Hagmayer et al., 2021; Zúñiga-Vega et al., 2007), studies on other *Poeciliopsis* species do not provide support for a potential benefit in fast-flowing (Frías-Alvarez et al., 2014; Frías-Alvarez & Zúñiga-Vega, 2016) or high predation environments (Hagmayer, Furness, Reznick et al., 2020).

The second category includes hypotheses that argue superfetation may bestow benefits to females by facilitating multiple paternity (Zúñiga-Vega et al., 2010). There are two ways in which superfetation may achieve this. First, superfetatious females have more moments in time in which they initiate and fertilize new broods, compared with non-superfetatious species (Yamaguchi et al., 2004, 2006). This allows them to more easily 'trade-up' by remating with males regarded to be of better quality (e.g. based on behavioural or phenotypical cues) than those previously mated with (Halliday, 1983; Jennions & Petrie, 2000; Pitcher et al., 2003) and then use the sperm derived from the most recent mating event to fertilize their next brood ('last male sperm precedence'; Halliday, 1983) resulting in a high probability that the temporally overlapping broods are sired by different fathers. Second, in live-bearing species that provide maternal provisioning after fertilization, embryos could potentially influence this investment during gestation, creating potential for genomic conflicts (Haig, 1993; Zeh & Zeh, 2000, 2003). Theory predicts that the emergence of these genomic conflicts should drive a shift from a reliance on pre-copulatory mate choice to increasing levels of polyandry in

conjunction with post-zygotic mechanisms of sexual selection (Zeh & Zeh, 2000, 2003). It has been postulated that superfetation could promote polyandry by diminishing the probability of a single male monopolizing a female's offspring, instead creating opportunities for offspring to be sired by different males. These females could then rely on the expression of the paternal genomes to induce differential maternal investment among the embryos and, in extreme cases, divert resources from genetically defective or incompatible embryos to viable or compatible ones (Crespi & Semeniuk, 2004; Haig, 1990, 1993; Pollux et al., 2014; Wilkins & Haig, 2003; Zeh & Zeh, 2000).

Finally, it is noteworthy to emphasize that these two broad hypotheses are not mutually exclusive. For example, it is possible that superfetation initially evolved because it conveyed an adaptive benefit to pregnant females, but once established acquired an additional function to facilitate polyandry. Whereas several studies have investigated the potential adaptive benefit of superfetation in performance-demanding conditions (Fleuren et al., 2019; Thibault & Schultz, 1978; Travis et al., 1987; Zúñiga-Vega et al., 2007), the idea that superfetation may influence polyandry has not yet been subject to similar investigation. Here, we study patterns of multiple paternity in truly superfetatious animals. We aim to test if, and two what extent, superfetation is associated with the occurrence of unique fathers in the simultaneously overlapping broods of pregnant females. We study this in two live-bearing fish species (family Poeciliidae), *Poeciliopsis retropinna* and *P. turrubarensis* that co-occur in freshwater streams in Costa Rica. These closely related species have a similar degree of superfetation, often carrying two simultaneously developing broods, which makes them an excellent model to test for differences in sire diversity between overlapping broods. Specifically, in this study, we investigate (a) to what extent overlapping broods are sired by unique fathers and/or share the same sires; and (b) to what extent the sires in the overlapping broods contribute to reproductive skew (defined as the partitioning of reproduction among sires).

2 | METHODS

2.1 | Study species

Poeciliopsis (subgenus *Aulophallus*) *retropinna* (Figure S1a) and *Poeciliopsis turrubarensis* (Figure S1b) are found in middle America, where they occur in Pacific-slope drainages south of the Trans-Mexican Volcanic Belt (Mateos et al., 2002). *P. retropinna* occurs from the Río Grande de Térraba (Costa Rica) to the Río Chiriquí drainage (Panama) (Bussing, 2002; Mateos et al., 2002). *Poeciliopsis turrubarensis* has a somewhat wider distribution and is found from Jalisco, Mexico to the Rio Dagua in Colombia (Mateos et al., 2002). In locations where they co-occur, adult *P. retropinna* occupy slightly deeper and faster flowing microhabitats in the river compared to *P. turrubarensis* (Hagmayer et al., 2021). Both species live in social groups, where males are persistently pursuing females in an attempt to copulate with them. Details on male mating behaviour of *P. retropinna* and *P. turrubarensis*, obtained by means of underwater visual census (following Furness et al., 2020), are given in Figure S3. The males have small bodies, long genitalia (i.e. copulatory organs called gonopodia) and lack bright coloration, conspicuous ornamental display traits and courtship behaviour, a combination of traits that is typically associated with sneak or coercive mating behaviour (Furness, Avise et al., 2021, Furness, Hagmayer et al., 2021; Pollux et al., 2014). Both species exhibit superfetation, carrying on average two broods per female, both in *P. turrubarensis* (Zúñiga-Vega et al., 2007) and *P. retropinna* (Hagmayer, Furness & Pollux, 2020; Hagmayer, Furness, Reznick et al., 2020).

2.2 | Sampling

Specimens of *Poeciliopsis retropinna* and *P. turrubarensis* were collected from the Terraba-General and Coto drainages (Province Puntarenas, Costa Rica) from February to March 2017 (dry season), using seine and cast nets. To minimize the potential effect of local environmental conditions on multiple paternity, fishes were collected from different locations (Table 1; Figure S2). In each location, we collected gravid females to quantify multiple paternity, plus additional individuals to estimate population allele frequencies (Table 1). This resulted in the collection of 105 individuals for *P. retropinna* and 142 individuals for *P. turrubarensis*, from five and six different locations, respectively, resulting in 18–25 individuals per sampling location (Table 1). All collected specimens were immediately euthanized in the field using MS-222 (Tricaine methanesulfonate) and preserved in 96% ethanol. The fish were subsequently transported to Wageningen University (the Netherlands) and stored at 4°C until further processing.

Of these 105 *P. retropinna* and 145 *P. turrubarensis*, we selected 25 pregnant females per species for life history measurements and molecular paternity analyses. We measured their total length (TL), standard length (SL), total wet mass, fecundity (total number of embryos in utero), wet mass of the reproductive tissue (ovary with embryos) and the reproductive allocation (RA; calculated by dividing the wet mass of the ovary by the total wet mass of the female) (Table S1). The number of broods per female was determined by staging the developing embryos, following Haynes (1995) classification for poecilid fishes. All the embryos (i.e. 420 embryos for *P. retropinna* and 788 for *P. turrubarensis*) were genotyped using microsatellites to quantify multiple paternity.

2.3 | DNA extraction

To ensure that sufficient DNA could be extracted from the embryos, we selected pregnant females with broods that were at least in developmental stage 10–15 (Haynes, 1995). We then extracted DNA from all their embryos (i.e. N_{total} = 420 embryos for *P. retropinna*; N_{total} = 788 embryos for *P. turrubarensis*, respectively). Entire embryos and tailfin clips from adults were taken and dried for 10 min

TABLE 1 Sampling locations, sampling dates, total number of field-collected individuals and number of analyzed pregnant females per location for the two study species *Poeciliopsis retropinna* and *P. turrubarensis* (family Poeciliidae)

Species	Sampling location[a]	Sampling date	Latitude	Longitude	Total number of field-collected individuals[b]	Number of pregnant females analyzed[c]
P. retropinna	Rio Ceibo	16.03.2017	9.216445	−83.3154	21	4
P. retropinna	Rio Coloradito	05.03.2017	8.598738	−82.8777	21	8
P. retropinna	Rio Conte	07.03.2017	8.438241	−83.0429	24	4
P. retropinna	Rio Pedigroso	12.03.2017	9.356514	−83.7196	18	2
P. retropinna	Rio Sucio	03.03.2017	8.809893	−82.9105	21	7
					105	25
P. turrubarensis	Rio Canaza	08.03.2017	8.649891	−83.1815	25	2
P. turrubarensis	Rio Coloradito	05.03.2017	8.598738	−82.8777	21	2
P. turrubarensis	Rio Conte	07.03.2017	8.438241	−83.0429	23	2
P. turrubarensis	Rio Incendio	07.03.2017	8.443007	−82.9956	25	6
P. turrubarensis	Rio Tigre	10.03.2017	8.547105	−83.3336	24	8
P. turrubarensis	Rio Vacca	10.03.2017	8.433390	−82.9665	24	5
					142	25

[a]Photographs of the sampling locations are given in Figure S2.
[b]These individuals were used to calculate location specific allele frequencies.
[c]The number of embryos analyzed per female is given in Figures 1 and 2.

at room temperature (20–25°C) and stored at −20°C before DNA extraction. For the DNA extraction, we used the following adapted protocol from the Wizard Genomic DNA purification kit (Promega). 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 16 000 g for 1 min to form a pellet. Next, the supernatant was decanted and the pellet (DNA) was washed with 300 µl of 70% cooled ethanol (0°C), mixed, centrifuged at 16 000 g for 2 min and dried at room temperature for 20–30 min. Finally, the pellet was dissolved in 25 µl Tris (10 mM pH 8.0) for embryonic samples and in 50 µl Tris (10 mM pH 8.0) for tailfin samples. The stock DNA was diluted to 20 ng/µl for further analyses.

2.4 | Microsatellite selection and development

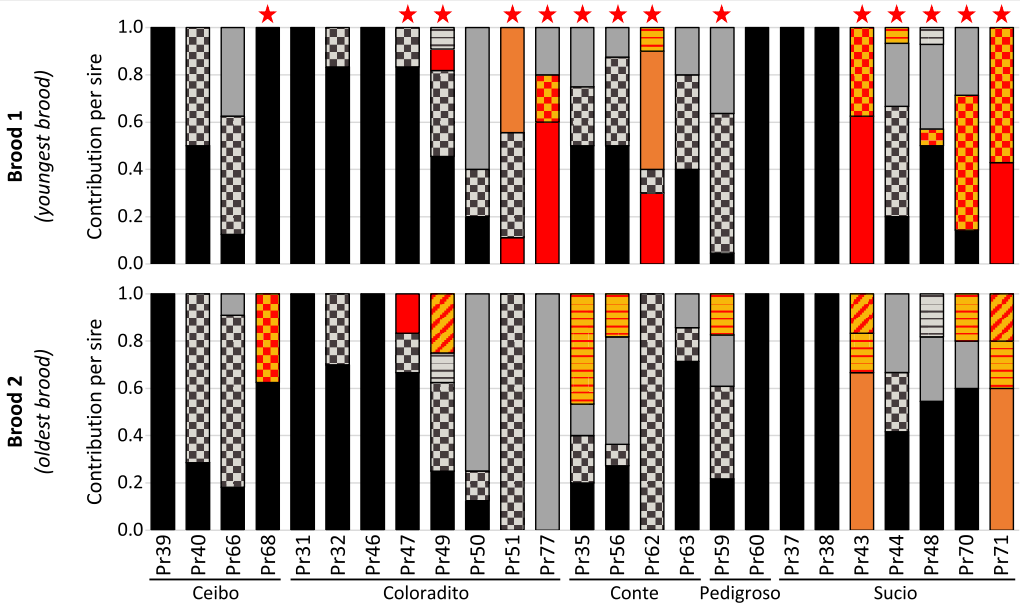
We assessed paternity in *P. retropinna and P. turrubarensis* using microsatellite markers. We first tested existing microsatellite markers developed for other poeciliid species (Kelly et al., 1999; Parker et al., 1998; Soucy & Travis, 2003; Tonhatti et al., 2014; Walter et al., 2004; Yue & Orban, 2004) and checked for cross-amplification and polymorphism in our two study species. Four of the tested markers cross-amplified in *P. turrubarensis*, were highly polymorphic and hence used to estimate multiple paternity in *P. turrubarensis* (Table S2). Six markers that cross-amplified in *P. retropinna* were moderately polymorphic. Therefore, we developed three additional new microsatellite markers using a recently published genome for *P. retropinna* (van Kruistum et al., 2020, 2021). We identified a total of 59 repetitive regions and subsequently developed eight putative microsatellite markers based on three criteria. First, we selected dinucleotide repeats, because these loci generally contain more repeat units than tri-, tetranucleotide repeats (Kelkar et al., 2008). Second, we selected repeats that showed the least interruptions/mutations within repeats. Third, we selected markers with at least 10 repeats, as longer markers are generally more variable (Kelkar et al., 2008). For the eight selected markers, primers were designed and tested on 11 female *P. retropinna* samples from the five sampling locations to check for polymorphism, allele frequency distribution and heterozygosity. Of these eight microsatellite markers, three showed a high degree of polymorphism, a high degree of heterozygosity, and the most balanced allele frequency distribution. Therefore, these three newly developed microsatellite markers (Pretr7-2, Pretr33-1, Pretr49-1; for marker specific DNA sequences see Table S3) were used for further analysis together with the six markers from the literature, resulting in a total of nine microsatellite loci to detect multiple paternity in *P. retropinna* (Table S2).

2.5 | Microsatellite analysis

Each microsatellite locus was amplified by a polymerase chain reaction in 15 µl, with annealing temperatures and number of cycles shown in Table S2. We checked PCR products by gel

(a)

	Pr39	Pr40	Pr66	Pr68	Pr31	Pr32	Pr46	Pr47	Pr49	Pr50	Pr51	Pr77	Pr35	Pr56	Pr62	Pr63	Pr59	Pr60	Pr37	Pr38	Pr43	Pr44	Pr48	Pr70	Pr71
Total # offspring	*12*	*13*	*19*	*16*	*9*	*16*	*12*	*12*	*19*	*13*	*16*	*9*	*27*	*19*	*11*	*12*	*45*	*24*	*15*	*11*	*14*	*27*	*25*	*12*	*12*
# offspring brood 1	7	6	8	8	4	6	7	6	11	5	9	5	12	8	10	5	22	10	7	6	8	15	14	7	7
# offspring brood 2	5	7	11	8	5	10	5	6	8	8	7	4	15	11	1	7	23	14	8	5	6	12	11	5	5
Total # sires	*1*	*2*	*3*	*2*	*1*	*2*	*1*	*3*	*5*	*3*	*3*	*3*	*4*	*4*	*4*	*3*	*4*	*1*	*1*	*1*	*5*	*4*	*4*	*4*	*5*
# shared sires brood 1 & 2	1	2	3	1	1	2	1	2	3	3	1	1	3	3	1	3	3	1	1	1	0	3	3	2	0
# unique sires brood 1	0	0	0	0	0	0	0	0	**1**	0	**2**	**2**	0	0	**3**	0	0	0	0	0	**2**	**1**	**1**	**1**	**2**
# unique sires brood 2	0	0	0	**1**	0	0	0	**1**	**1**	0	0	0	**1**	**1**	0	0	**1**	0	0	0	**3**	0	0	**1**	**3**



(b)

	Pr39	Pr40	Pr66	Pr68	Pr31	Pr32	Pr46	Pr47	Pr49	Pr50	Pr51	Pr77	Pr35	Pr56	Pr62	Pr63	Pr59	Pr60	Pr37	Pr38	Pr43	Pr44	Pr48	Pr70	Pr71
Jaccard index	-	*0*	*0*	*.50*	-	*0*	-	*.33*	*.40*	*0*	*.67*	*.67*	*.25*	*.25*	*.75*	*0*	*.25*	-	-	-	*1*	*.25*	*.25*	*.50*	*1*
Bray-Curtis index	-	*.21*	*.28*	*.38*	-	*.13*	-	*.17*	*.30*	*.15*	*.56*	*.80*	*.47*	*.51*	*.90*	*.31*	*.35*	-	-	-	*1*	*.28*	*.16*	*.66*	*1*
Skew per female	-	*.05*	***.29***	***.28***	-	***.20***	-	***.44***	*.33*	***.37***	***.38***	*.23*	*.05*	*.14*	*.22*	*.23*	***.28***	-	-	-	*.25*	*.20*	***.36***	*.14*	*.20*
Skew brood 1	-	0	.18	-	-	.31	-	.31	.30	.24	.18	.24	.11	.18	.31	.07	**.31**	-	-	-	.06	.25	.36	.22	.02
Skew brood 2	-	.16	**.42**	.06	-	.14	-	.33	.11	**.44**	-	-	.21	.22	-	.40	.10	-	-	-	.33	.04	.18	.24	.24

FIGURE 1 Patterns of multiple paternity in 25 pregnant females of the superfetatious species *Poeciliopsis retropinna* (family Poeciliidae) collected from different rivers in Costa Rica (see Sampling location). Each of the 25 females (see Female ID) carried two simultaneous overlapping broods that were in a different stage of embryonic development: brood 1 (the youngest brood) and 2 (the oldest brood). (a) The table shows for each female the brood size, number of unique sires (i.e., sires that are only present in one of the two broods; indicated in bold red) and the number of shared sires (i.e., sires that are found in both broods) in brood 1 and 2. The graph shows for each female the proportional contribution of each of these sires. Within each female, different colours in the bars indicate different sires: shared sires between the two broods are represented by black/grey colours and unique sires are represented by orange/red colours. A red star at the top of the bars indicates the presence of a unique sire in one, or both, of the broods of a female, showing that in *P. retropinna* 14 out of the 25 females (56%) carried a brood that was sired by unique fathers. (b) Indices of reproductive skew: i.e., the Jaccard index, Bray-Curtis index and paternity skew per female, as well as the skew within each brood separately. When the observed paternal contribution among offspring differed significantly from the expected (equal) contribution (goodness-of-fit χ^2-tests, $p < 0.05$) this is shown in bold, meaning that paternity was significantly skewed for those broods or females

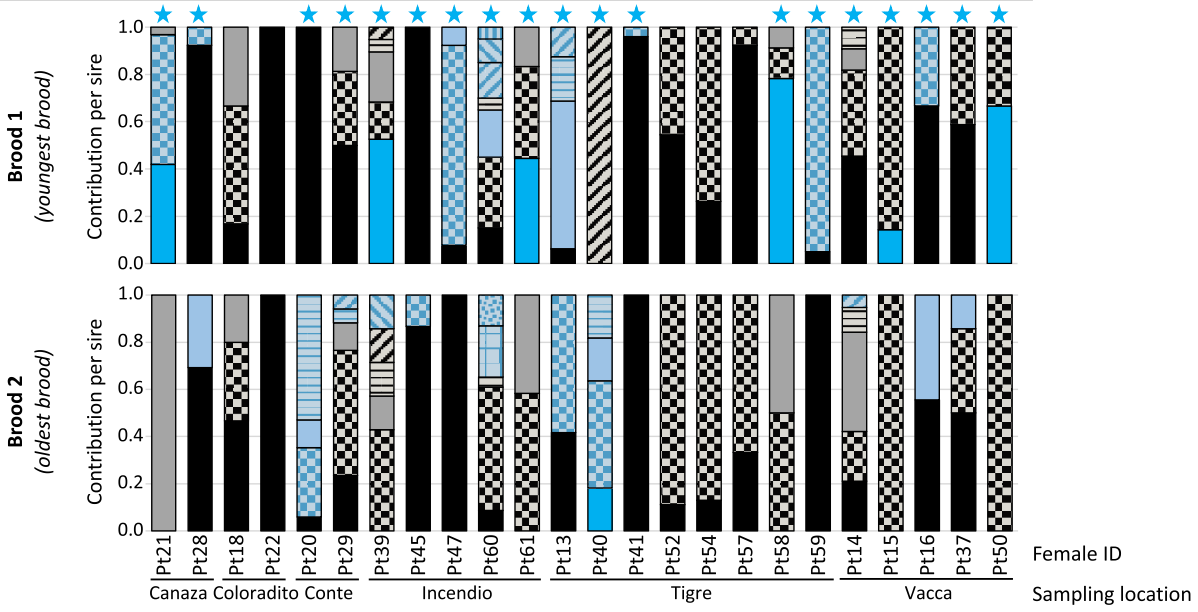
electrophoresis, and diluted samples based on band thickness. We used 1 μl of the diluted and pooled PCR samples combined with 9 μl ladder mix (0.5% (v/v)) Liz500 size ladder (GeneScan) to perform capillary gel electrophoresis using an ABI3730 Sequencer (Applied Biosystems). Fragment analysis was made using GeneMapper (Applied Biosystems). We scored peaks with automatic binning but checked all samples manually and rescored when automatic scoring errors occurred. Allele count, range, observed, and expected heterozygosity were calculated based on 18–25 field-collected individuals per population using GenAlex version 6.5 (TablesS4 and S5; add-in for Excel, Peakall & Smouse, 2006, 2012).

2.6 | Paternity analysis

Molecular paternity assignments were calculated based on the genotype of the mother and her offspring with population-specific allele frequencies that were obtained from the genotypes of 18 to 25 additional field-collected specimens per location, using nine microsatellite markers in *P. retropinna* and four microsatellite markers in *P. turrubarensis*. Multiple paternity was estimated using two software programs: GERUD version 2.0 (Jones, 2005) and COLONY version 2.0 (Jones & Wang, 2010). GERUD is an exclusion-based method, that estimates the minimum number of sires for multi-locus data using codominant markers, such as microsatellites (Jones, 2005). It

(a)

	Pt21	Pt28	Pt18	Pt22	Pt20	Pt29	Pt39	Pt45	Pt47	Pt60	Pt61	Pt13	Pt40	Pt41	Pt52	Pt54	Pt57	Pt58	Pt59	Pt14	Pt15	Pt16	Pt37	Pt50
Total # offspring	*46*	*26*	*27*	*21*	*34*	*33*	*26*	*34*	*18*	*43*	*30*	*28*	*37*	*36*	*20*	*42*	*19*	*39*	*40*	*30*	*24*	*21*	*31*	*19*
# offspring brood 1	31	13	12	11	17	16	19	19	13	20	18	16	26	25	11	19	13	23	20	11	14	3	17	9
# offspring brood 2	15	13	15	10	17	17	7	15	5	23	12	12	11	11	9	23	6	16	20	19	10	18	14	10
Total # sires	*3*	*3*	*3*	*1*	*4*	*5*	*6*	*2*	*3*	*9*	*3*	*5*	*5*	*2*	*2*	*2*	*2*	*3*	*2*	*5*	*2*	*3*	*3*	*2*
# shared sires brood 1 & 2	1	1	3	1	1	3	4	1	1	3	2	1	0	1	2	2	2	2	1	4	1	1	2	1
# unique sires brood 1	**2**	**1**	0	0	0	0	**1**	0	**2**	**4**	**1**	**3**	**1**	**1**	0	0	0	**1**	**1**	0	**1**	**1**	0	**1**
# unique sires brood 2	0	**1**	0	0	**3**	**2**	**1**	**1**	0	**2**	0	**1**	**4**	0	0	0	0	0	0	**1**	0	**1**	**1**	0



(b)

	Pt21	Pt28	Pt18	Pt22	Pt20	Pt29	Pt39	Pt45	Pt47	Pt60	Pt61	Pt13	Pt40	Pt41	Pt52	Pt54	Pt57	Pt58	Pt59	Pt14	Pt15	Pt16	Pt37	Pt50
Jaccard index	*.67*	*.67*	*0*	-	*.75*	*.40*	*.33*	*.50*	*.67*	*.67*	*.33*	*.80*	*1*	*.50*	*0*	*0*	*0*	*.33*	*.50*	*.20*	*.50*	*.67*	*.33*	*.50*
Bray-Curtis index	*.97*	*.31*	*.30*	-	*.94*	*.33*	*.59*	*.13*	*.92*	*.57*	*.17*	*.94*	*1*	*.04*	*.43*	*.13*	*.59*	*.78*	*.95*	*.40*	*.14*	*.44*	*.14*	*.67*
Skew per female	*.01*	***.51***	*.03*	-	***.33***	***.41***	***.34***	***.44***	***.32***	***.51***	***.21***	*.21*	***.62***	***.47***	*.08*	***.28***	***.18***	*.07*	*.00*	*.24*	***.41***	***.30***	***.27***	*.12*
Skew brood 1	**.30**	.42	.14	-	-	.13	**.43**	-	**.54**	.25	.11	**.44**	-	**.46**	.01	**.18**	**.42**	**.48**	**.45**	**.30**	**.34**	.10	.03	.10
Skew brood 2	-	.13	.10	-	**.35**	**.44**	.25	**.35**	-	**.42**	.03	.03	.18	-	**.38**	**.35**	.10	0	-	.29	-	.01	.16	-

FIGURE 2 Patterns of multiple paternity in 24 pregnant females of the superfetatious species *Poeciliopsis turrubarensis* (family Poeciliidae) collected from different rivers in Costa Rica (see Sampling location). Each of the 24 females (see Female ID) carried two simultaneous overlapping broods that were in a different stage of embryonic development: brood 1 (the youngest brood) and 2 (the oldest brood). (a) The table shows for each female the brood size, number of unique sires (i.e., sires that are only present in one of the two broods; indicated in bold blue) and the number of shared sires (i.e., sires that are found in both broods) in brood 1 and 2. The graph shows for each female the proportional contribution of each of these sires. Within each female, different colours in the bars indicate different sires: shared sires between the two broods are represented by black/grey colours and unique sires are represented by blue colours. A blue star at the top of the bars indicates the presence of a unique sire in one, or both, of the broods of a female, showing that in *P. turrubarensis* 19 out of the 24 females (79%) carried a brood that was sired by unique fathers. (b) Indices of reproductive skew: i.e., the Jaccard index, Bray-Curtis index and paternity skew per female, as well as the skew within each brood separately. When the observed paternal contribution among offspring differed significantly from the expected (equal) contribution (goodness-of-fit χ^2-tests, $p < 0.05$) this is shown in bold, meaning that paternity was significantly skewed for those broods or females

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). Missing data are not accepted in GERUD, therefore offspring that lacked data for one or more markers were excluded from this analysis. In addition, we calculated the expected number of sires in COLONY by maximum likelihood methods that use simulated annealing to search for a global optimum (Jones & Wang, 2010). The program uses multi-locus data and considers all mothers and embryos jointly for paternity assignments (Jones & Wang, 2010). We performed a full-likelihood analysis in COLONY per sampling location, as population allele frequencies are used to determine the number of compatible fathers. 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 we did not include a prior for sibship size in the analysis. COLONY can run with missing data, but with limited genetic information paternity estimates become less accurate (Sefc & Koblmüller, 2009), thus we excluded samples for which we had genotype data of less than three markers (this rarely occurred and only in offspring samples with lower quality DNA). Finally, we checked the correlation between the number of sires obtained with the two different programs (GERUD and COLONY) using a Spearman rank correlation coefficient in R v 4.1.0 (R Core Team, 2019).

2.7 | Paternity skew

Reproductive skew refers to the partitioning of reproduction among same sex individuals, or, in other words, the degree to which reproduction is shared equally among males (low skew) or monopolized by a few or even a single male (high skew). We assessed paternity skew for each female by quantifying the (relative) contribution of sires, in three ways. First, we estimated the difference in paternal contribution between broods using two β-diversity dissimilarity indices: (a) the Jaccard-index (Jaccard, 1900) and (b) Bray-Curtis-index (Bray & Curtis, 1957). These indices are widely applied in biodiversity research and are usually used to estimate the difference in species diversity between two communities in a certain area (Anderson et al., 2006), but here we used them to quantify the difference in sire diversity between the two overlapping broods of each female. The Jaccard-index quantifies between-brood heterogeneity in paternity based solely on sire occurrence and is defined as the proportion of unshared sires to the total number of sires of two broods (Jaccard, 1900):

$$d_J = \frac{(b+c)}{(a+b+c)},$$

where a is the number of sires shared, b the number of sires in brood 1 that do not occur in brood 2, and c the number of sires in brood 2 that do not occur in brood 1.

The Bray-Curtis-index additionally includes information on the relative sire abundance and is defined as the proportional dissimilarity between broods based on sire abundances (Bray & Curtis, 1957):

$$d_{BC} = \frac{\sum_{k=1}^{p} |\omega_{1k} - \omega_{2k}|}{\sum_{k=1}^{p} (\omega_{1k} + \omega_{2k})},$$

where ω_{1k} is the abundance of sire k in brood 1, ω_{2k} the abundance of sire k in brood 2, and p the total number of sires across both broods. Second, we estimated whether the contribution of sires was skewed towards one or more sires, by calculating the paternity skew (a) within each brood separately and (b) within each female (i.e. with the sum of all offspring of both overlapping broods combined). The paternity skew was calculated following Neff et al. (2008) using the data obtained in COLONY. We tested whether the observed contribution of sires in a brood significantly deviated from the expected contribution if there was no skew (i.e., equal contribution) by means of χ^2-tests for goodness of fit (Green et al., 2017; Yue & Chang, 2010). Finally, to test whether the paternal contribution of males to successive overlapping broods within females declined over time, we modelled the proportional contribution of males to the two consecutive broods using generalized linear mixed models (i.e. the glmer function from the lme4 package) with a logit link for the binomial-distributed response in R v 4.1.0 (R Core Team, 2019). Analyses were performed separately for each of the two study species and included time as a fixed factor (2 levels: Brood 2 and Brood 1). To control for the effect of brood size in the analyses, paternal contribution was weighted by brood size using the 'weights' argument. Male- and female identity were included as random effects to correct for between male and between female variation in paternal contribution that is not accounted for by the fixed effects.

3 | RESULTS

3.1 | Poeciliopsis retropinna

3.1.1 | Multiple paternity

All 25 *P. retropinna* females that were analysed for multiple paternity had two broods (meaning that we studied a total of 50 broods in *P. retropinna*), with an average (± *SE*) brood size of 8.40 ± 0.59 (range: 1–23 embryos per brood; Figure 1a). Multiple paternity was observed in 15 out of 25 (60%) *P. retropinna* females when it was calculated in GERUD, and in 19 out of 25 females (76%) when it was estimated with COLONY. The minimum number of sires obtained with GERUD was 1.80 ± 0.16 (range: 1–4), and the estimated number of sires obtained with COLONY was on average 2.92 ± 0.27 (range: 1–5) per female (Figure 1a). The sires in two overlapping broods within a female can either be shared among broods (i.e., shared sires; in Figure 1a represented by gray/black bar colours) or only be present in one of the two broods (i.e., unique sires; represented by orange/red bar colours). Figure 1a shows that 14 out of the 25 *P. retropinna* females (56%) were carrying at least one brood that was sired by unique fathers (indicated by a red star at the top of the bars).

3.1.2 | Paternity skew

For each female, the proportional contributions of the sires in the 2 broods are visualized in Figure 1a. Three measures of paternity skew were calculated for the 19 *P. retropinna* females with multiple paternity (Figure 1b). First, the mean (± *SE*) Jaccard-index, which varies from 0 (i.e., if all sires are shared among the two broods) to 1 (i.e., if all sires differ between the two broods), was 0.37 ± 0.07 (range: 0–1) (Figure 1b). Second, the mean (± *SE*) Bray-Curtis index (similar to the Jaccard-index, but which takes the sire abundance into account), which varies from 0 (indicating that the same sires are shared among the two broods with an equal distribution) to 1 (indicating that no sires are shared among the two broods) was 0.45 ± 0.06 (range: 0.13–1.00) (Figure 1b). Third, the mean (± *SE*) paternity skew was 0.24 ± 0.02 (range: 0.05–0.44; Figure 1b), with 8 out of the 19 (42%) females with multiple paternity having observed paternal contributions that differed significantly from an expected equal contribution (goodness-of-fit χ^2-tests, $p < 0.05$), indicating that in these females paternity was significantly skewed (Figure 1b). Finally, paternity skew was also calculated for each of the broods separately showing that in only 3 out of 34 (9%) broods with multiple paternity significant skew was observed (goodness-of-fit χ^2-tests, $p < 0.05$; Figure 1b).

3.2 | Poeciliopsis turrubarensis

3.2.1 | Multiple paternity

In *P. turrubarensis*, 24 out of 25 females had two broods, and one female had three broods (meaning that we studied a total of 51 broods in *P. turrubarensis*; Table S1). To enable comparisons among females, we only report the 24 females that carried two overlapping broods (Figure 2). The average (± *SE*) brood size of *P. turrubarensis* was 15.08 ± 0.82 embryos (range: 3–31 embryos per brood; Figure 2a). Almost all *P. turrubarensis* females had multiple paternity, it was observed in 22 (92%, GERUD) and 23 (96%, COLONY) out of 24 females, respectively. The minimum number of sires calculated with GERUD was on average 2.48 ± 0.18 (range: 1–5), and the estimated number of sires obtained with COLONY was on average 3.40 ± 0.34 (range: 1–9) per female. The majority of the *P. turrubarensis* females carried unique sires, with 19 of the 24 females (>79%) having at least one brood that was sired by unique fathers (indicated by a blue star at the top of the bars in Figure 2a).

3.2.2 | Paternity skew

Three measures of reproductive skew were calculated for the 23 females that had multiple paternity, revealing a mean (a) Jaccard-index of 0.45 ± 0.06 (range: 0–1), (b) Bray-Curtis index of 0.52 ± 0.07 (range: 0.04–1) and (c) paternity skew of 0.28 ± 0.04 (range: 0–0.62), with 15 out of the 23 (65%) females with multiple paternity showing significant skew (Figure 2b; goodness-of-fit χ^2-tests, $p < 0.05$). Finally, paternity skew was also calculated for each of the broods separately, showing that in only 17 out of 37 (46%) broods with multiple paternity a significant skew was observed (goodness-of-fit χ^2-tests, $p < 0.05$; Figure 2b).

4 | DISCUSSION

4.1 | Unique sires among broods

We found unique sires between the two temporally overlapping broods of most pregnant females, that is in 56% of *Poeciliopsis retropinna* and 79% of *P. turrubarensis* females. The high incidence of unique sires among the overlapping broods raises the question whether superfetation could potentially contribute to multiple paternity in females. Yamaguchi et al. (2004, 2006) argued that in the American mink and European badger, the ability of females to continue ovulation after successful matings (a reproductive strategy that is similar to superfetation and is referred to as 'superfertilization', sensu Roellig et al., 2011) facilitates the fertilization of ova from different ovulations by different males. They hypothesized that superfertilization is a female reproductive strategy that greatly extends the window of opportunity for mating, increasing a female's temporal access to more males and hence diminishing the chances of any single male monopolizing paternity (Yamaguchi et al., 2004, 2006). A similar argument was made for superfetatious live-bearing fishes of the family Poeciliidae (Pollux et al., 2014; Zúñiga-Vega et al., 2010). In this family, superfetation is associated with a more frequent production of (smaller) broods overtime (Reznick & Miles, 1989). This results in the *in utero* presence of simultaneous, temporally overlapping broods that have been fertilized at different moments in time and, hence, are in different developmental stages. By dividing offspring into multiple, temporally overlapping broods, each fertilized at different points in time, superfetation may promote polyandry by increasing a female's ability to create multiple-paternity broods. While it is generally assumed that superfetation increases opportunities for offspring from temporally overlapping broods to be sired by different males, concrete evidence (e.g., molecular evidence of multiple paternity) to support this is still lacking. An important first step is to show that temporally overlapping broods are indeed (at least partially) sired by different fathers. The high incidence of unique sires in overlapping broods in *P. retropinna* and *P. turrubarensis* provides the first evidence to support the idea that, by spreading offspring production over time and creating several (smaller) overlapping broods that are each fertilized at different points in time, superfetation could in principle contribute to multiple paternity in live-bearing fishes.

4.2 | Shared sires among broods

The presence of unique sires between temporally overlapping broods, however, was not observed in every female; some females carried overlapping broods that shared the same sires (Figures 1 and 2). The interbrood interval is approximately two weeks for both species (BJA Pollux, personal observation). Given this 2-week period between the production of broods, we expected that different sires would contribute to the two overlapping broods of most, if not all, females. This raises the question why we did not always find unique sires between overlapping broods? One potential explanation for this would be that these females re-mated two weeks later with exactly the same male(s). However, given the high densities and high mating frequencies in the field (e.g. Figure S3) we deem it highly unlikely that these females re-mated with only these same males. A more plausible explanation is that the overlap in sires between broods stems from sperm storage, which seems to be not only omnipresent, but also long-term (up to 10 months) in live-bearing poeciliids (Evans & Pilastro, 2011; Olivera-Tlahuel et al., 2017; Potter & Kramer, 2000). That sperm storage can lead to the same males fertilizing successive broods was shown in a study by Lopez-Sepulcre et al. (2013) investigating reproductive success in wild guppy (*Poecilia reticulata*) populations (Lopez-Sepulcre et al., 2013). They found that males sired offspring for (up to ten) months after their own death. This idea is further supported by studies in several other poeciliid species showing that only one copulation is necessary to fertilize several successive broods (Constantz, 1984; Greven, 2011; Hildemann & Wagner, 1954; Winge, 1937).

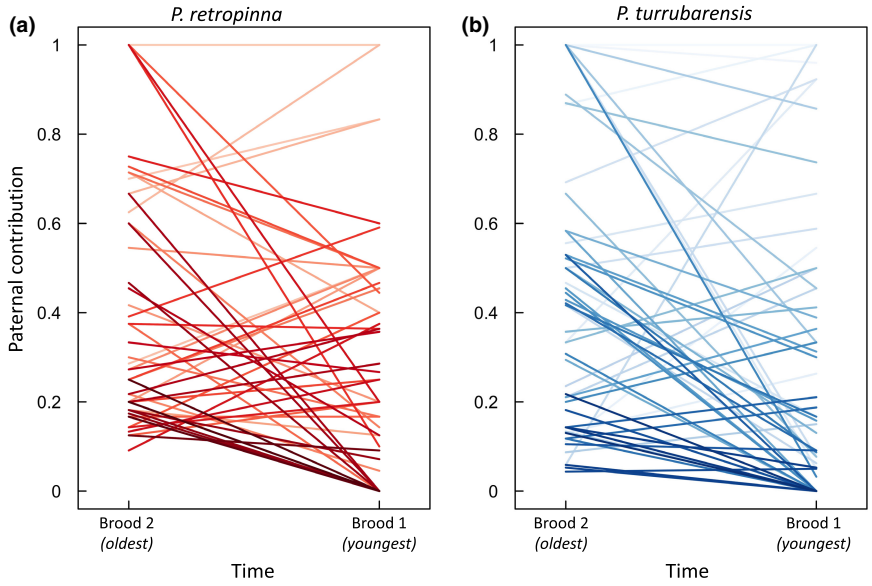


FIGURE 3 Temporal decline in paternal contribution to successive, temporally overlapping broods in the superfetatious live-bearing (a) *P. retropinna* and (b) *P. turrubarensis* (family Poeciliidae). The panels show that males that contributed to the oldest brood (brood 2) had a significantly lower proportional contribution in the subsequent brood (brood 1), both for *P. retropinna* (a mean decline of 7.94%; $z = -2.158$, $p = 0.0309$) and *P. turrubarensis* (21.27%; $z = -7.535$, $p < 0.0001$)

4.3 | Multiple sires within vs. among broods

We also observed multiple paternity within broods in 34 out of 50 (68%) broods in *P. retropinna* and 37 out of 48 (77%) broods in *P. turrubarensis* (Figures 1 and 2). The occurrence of multiple paternity within broods is a common phenomenon in poeciliid fishes, both in superfetatious (Schrader et al., 2011; Soucy & Travis, 2003) and non-superfetatious species (Dekker et al., 2020; Girndt et al., 2012; Neff et al., 2008; Simmons et al., 2008; Tatarenkov et al., 2008; Zane et al., 1999). From an evolutionary perspective, multiple paternity may arise because it is favoured as a 'bet-hedging' strategy (Fox & Rauter, 2003; Garcia-Gonzalez et al., 2015). For instance, multiple paternity reduces the risk that offspring are solely being sired by a male of low genetic quality (Fox & Rauter, 2003; Watson, 1991; Yasui & Garcia-Gonzalez, 2016). In addition, multiple paternity can increase the genetic variation in offspring, thereby potentially increasing offspring survival by ensuring that some offspring survive in different environmental conditions (Fox & Rauter, 2003; Garcia-Gonzalez et al., 2015; Loman et al., 1988; Yasui, 1998). We found that the mean number of sires observed within pregnant females was higher than the mean number of sires within the separate broods (*P. retropinna*: 2.92 sires within mothers vs. 2.36 within separate broods; *P. turrubarensis*: 3.40 sires within mothers vs. 2.56 sires within broods). This suggests that, although multiple paternity mostly arises from within-brood multiple paternity in *P. retropinna* and *P. turrubarensis*, superfetation can contribute to a higher level of polyandry within females.

4.4 | Paternity skew within vs. among broods

The paternal contribution in most females of *P. retropinna* and *P. turrubarensis* was unequally divided among their offspring, with a significant skew observed in 42% and 65% of the females, respectively. Paternity skew was less frequently observed within broods (in only 9% and 46% of the broods, respectively), indicating that paternity skew mostly originates from differences in paternal contribution between broods, rather than unequal contribution within broods. For instance, a sire that is present in both overlapping broods might sire only 10% of the offspring in one brood, while siring 70% of the offspring in the other brood. The difference in sire contribution between broods is also shown by the Bray-Curtis index (in which an index of 0 indicates that all sires are shared among the two broods with an equal distribution, and an index of 1 means that no sires are shared among the two broods). This index was on average 0.45 ± 0.06 (range 0.13–1.00) for *P. retropinna* and 0.52 ± 0.07 (range: 0.04–1.00) for *P. turrubarensis*, suggesting that the contribution of sires differs strongly between broods. These findings suggest that superfetation may increase paternity skew, with sires that are shared between overlapping broods often showing a different contribution to each of these broods.

The observed patterns in paternity skew between broods (i.e., shared sires but a proportional paternal contribution that differs between broods) could potentially be explained by sperm storage in combination with 'last male sperm precedence' (i.e. sperm competition favouring the most recently mated male) and/or cryptic female choice (nonrandom paternity biases resulting from female morphology, physiology or behaviour that occur during or after mating; Pitnick & Brown, 2000). In most mammals, the duration of sperm storage usually lasts a few hours to a few days, however, in many non-mammalian vertebrates, such as birds, reptiles and fishes, sperm storage can last for weeks, months, or even years (Holt & Fazeli, 2016). In poeciliid fishes, last male sperm precedence is a widely observed phenomenon, and can be caused not only by the fact that fresher sperm has a competitive advantage over longer stored sperm (Gasparini et al., 2018; Winge, 1937), but also by females that re-mate with a more attractive partner, i.e. by "trading-up" (Evans et al., 2004; Evans & Magurran, 2001; Jennions & Petrie, 2000; Pitcher et al., 2003). In an elegant laboratory experiment using artificial insemination in *P. reticulata*,

Gasparini et al. (2018) observed a strong paternity bias in favour of freshly inseminated sperm over stored sperm from previous copulatory events. If this 'last male sperm precedence' is a general feature of poeciliid fishes, then we would expect males in our study, that contribute to a particular brood, to show a lower contribution to the next brood. To test this, we looked for males that contributed to the oldest brood (brood 2) and then compared their contribution to the youngest brood (brood 1). We found that the contribution of males significantly decreased from the oldest to the youngest brood by 7.94% in *P. retropinna* (GLMM: $z = -2.158$, $p = 0.0309$; Figure 3a) and 21.27% in *P. turrubarensis* (GLMM: $z = -7.535$, $p < 0.0001$; Figure 3b). We propose that this observed temporal decline in paternal contribution of individual sires to overlapping broods can potentially contribute to a higher skew in females with superfetation.

4.5 | Potential for genomic conflict

The conflict theory predicts that by increasing the opportunity for multiple paternity superfetation could intensify genomic conflicts (Furness et al., 2019; Pollux et al., 2014; Zeh & Zeh, 2000, 2003). When offspring of a single female (or a single brood) are sired by multiple males, differences in relatedness between offspring arise that can enhance postzygotic conflicts over resource transfer from mother to offspring (referred to as parent-offspring conflicts; Macnair & Parker, 1978; Parker et al., 2002; Trivers, 1974; Zeh & Zeh, 2000; Zeh & Zeh, 2003). Such postzygotic (genomic) conflicts are known to occur among siblings within broods (Haig, 1993). Here, we show that superfetation is associated with the occurrence of unique sires in temporally overlapping broods. This suggests that genomic conflicts could potentially occur between simultaneously developing offspring that are in different broods. A study by Schrader and Travis (2011) in the live-bearing fish *Heterandria formosa* (Poeciliidae) provided preliminary experimental evidence for such asymmetric sibling competition. They hypothesized that early broods mainly have to compete with a small number of siblings that are also less developed, whereas later broods have to compete with not only more, but also more developed siblings. They showed that *H. formosa* offspring born in early broods were significantly larger than offspring from later broods (Schrader & Travis, 2011). The potential for this kind of genomic conflict depends on the extent of physiological interaction between mother and developing foetuses (Zeh & Zeh, 2000; Crespi & Semeniuk, 2004) and may therefore be greater for the matrotrophic *P. retropinna* than for lecithotrophic *P. turrubarensis* (Furness et al., 2019; Pollux et al., 2014; Reznick et al., 2002). Finally, it is possible that in eutherian mammals, phenomena that are conceptually similar to superfetation, such as superfertilization in the American mink and European badger, and superconception in the European brown hare, may likewise intensify genomic conflicts among offspring in different temporally overlapping broods during pregnancy.

4.6 | Conclusion and suggestions for future studies

Our study revealed a high incidence of unique sires in the simultaneous, temporally overlapping broods of pregnant females in *P. retropinna* and *P. turrubarensis*. We found that the mean number of sires within females was higher than the number of sires within the separate broods. We further found that paternity skew predominantly arises from different proportional paternal contribution between broods, rather than within broods. Together, these two findings tentatively suggest that superfetation may promote polyandry and reproductive skew within females.

These findings are derived from two species in the genus *Poeciliopsis*, representing only 1 of at least 4 (possibly 5) independent origins of superfetation in the family Poeciliidae (Furness et al., 2019). To evaluate the generality of these findings, future studies should include species from other origins of superfetation. In this study we investigated two species that carried on average two simultaneous, overlapping broods. There is, however, pronounced variation among species in the level of superfetation across the Poeciliidae, with the maximum number of simultaneous broods per female in a given species ranging from 2 up to 14 (Furness, Avise et al., 2021, Furness, Hagmayer et al., 2021; Pires, Banet et al., 2011; Pires, Bassar et al., 2011). To study how higher levels of superfetation might affect multiple paternity and paternity skew, future studies should include species that can carry more simultaneous broods (e.g. 5–7 broods in *Heterandria formosa*, *Poecilia branneri*, *Xenodexia ctenolepis*, Pires, Banet et al., 2011; Pires, Bassar et al., 2011; up to 14 broods in *Phalloptychus Januarius*, Pollux & Reznick, 2011).

AUTHOR CONTRIBUTIONS

MLD and BJAP conceived the project idea. MLD, AH, AIF and BJAP planned the fieldwork and collected the samples. MLD, KL and LMvS carried out the dissections, developed the microsatellite markers and performed the molecular analyses. MLD analysed the data. MLD and BJAP wrote the first draft of the manuscript. All authors critically reviewed the initial manuscript, provided helpful input and approved the final manuscript.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The DNA sequence of the published *Poeciliopsis retropinna* genome is available at Genbank accession number: PRJNA555005 (van Kruistum et al., 2020). The specific sequences of the newly developed microsatellite markers for *P. retropinna* are available in the supplementary data. Microsatellite genotypes of all individuals used in the current study is archived online on Dryad: Dekker et al. (2022), Dryad, Dataset, https://doi.org/10.5061/dryad.m0cfxpp5z.

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