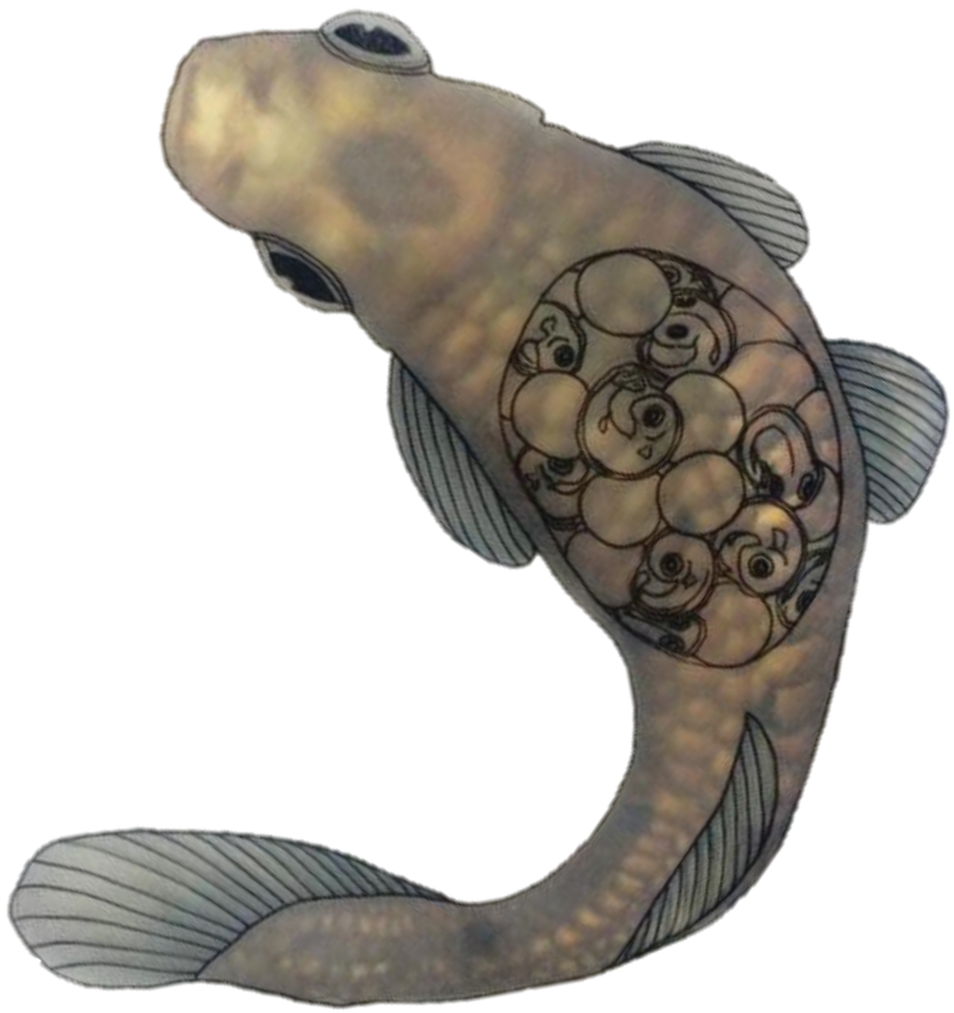
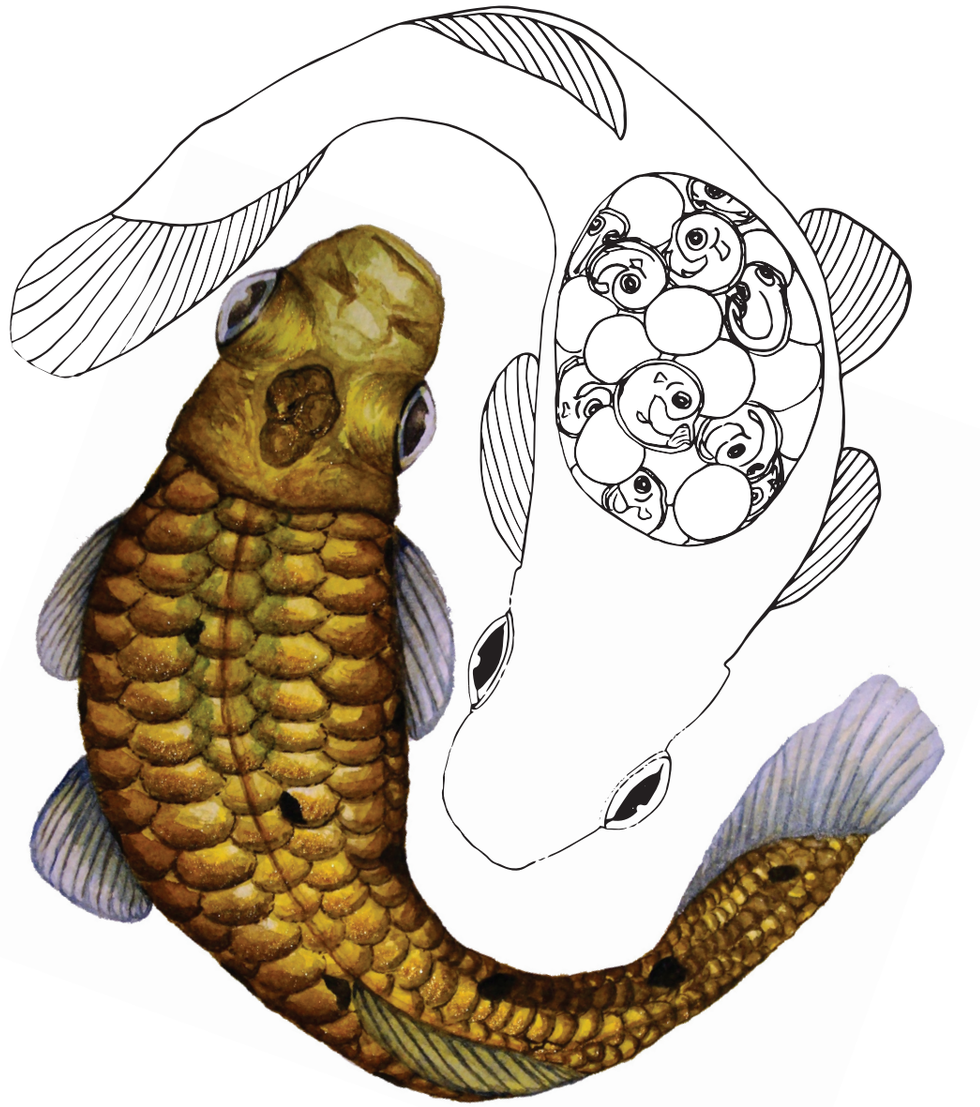


SWIMMING PERFORMANCE AND MORPHOLOGY OF PREGNANT FISH

Elsa Magnolia Quicazán Rubio

2019

# SWIMMING PERFORMANCE AND MORPHOLOGY OF PREGNANT FISH



Elsa Magnolia Quicazán Rubio

# Swimming performance and morphology of pregnant fish

**Elsa Magnolia Quicazán Rubio**

**Thesis committee**

**Promotor**
Prof. Dr J. L. van Leeuwen
Professor of Experimental Zoology
Wageningen University & Research

**Co-promotor**
Dr B. J. A. Pollux
Assistant professor, Experimental Zoology Group
Wageningen University & Research

**Other members**
Prof. Dr M. Naguib, Wageningen University & Research
Dr L. A. J. Nagelkerke, Wageningen University & Research
Dr S. van Wassenbergh, Muséum National D'Histoire Naturelle, Paris, France
Prof. Dr M. K. Richardson, Leiden University

This research was conducted under the auspices of the Graduate School of the Wageningen Institute of Animal Sciences (WIAS).

# Swimming performance and morphology of pregnant fish

Elsa Magnolia Quicazán Rubio

**Thesis**
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A. P. J. Mol,
In the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 18 September 2019
at 4 p.m. in the Aula.

Elsa Magnolia Quicazán Rubio
Swimming performance and morphology of pregnant fish, 164 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2019)
With references, with summaries in English, Dutch and Spanish.

ISBN 978-94-6395-033-6
DOI 10.18174/495855

# Contents

# Chapter 1

## General Introduction

Viviparity in fish already occurred around 370 million years ago, as demonstrated by the finding of a fossil of a developed fetus of *Materpiscis attenboroughi* attached to its mother by an umbilical cord [1]. Thus, it is very likely that fish are the first vertebrates to have developed internal fertilization and viviparity. But the shape of fish is constrained by the higher density of the water compared to that of air, which favours hydrodynamic shapes to lower resistance when moving forward. Fish also require a well-developed and extensive muscle system to propel their bodies through the dense liquid. Thus, pregnant fish not only face the demands that come from feeding and transporting their descendants, they also experience the constraints of living in an aqueous environment, all while avoiding being predated upon. Therefore, the question that arises here is, how did pregnant fish overcome the constraints imposed by living in water and internally carrying their embryos?

In this thesis, I explore the internal and external morphology adopted by live-bearing female fish and their possible effect on the female's locomotion. Several techniques, from photogrammetry, force measurements, high-speed video recording and analysis, histology, and real-time quantitative PCR are combined to contribute to the scientific knowledge of the effects of pregnancy on the swimming performance of females.

## 1. Using fish as a model to understand the effects of pregnancy on locomotion:

### 1.1. Fish swimming

Fish swimming is a combination of periodic and aperiodic movements. The periodic movements can be classified, depending on the way that they are powered, as body and caudal fin (BCF) and median and paired fins (MPF) swimming [2]. BCF swimming involves the undulation of the body and caudal fin, whereas in the MPF swimming the body is approximately straight while the fins make undulatory or oscillatory movements [3]. Within BCF swimming, four swimming modes can be distinguished based on the undulation pattern along the body [4,5] from serpentine undulation (eel) to undulations that are only visible on the posterior region of the body (tuna): anguilliform, carangiform, subcarangiform, and thunniform. The aperiodic swimming modes, fast-starts and turns, are used alone or in alternation with the near cyclic movements depending on the situation.

Fish power their movements by differentially recruiting their axial muscles generating a wave along their body. The muscles of fish are arranged in myotomes which are laterally symmetric packages of muscle fibres that are subdivided in a dorsal and a ventral portion at each side [6]. Three main types of muscle fibres are distinguished by their colour: red, pink and white [7]. The red fibres are slow, aerobic and virtually inexhaustible [8]. They are usually located just underneath the skin forming a thin layer. The white fibres are fast and anaerobic, have a higher power output, and exhaust quickly [4]. The pink fibres are located between the red and the white ones and their activity is intermediate between the other two groups [8]. Each swimming motion requires the

recruitment of an ensemble of fibre types, e.g. the fast-start, an explosive manoeuvre, requires the recruitment of the entire mass of white muscle fibres to catch a prey or escape from a predator [9].

### 1.2. Possible causes of a lower locomotor performance in reproductive fish

Both egg-carrying and pregnancy negatively affect the locomotor performance of fish [10–12], and the causes for it can be divided into two non-mutually exclusive categories: physiological and physical. First, the extra energy required to provision eggs before fertilization (in oviparous animals) or nourish developing embryos during gestation (in viviparous animals) might lead to lower energetic investments towards the locomotor muscles, negatively affecting their contractile properties and power output [12]. In addition, during the later stages of pregnancy, embryos increase their mass-specific oxygen requirement. The females of some species supply the greater need for oxygen by spending more time on aquatic surface respiration when exposed to hypoxic conditions, and this behaviour might increase their risk of predation [13]. The physical causes are likely determined by the aquatic medium in which they live. The additional reproductive mass may negatively affect a female's ability to accelerate, while a greater body volume could increase body drag and limit axial bending, negatively influencing a female's general swimming performance [10–12].

### 1.3. Poeciliidae family as a model group to study the effect of pregnancy on locomotion

The neotropical fish family Poeciliidae, represents one of the origins of viviparity in the Actinopterygii fishes (ray-finned) [14]. It is a monophyletic group of about 220 species that presents a wide variety of life-history traits [15]. Adult body size of this group is small and the species are relatively easy to rear in captivity. Also, its individuals have short life-cycles, and their natural habitat is accessible. All of this makes this family a convenient model for laboratory and field studies. Among the ample variety of topics researched in this group are the population and community ecology, life-history evolution, viviparity, sexual selection, behaviour, phenotypic plasticity and physiology, to list just a few of them. Its species present a wide variation of maternal provisioning that range from feeding their internally developing embryos exclusively from the nutrients that have been stored in the egg-yolk prior to fertilization (lecithotrophy), to almost exclusively nourishing them through a placenta (placentotrophy) [16,17]. This variety of reproductive strategies within the family makes it a good model to study the effects of reproduction on the locomotor performance of females [10,11,18].

## 2. Exploring the effects of pregnancy on swimming performance

### 2.1. How does a placenta affect female body shape during pregnancy?

A live-bearing mode of reproduction may increase the volume and mass of the female throughout the reproductive cycle increasing the costs of locomotion. Placentation may have evolved to reduce these costs [19]. In Poeciliid placentotrophic species, the mother transfers the nutrients to the embryo through a simple structure that also takes care of the osmotic balance of the embryo's environment, the exchange of gases, and the protection of the embryo from immunological rejection [16,20]. The gradual transfer of nutrients from the mother to the embryos through the placenta implies a lower reproductive allocation (RA, the proportion of body mass dedicated to reproduction) at the beginning of gestation in placentotrophic fish. This may reduce the mass and volume of the female at the early stages of pregnancy, keeping its body more streamlined than the one of non- placental species, and presumably reducing the total locomotor costs of reproduction. The inference that the placenta may have evolved to reduce the locomotor costs of reproduction is known as the *Locomotor cost hypothesis* [19]. Within the family Poeciliidae, placentotrophy has evolved independently several times, which makes this family a good model to study the possible adaptive benefit that the placenta would have on the morphology of females [19].

### 2.2. How does a pregnancy affect the drag on a female's body?

The growing embryos affect the body shape of females by increasing their wetted and cross-sectional areas and volume, which may increase the production of drag [10–12,18,21–23] and presumably the metabolic costs of swimming [23,24]. Reproductive allocation (RA) rises during pregnancy as embryos grow. In the guppy (*Poecilia reticulata*), RA also increases within a population when high predation leads to higher fecundity, understood as higher numbers of offspring [10]. In both cases, drag supposedly grows causing a decrease of the maximum swimming speed [11], which may negatively affect the fitness of the female by making her an easier predation target [25]. Therefore, drag may be one of the main mechanisms influencing the swimming performance of pregnant females. As mentioned above, some reproductive strategies such as placentotrophy, presumably decrease reproductive allocation, contributing to thinner body forms either during the early stages of pregnancy, or throughout it. This could give a selective advantage to females with slender bodies, yet little is known about how reproductive investment affects drag production and how the surrounding flow interacts with the body of the pregnant fish to produce drag. Until now, the effect of pregnancy on the production of drag on a swimming animal has only been examined during passive coasting in pre- and post-parturition females of the bottlenose dolphin, *Tursiops truncatus*, [26]. During coasting, females keep a straight body while moving forward [27]. The drag, derived from body deceleration observed in videos, increased during pregnancy and was associated with diminished

locomotor performance [26].

The Poeciliidae family presents considerable increases in reproductive investment during gestation, ranging from 4.1% to over 35% of their body weight [15]. Exploring how this investment relates to the production of drag allows for a very basic assessment of the locomotor costs associated with different levels of RA. Poeciliid fish use a wide range of motions [28] including the burst-and-coast swimming style, alternating coasting and undulatory movements. The study of drag during undulatory movements presents technical difficulties for untethered individuals, including considerable computational efforts, and unavoidable inaccuracies imposed by the turbulent nature of the flow. On the other hand, the measurement of drag during coasting on life or model animals is a valid first approach to elucidate the differences in drag between different levels of reproductive allocation.

### 2.3. How does a pregnancy affect the escape response of fish?

Escape responses are the main defence of many taxa against predator attacks [29] and they are modulated by biomechanical, neurological, physiological, morphological and behavioural parameters [3]. Pregnant females may be more sensitive to predation, not only because they carry eggs and embryos, which are highly nutritious resources [30], but also because their morphological and physiological changes may hinder their swimming performance. For example, in some species of the Poeciliidae family, such as *Gambusia affinis*, *Poecilia reticulata*, *Poeciliopsis turneri, Heterandria formosa* and *Phalloptychus januarius* [10,11,18,31,32], pregnancy negatively affects the escape performance of females, and the effects increase towards the end of the gestation period. As explained above, reproductive strategies such as placentotrophy and others present in the Poeciliidae family presumably affect the body shape of the female during pregnancy [19]. The alteration of body shape influences the locomotor performance of fish [8,24], and therefore it is important to explore how different reproductive strategies affect behaviours crucial to survival, such as the escape response. This information lays the basis for understanding the possible advantages of reproductive traits, such as placentation, in performance, and eventually, their evolution.

### 2.4. How does pregnancy affect muscles morphology and the expression of genes that are related to muscle function?

Fish skeletal muscle has a high phenotypic plasticity [33], and it is very adaptable [34]. The plasticity is kept through life and adult muscle tissue can alter its contractile and metabolic properties by changing its protein composition [35] in response to environmental conditions, migration and spawning [12,35,36]. Training in teleost fish, for example, alters the cell diameter [33] and number of fibres in the aerobic muscle [35,37–40], and alters the activity of the AMP-activated protein kinase which is a sensor of cellular energy status in the skeletal muscle and other tissues and organs [41]. Atlantic salmon subjected to fasting

and refeeding upregulates IGFBP-4, a binding protein that, at least for mammals [42], is part of the central pathways regulating protein synthesis in skeletal muscle. Yet, a rigorous test of the function of this and any other gene/protein related to the function of the fish muscles in specific lineages, in this case the Poeciliidae family, is still needed [36]. Muscle physiology is also affected by reproduction. Spawning affects the muscle condition and activation in the short-horn sculpin which is reflected in lower swimming performances as spawning approaches [12]. So far, no information is available for live-bearing fish about the muscle activity, and the possible morphological and physiological adaptations to their reproductive mode.

## 3. The aims and order of the research chapters

I propose that physical constraints of aquatic locomotion influence the evolution of the reproductive traits of fish. *The aim of this thesis is to improve the understanding of the relationship between the morphological changes caused by pregnancy and the swimming performance of fish.* I apply biomechanical experimental techniques to study individuals from the family Poeciliidae, a diverse clade of small live-bearing fish.

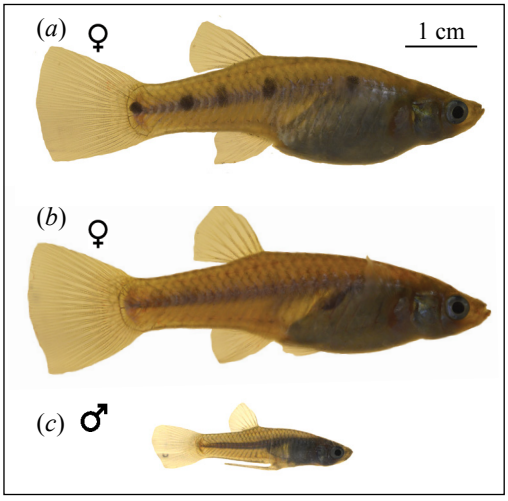



**Figure 1.** Photos of adult *Poeciliopsis gracilis* (*a*) Pregnant female, (*b*) virgin female, and (*c*) male.

In this study, we start out with an analysis of the effect of having a placenta on the body shape of pregnant fish. The placenta, as mentioned before, is expected to reduce the locomotor costs of pregnant females by allowing slender bodies at the beginning of pregnancy. In **Chapter 2**, we test this hypothesis by comparing the three-dimensional body shape changes between a placentotrophic and non-placentotrophic species. We developed and used a non-invasive photogrammetry method to document the three-dimensional shape and volume changes through pregnancy of the closely related species *Poeciliopsis*

*turneri* (placentotrophic) and *Poeciliopsis gracilis* (lecithotrophic, figure 1). This study provides the first empirical evidence for a possible adaptive morphological advantage of the placenta in live-bearing fish and could be a good starting point for future research on additional independent placental lineages. In both reproductive strategies, the growing embryos increase the volume of the female and consequently its body surface and cross-sectional area, which could increase body drag [10–12,18,26,43]. However, the effects of pregnancy and the level of reproductive investment on the production of drag have not been evaluated so far in live-bearing fish. This motivated the study presented in **Chapter 3**, in which we examined the effect of the reproductive allocation increase (RAI, the increase in the proportion of body mass dedicated to reproduction) on the body drag of pregnant fish. We generated 3D printed models of females of *Poeciliopsis gracilis* in a straight body posture with different levels of RAI. We measured the drag and visualized the flow around them in a flow tunnel at different speeds. Using our experimental data, we generated a curve fit of drag over all RAI and speeds, and we generated equations that describe the scaling of the drag of a straight body aligned with the flow. This allowed us to describe what would potentially happen beyond the limits tested. This chapter offers a basis for future studies on the effect of reproductive strategies on the production of drag.

With this knowledge in mind, we aimed to find out the effects of pregnancy on the escape response of *Poeciliopsis gracilis*. Escape responses are the main defence against predator attacks [44]. In some species, pregnancy negatively affects the escape performance of females and the effect increases as the pregnancy advances and it worsens with higher reproductive allocations [10,18,31], which may increase the risk of predation [31]. To examine the effect of pregnancy on *Poeciliopsis gracilis,* in **Chapter 4**, we elicited and measured the escape response of pregnant and virgin females before and just after giving birth, and filmed it with three orthogonal high-speed cameras. We evaluated the three-dimensional manoeuvre using an automatic tracking system, that provides high detail of the movements of the longitudinal axis and centre of mass of the fish through the manoeuvre. At the end of the chapter, we highlight the importance of understanding how biological characteristics of the species and technological constraints of the experiments may affect the study outcomes.

Pregnancy may also affect the morphology and physiology of fish muscles. But for gravid fish nothing is known about the disposition of muscles and muscle fibres, nor about the transcription levels of the genes involved in different processes related to muscle physiology and metabolism. In **Chapter 5**, we made transverse sections of the abdominal region of pregnant and virgin females of *Poeciliopsis gracilis*. We used Crossmon's Trichrome and hematoxylin-eosin staining to identify the muscles in cross-section; standard immune-histochemistry to characterise the slow, intermediate, and fast fibres; and enzyme-histochemistry techniques to identify the levels of (an)aerobic activity of the fibres. Additionally, we performed real-time quantitative PCR on the

muscles in the caudal peduncle and quantified the transcription levels of genes that are particularly expressed in muscles. Then we explored whether pregnancy would affect (i) the distribution and cross-sectional area of the epaxial, hypaxial and lateralis superficialis muscles and the number and cross-sectional area of the fibres within each muscle; (ii) the location of the fast, intermediate and slow muscle fibres; and (iii) the expression of genes that are particularly important in muscle function. This is the first time that the muscle morphology and the activity of genes that express muscle function have been described for gravid fish. Furthermore, this chapter explores for the first time whether the muscles of a live-bearing fish change due to pregnancy and provides suggestions for future studies.

Finally, **Chapter 6** synthetizes the results from this thesis and discusses the contribution to the scientific knowledge of the effects of pregnancy on the swimming performance of females as well as potential avenues towards the understanding of how the trade-offs between locomotion and reproduction might have influenced the reproductive strategies adopted by viviparous fish.

## References


1. Long JA, Trinajstic K, Young GC, Senden T. 2008 Live birth in the Devonian period. *Nature* 453, 650–653. (doi:10.1038/nature06966)
2. Webb PW. 1994 The biology of fish swimming. In *Mechanics and physiology of animal swimming* (eds L Maddock, Q Bone, JMV Yayner), pp. 45–62. Cambridge: Cambridge University Press.
3. Domenici P, Blake RW, editors. 2000 *Biomechanics in animal behabiour*. Oxford: Bios Scientific, Oxford.
4. Blake RW. 1983 Fish locomotion Part 1. In *Fish Locomotion*, Cambridge: Cambridge University Press.
5. Blake RW. 2004 Fish functional design and swimming performance. *J. Fish Biol.* **65**, 1193–1222. (doi:10.1111/j.1095-8649.2004.00568.x)
6. Altringham JD, Ellerby DJ. 1999 Fish swimming: patterns in muscle function. *J. Exp. Biol.* **202**, 3397–403.
7. Spierts ILY, van Leeuwen JL. 1999 Kinematics and muscle dynamics of C- and S-starts of carp (*Cyprinus carpio* L.). *J. Exp. Biol.* **202**, 393–406.
8. Videler JJ. 1993 *Fish swimming*. Dordrecht: Springer Netherlands. (doi:10.1007/978-94-011-1580-3)
9. Domenici P, Blake RW. 1997 The kinematics and performance of fish fast-start swimming. *J. Exp. Biol.* **200**, 1165–1178.
10. Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* **164**, 38–50. (doi:10.1086/421412)
11. Plaut I. 2002 Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
12. James RS, Johnston IA. 1998 Influence of spawning on swimming performance and muscle contractile properties in the short-horn sculpin. *J. Fish Biol.* **53**, 485–501. (doi:

10.1006/jfbi.1998.0722)
13. Timmerman CM, Chapman LJ. 2003 The effect of gestational state on oxygen consumption and response to hypoxia in the sailfin molly, *Poecilia latipinna*. *Environ. Biol. Fishes* **68**, 293–299. (doi:10.1023/A:1027300701599)
14. Blackburn DG. 2015 Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *J. Morphol.* **276**, 961–990. (doi:10.1002/jmor.20272)
15. Pires MN, Banet AI, Pollux BJA, Reznick DN. 2011 Variation and evolution of reproductive strategies. In *Ecology and Evolution of Poeciliid Fishes* (eds JP Evans, A Pilastro, I Schlupp), pp. 28–37. Chicago: The University of Chicago Press.
16. Banet AI, Au AG, Reznick DN. 2010 Is mom in charge? Implications of resource provisioning on the evolution of the placenta. *Evolution* **64**, 3172–3182. (doi:10.1111/j.1558-5646.2010.01059.x)
17. Pires MN, Arendt J, Reznick DN. 2010 The evolution of placentas and superfetation in the fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae: subgenera *Micropoecilia* and *Acanthophacelus*). *Biol. J. Linn. Soc.* **99**, 784–796. (doi:10.1111/j.1095-8312.2010.01391.x)
18. Belk MC, Tuckfield RC. 2010 Changing costs of reproduction: age-based differences in reproductive allocation and escape performance in a livebearing fish. *Oikos* **119**, 163–169. (doi:10.1111/j.1600-0706.2009.17742.x)
19. Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009 Evolution of placentas in the fish family Poeciliidae: An empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **40**, 271–289. (doi:10.1146/annurev.ecolsys.110308.120209)
20. Pires MN, Bassar RD, McBride KE, Regus JU, Garland TJ, Reznick DN. 2011 Why do placentas evolve? An evaluation of the life-history facilitation hypothesis in the fish genus *Poeciliopsis*. *Funct. Ecol.* **25**, 757–768. (doi:10.1111/j.1365-2435.2011.01842.x)
21. Booth RK. 1998 Swimming performance of Anadromous Atlantic salmon, *Salmo salar* L., during their spawning migration in the Exploits River, Newfoundland, Canada. PhD dissertation. University of Waterloo, Waterloo, ON, Canada.
22. Fleuren M, Quicazan-Rubio EM, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS One* **13**, e0195976. (doi:10.1371/journal.pone.0195976)
23. Pettersson LB, Hedenström A. 2000 Energetics, cost reduction and functional consequences of fish morphology. *Proc. R. Soc. B Biol. Sci.* **267**, 759–764. (doi:10.1098/rspb.2000.1068)
24. Langerhans RB. 2009 Morphology, performance, fitness: functional insight into a post-Pleistocene radiation of mosquitofish. *Biol. Lett.* **5**, 488–491. (doi:10.1098/rsbl.2009.0179)
25. Langerhans RB, Reznick DN. 2010 Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics. In *Fish Locomotion*, pp. 200–248. (doi:10.1201/b10190-8)
26. Noren SR, Redfern JV, Edwards EF. 2011 Pregnancy is a drag: hydrodynamics, kinematics and performance in pre- and post-parturition bottlenose dolphins (*Tursiops truncatus*). *J. Exp. Biol.* **214**, 4151–4159. (doi:10.1242/jeb.059121)
27. McHenry MJ, Lauder GV. 2005 The mechanical scaling of coasting in zebrafish (*Danio rerio*). *J. Exp. Biol.* **208**, 2289–301. (doi:10.1242/jeb.01642)

28. Svendsen JC, Banet AI, Christensen RHB, Steffensen JF, Aarestrup K. 2013 Effects of intraspecific variation in reproductive traits, pectoral fin use and burst swimming on metabolic rates and swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *J. Exp. Biol.* **216**, 3564–74. (doi:10.1242/jeb.083089)
29. Domenici P. 2009 Escape responses in fish: kinematics, performance and behavior. In *Fish Locomotion: An Eco-ethological Perspective* (eds P Domenici, BG Kapoor), pp. 123–170. Enfield, NH: Enfield: Science Publishers.
30. Berglund A, Rosenqvist G. 1986 Reproductive costs in the prawn *Palaemon adspersus*: effects on growth and predator vulnerability. *Oikos* **46**, 349–354. (doi:10.2307/3565833)
31. Banet AI, Svendsen JC, Eng KJ, Reznick DN. 2016 Linking reproduction, locomotion, and habitat use in the Trinidadian guppy (*Poecilia reticulata*). *Oecologia* **181**, 87–96. (doi:10.1007/s00442-015-3542-9)
32. Fleuren M. 2017 Reproductive adaptations to reduce locomotor costs in viviparous fish (Poeciliidae). PhD thesis. Wageningen University and Research, Wageningen, The Netherlands. (doi:10.18174/424276)
33. Palstra AP, Rovira M, Rizo-Roca D, Torrella JR, Spaink HP, Planas J V. 2014 Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. *BMC Genomics* **15**, 1136. (doi:10.1186/1471-2164-15-1136)
34. Sänger AM. 1993 Limits to the acclimation of fish muscle. *Rev. Fish Biol. Fish.* **3**, 1–15.
35. Sänger AM, Stoiber W. 2001 Muscle fiber diversity and plasticity. In *Fish Physiology*, pp. 187–250.(doi:10.1016/S1546-5098(01)18008-8)
36. Johnston IA, Bower NI, Macqueen DJ. 2011 Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol.* **214**, 1617–1628. (doi:10.1242/jeb.038620)
37. Hinterleitner S, Huber M, Lackner R, Wieser W. 1992 Systemic and enzymatic responses to endurance training in two cyprinid species with different life styles (Teleostei: Cyprinidae). *Can. J. Fish. Aquat. Sci.* **49**, 110–115. (doi: 10.1139/f92-013)
38. Sänger AM. 1992 Effects of training on axial muscle of two cyprinid species: *Chondrostoma nasus* (L.) and *Leuciscus cephalus* (L.). *J. Fish Biol.* **40**, 637–646.
39. Meyer-Rochow VB, Ingram JR. 1993 Red-white muscle distribution and fibre growth dynamics: a comparison between lacustrine and riverine populations of the Southern smelt *Retropinna retropinna* Richardson. *Proc. R. Soc. B Biol. Sci.* **252**, 85–92.
40. Young PS, Cech JJ. 1993 Improved growth, swimming performance, and musculature development in exercised-conditioned young-of-the-year striped bass (*Morone saxatilis*). *Can. J. Fish. Aquat. Sci.* **50**, 703–707. (doi: 10.1139/f93-080)
41. Kahn BB, Alquier T, Carling D, Hardie DG. 2005 AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* **1**, 15–25.
42. Bower NI, Taylor R, Johnston IA. 2008 Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon. *J. Exp. Biol.* **211**, 3859–3870. (doi: 10.1242/jeb.024117)
43. Beamish FWH. 1978 Swimming Capacity. In *Fish Physiology* (eds WS Hoar, DF Randall), pp. 101–187. New York: Academic Press, Inc. (doi:10.1016/S1546-5098(08)60164-8)
44. Domenici P, Blagburn JM, Bacon JP. 2011 Animal escapology I: theoretical issues and

emerging trends in escape trajectories. *J. Exp. Biol.* **214**, 2463–2473. (doi:10.1242/jeb.029652)

# Chapter 2



## Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish

Mike Fleuren, Elsa M. Quicazan-Rubio, Johan L. van Leeuwen, and Bart J.A. Pollux

*Experimental Zoology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, The Netherlands*

Published in *PLoS ONE 13 (4): e0195976.* (2018)

**Abstract**

A live-bearing reproductive strategy can induce large morphological changes in the mother during pregnancy. The evolution of the placenta in swimming animals involves a shift in the timing of maternal provisioning from pre-fertilization (females supply their eggs with sufficient yolk reserves prior to fertilization) to post-fertilization (females provide all nutrients via a placenta during the pregnancy). It has been hypothesised that this shift, associated with the evolution of the placenta, should confer a morphological advantage to the females leading to a more slender body shape during the early stages of pregnancy. We tested this hypothesis by quantifying three-dimensional shape and volume changes during pregnancy and in full-grown virgin controls of two species within the live-bearing fish family Poeciliidae: *Poeciliopsis gracilis* (non-placental) and *Poeciliopsis turneri* (placental). We show that *P. turneri* is more slender than *P. gracilis* at the beginning of the interbrood interval and in virgins, and that these differences diminish towards the end of pregnancy. This study provides the first evidence for an adaptive morphological advantage of the placenta in live-bearing fish. A similar morphological benefit could drive the evolution of placentas in other live-bearing (swimming) animal lineages.

## 1. Introduction

The placenta, defined as an intimate apposition or fusion of maternal and foetal tissues for physiological exchange [1], has evolved many times independently throughout the animal kingdom (e.g. in invertebrates, fish, amphibians, reptiles and mammals; [2–6]), including at least eight times within the live-bearing fish family Poeciliidae [7–10]. Despite the repeated emergence of placentas among widely diverged animal lineages, it is still unclear what selective forces drive the evolution of placental organs. Three non-mutually exclusive adaptive hypotheses have been proposed to explain why the placenta may have evolved in Poeciliid fish: the resource availability hypothesis, the life history facilitation hypothesis and the locomotor cost hypothesis.

The resource availability hypothesis suggests that the evolution of the placenta and associated reduction in egg size at fertilization might allow females to attain a higher fitness through increased litter sizes. A critical assumption of this hypothesis is that females must be able to abort embryos when facing adverse food conditions [11]. Recent empirical studies in Poeciliidae, however, show that they are not able to do this, suggesting that the conditions under which the placenta might be favoured by natural selection are, at least in this taxonomic group, restricted to environments characterized by high and stable resource conditions [12–15].

The life history facilitation hypothesis states that the placenta might evolve to

facilitate the evolution of other life history traits, for example to enable organisms to mature at an earlier age or to produce more or larger offspring that have a higher early-life survivorship [16–21]. However, recent studies in Poeciliidae show that there are no consistent associations between placentation and life history traits, arguing against this hypothesis as a likely explanation for the evolution of the placenta in this taxonomic group [22–24].

Finally, the locomotor cost hypothesis argues that the placenta might evolve to offset some of the locomotor cost associated with a live-bearing mode of reproduction. The physical and physiological burden of a pregnancy negatively affects a female's locomotor performance in a broad range of live-bearing animals (e.g. scorpions, [25]; fishes, [26–29]; reptiles, [30,31]; and mammals, [32,33]). In aquatic animals an increase in abdominal volume may locally limit axial bending and, furthermore, enlarge frontal surface area thereby increasing the drag forces on the body [26,33–35]. An increase in body mass during pregnancy could reduce the ability to rapidly accelerate [27]. It has been postulated that the evolution of the placenta reduces a female's mean reproductive allotment (RA, the proportion of female mass allocated to developing offspring) during gestation, thereby reducing the distention of the female's abdomen during the pregnancy without sacrificing her reproductive output [9,36,37]. The argument is that the evolution of the placenta coincides with a shift in the timing of maternal provisioning from pre-fertilization nutrient allocation by building up large amounts of yolk reserves in the eggs prior to fertilization, to the allocation of nutrients after fertilization (via a placental organ throughout the pregnancy). Livebearing species that allocate nutrients prior to fertilization will start with a high RA (and hence a high burden) at the beginning of their pregnancy, because they produce large fully-yolked eggs. Placental species on the other hand species will start with a low RA, because they produce smaller eggs that contain little to no resources and instead rely on nutrient provisioning during gestation. Theory thus predicts that placental females should have a lower reproductive burden (e.g. lower total volume and frontal surface area) at the start of the pregnancy that diminishes over the course of gestation (figure 1; [9,36,37]). This morphological advantage may improve their locomotor performance (e.g. predator evasion ability; [27]) and hence survival [38] without sacrificing reproductive output, i.e. the locomotor cost hypothesis [9,36,37]. While it is known that Poeciliidae increase in body mass and frontal surface area during pregnancy [26], it is still unclear if, when, and to what extent, the evolution of a placenta alleviates this reproductive burden during pregnancy (figure 1).

Here, we set out to test the morphological predictions of the locomotor cost hypothesis by comparing body shape changes during gestation in two closely-related sister species within the live-bearing fish genus *Poeciliopsis* (Family Poeciliidae). These species differ markedly in the way they provision their developing embryos: *Poeciliopsis gracilis* lacks a placenta and instead allocates all resources necessary for embryo development to

the eggs before fertilization (lecithotrophy), while *Poeciliopsis turneri* has a well-developed placenta (placentotrophy; [7,10]). Specifically, we test whether, compared to *P. gracilis*, the placental species *P. turneri* has (1) a lower body volume and frontal surface area at the beginning of the interbrood interval and in virgin controls, and (2) a stronger increase in volume and frontal surface area (i.e. have a steeper slope) when pregnancy progresses, indicating that the potentially beneficial reduction in body volume associated with placentation will be greatest at the beginning of the pregnancy and will gradually diminish towards the end of the interbrood interval (figure 1).

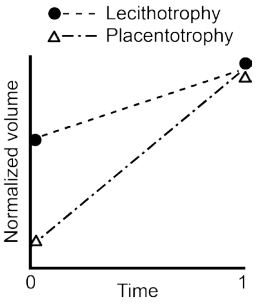



**Figure 1.** Predicted change in female body volume during pregnancy in two hypothetical lecithotrophic (non-placental) and placentotrophic (placental) live-bearing fish species, assuming an equal female length, offspring number and offspring size at birth (IB = 1): (1) the placental species (dash-dot line) will have a smaller volume during its entire pregnancy than the lecithotrophic species (dashed line) and (2) the relationship for the placental species will show a steeper slope than for the lecithotrophic species, indicating that the difference in body volume will be greatest at the beginning of the pregnancy and gradually diminish towards zero at end of the interbrood interval (redrawn after [9]). Similar plots could be constructed for frontal or wetted surface area. For heuristic purposes the temporal patterns are assumed linear, because the exact shape of the relationship between female volume and time is currently unknown [20].

## 2. Material and methods

A detailed description of the used species and their origins, fish rearing protocols and (pre-)experimental husbandry is provided in the Supporting Material S4. All procedures were approved by the Animal Ethics Committee of Wageningen University & Research (permit number 2013103). All efforts were made to minimize suffering.

*Time schedule and sample size* – We studied changes in body shape during the pregnancy of *Poeciliopsis gracilis* (lecithotrophic) and *Poeciliopsis turneri* (placentotrophic) by creating a series of 3D body reconstructions. For each female, these models were created at evenly spaced time points during one interbrood interval (IB), defined as the period between two parturitions starting the day after a female gave birth (hereafter referred to as IB = 0) and lasting until the next parturition (IB = 1). *Poeciliopsis turneri* was measured every second day and *P. gracilis* was measured every fourth day. This served to maintain an approximately equal number of measurements per individual, as the interbrood interval lengths varied between species due to differences in the level of superfetation.

Superfetation refers to a reproductive strategy in which females carry multiple broods at different developmental stages [39–41]. Assuming an equal embryo development time, species with a higher level of superfetation (i.e. more simultaneous overlapping broods) will have shorter interbrood intervals (defined as the period between two parturition events) compared to species with lower levels of superfetation [9,42]. Due to the presence of superfetation, IB = 1 does not represent the birth of embryos which eggs were fertilized at IB = 0, but that of an antecedent brood.

To avoid an effect of feeding on body shape (i.e. abdominal extension), the feeding schedules of both species included a 16–24 h food deprivation period prior to the measurements (see Supporting Material S1 for further information regarding feeding). Our final dataset comprised 246 three-dimensional body models for 10 pregnant (plus 10 virgin control) *P. gracilis* and 14 pregnant (plus 14 virgin control) *P. turneri*. Of these 246 data points, six *P. turneri* models were omitted preceding analysis because these females were fed shortly before imaging.

*Creation of three-dimensional body models* – To create a single body model, a fish was first transferred to a small tank (8 × 8 × 8 cm) with scale bars for image calibration on all walls. Orientation of the female was limited by a separate movable divider. Three photos were taken simultaneously with three Nikon D3200 DSLR cameras (Nikon, Tokyo, Japan; sensor resolution 24 Mpix, equipped with Micro-Nikkor f = 55 mm lenses), synchronized with a remote trigger (JinJiaCheng Photography Equipment Co., Ltd., Shenzhen, China) and with LED lights behind glass fibre cloths opposite to the cameras providing diffuse back lighting. The three orthogonally placed cameras yielded a lateral, ventral and rostral/caudal view of the fish. Multiple sets of photos were taken during one measurement session; for further analysis a set of three synchronized pictures was selected in which the fish was in a straight and minimally rotated position.

These photos were subsequently processed with an in-house developed program in MATLAB 2013a (MathWorks, Natick, MA, United States), adapted from a program previously described by [43]. The longitudinal axis of the fish was defined by a straight line between the most anterior point of the snout and the most posterior part of the caudal peduncle (Standard Length, $L_{SL}$; white lines in figure 2A). The longitudinal axis of the fish consisted of on average 3122 (SE ± 14) pixels and 3193 (SE ± 15) pixels in the lateral and ventral views respectively. Outlines of trunk and eyes were manually digitized (figure 2A, blue and red lines respectively) as was the position of the abdomen of the fish (lateral view only; delimited by the dorsal edge of the vertebral column, swim bladder and the bottom of the abdomen; figure 2A, orange line). After cubic spline interpolation of the outlines, the position of the outlines with respect to the longitudinal axis was measured at 251 equidistant points along the longitudinal axis. Using cubic spline interpolation, these points were subsequently converted into ellipse-like cross-sections, that differed in

shape depending on whether the section was located in the abdominal region (figure 2B). In the abdominal region, the minor axis is shifted to half-way the abdominal polygon at that section (default at centre of major axis). Cubic spline interpolations were also used to create a 3D-model of the eyes (with a cubic spline resembling a super-ellipse), which was then stitched to the trunk to create a full 3D-model (figure 2C).

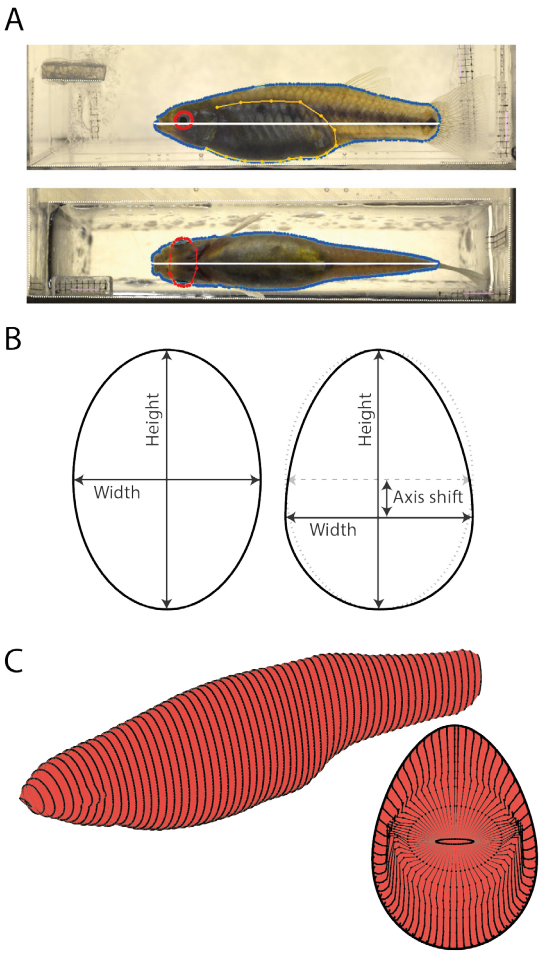



**Figure 2.** Morphological measurement and 3D model construction. **(A)** Lateral and ventral photographs in which the trunk (green), abdomen (orange) and eyes (red) are outlined by manually indicated polygons. The longitudinal axis is depicted by white lines. **(B)** At 251 equidistant points along the longitudinal axis, the width and height of the polygons are converted into ellipse-like cross-sections; in the abdominal area, the vertical position of the horizontal axis is shifted. **(C)** Stitching the cross-sections of trunk and eyes results in a 3D model from which volume, wetted surface area and frontal surface area (projection at the right) can be calculated. For illustrative purposes these examples only consist of one-fourth of the number of cross-sections.

From these 3D models maximum height, maximum width, frontal surface area (area of frontal view projection, figure 2C), wetted surface area (total body surface area), and volume were calculated. Maximum height was determined for the whole body, while maximum width was determined for the abdominal region only as the level of opercular distention (the moment in the breathing cycle) caused the position of the widest point to fluctuate in the slender-most fish. These fluctuations only had a minimal effect on the measured total body volume (on average 0.53% with respect to the instance with the least distention). To correct for the effect of intra- and interspecies differences in body size, maximum values of all one-dimensional parameters were normalized by dividing the values by $L_{SL}$, surface areas by dividing by $L^2_{SL}$ and volume was normalized by dividing by $L^3_{SL}$.

*Litter wet mass* – To get an estimate of the partial reproductive allotment at IB = 1, all newborns from the litter were caught on the day of delivery and euthanized with a lethal dose of MS-222 (Tricaine-S; Western Chemical Inc., Ferndale, WA, United States). Total litter wet mass was measured after carefully removing excess liquid with a paper towel on a Mettler AE200 analytic balance (scale accuracy 0.0001 g; Mettler-Toledo B.V., Tiel, The Netherlands). Litter wet mass provides a better approximation of reproductive burden than litter dry mass, because the water content of the embryos contributes to the total volume of the brood. Not all *P. gracilis* litters could be weighed; however, since offspring size did not differ between females (Mixed model, $F_{9,19} = 1.31$, $P = 0.2953$) total litter wet mass was instead estimated using a linear fit between offspring number and measured litter wet mass (wet mass (g) = $0.0078 \cdot n_{\text{new-borns}}$; $R^2 = 0.9469$).

*Statistical analysis* – The change in morphological parameters was modelled as a two-level longitudinal growth model [44], using the Mixed procedure in SAS version 9.3 (SAS Institute, Cary, NC, United States) under restricted maximum likelihood (REML). This multi-level modelling (MLM) method compares individual growth trajectories between species, allows time to be processed as a continuous variable and is able to handle unbalanced and missing data [44,45]. The model consists of two levels, the level-one model (Equation 1) that represents individual change trajectories, and the level-two model (Equation 2, 3) that provides intercept and slope term for the sample average. For each individual (i) and time point (j), the measured parameter is a function of the individual intercept ($\alpha_i$), the individual growth trajectory ($\beta_i \cdot T_i$) and a random error term for that specific individual and time point ($\varepsilon_{ij}$). Litter wet mass ($w_i$) at IB = 1 was added as a covariate in the level-two model for slope (Equation 3), as arguably larger broods result in increasingly larger morphological parameters due to a higher growth rate. This also allows comparison of the morphologies without the effect of offspring wet mass. The common intercept ($\gamma_{11}$), linear slope ($\gamma_{21}$) an covariate ($\gamma_{23}$) terms in equations 2 and 3 represent

the values for *P. gracilis* while the $\gamma_{12}$, $\gamma_{22}$ and $\gamma_{24}$ terms represent the added difference for *P. turneri* for intercept, linear slope and covariate values respectively; $\zeta_{1i}$ and $\zeta_{2i}$ factor individual random error terms. 'Variance components' was used as covariance structure (default in SAS Proc Mixed), denominator degrees of freedom were calculated with Kenward-Roger and significance level alpha was set to 0.05 (default in SAS Proc Mixed). To compare model parameter estimates, post-hoc tests were performed using 'contrast' and 'lsmeans' statements. Virgin data were analysed using a similar MLM method, albeit with a simpler model. Because we did not expect any time-dependent effects, and the virgin controls did not have litter wet mass to use as a covariate, the model consisted solely of an effect of species.

Transformation of the data occasionally resulted in slightly better fits as indicated by marginally higher $R^2$-values from linear fits (using Proc GLM in SAS version 9.3), but using transformed data did not change the outcomes of the previously mentioned statistical models. Furthermore, we did not have any a priori expectations for the curve of the line. Therefore, we opted to use the original untransformed data and a linear depiction of change.

$$Y_{ij} = \alpha_i + \beta_i \cdot T_j + \varepsilon_{ij} \quad \text{(Eq.1)}$$

$$\alpha_i = \gamma_{11} + \gamma_{12} \cdot S_i + \zeta_{1i} \quad \text{(Eq.2)}$$

$$\beta_i = (\gamma_{21} + \gamma_{22} \cdot S_i) + (\gamma_{23} + \gamma_{24} \cdot S_i) \cdot w_i + \zeta_{2i} \quad \text{(Eq.3)}$$

## 3. Results

Type 3 tests for Fixed Effects for both the pregnant and the virgin MLM model can be found in Table S1. All fixed effects in the model were significant, for all measured morphological parameters.

*Morphological changes during pregnancy* – At the beginning of the interbrood interval (IB = 0), *Poeciliopsis gracilis* females have an overall larger normalized body size than females of *Poeciliopsis turneri*. Except for maximum width (MLM contrast of intercepts: $F_{1,21.2}$ = 2.52, $P$ = 0.1275; figure 3A), females of *P. gracilis* have a higher maximum height ($F_{1,21.4}$ = 15.46, $P$ = 0.0007; figure 3B), frontal surface area ($F_{1,21.6}$ = 12.49, $P$ = 0.0019; figure 3C), wetted surface area ($F_{1,24.8}$ = 18.17, $P$ = 0.0003; figure 3D) and volume ($F_{1,24.3}$ = 12.10, $P$ = 0.0019; figure 3E) than females of *P. turneri*.

We found that *P. turneri* increases in body size faster than females of *P. gracilis*, as indicated by the steeper slopes of the former species in maximum width (MLM contrast

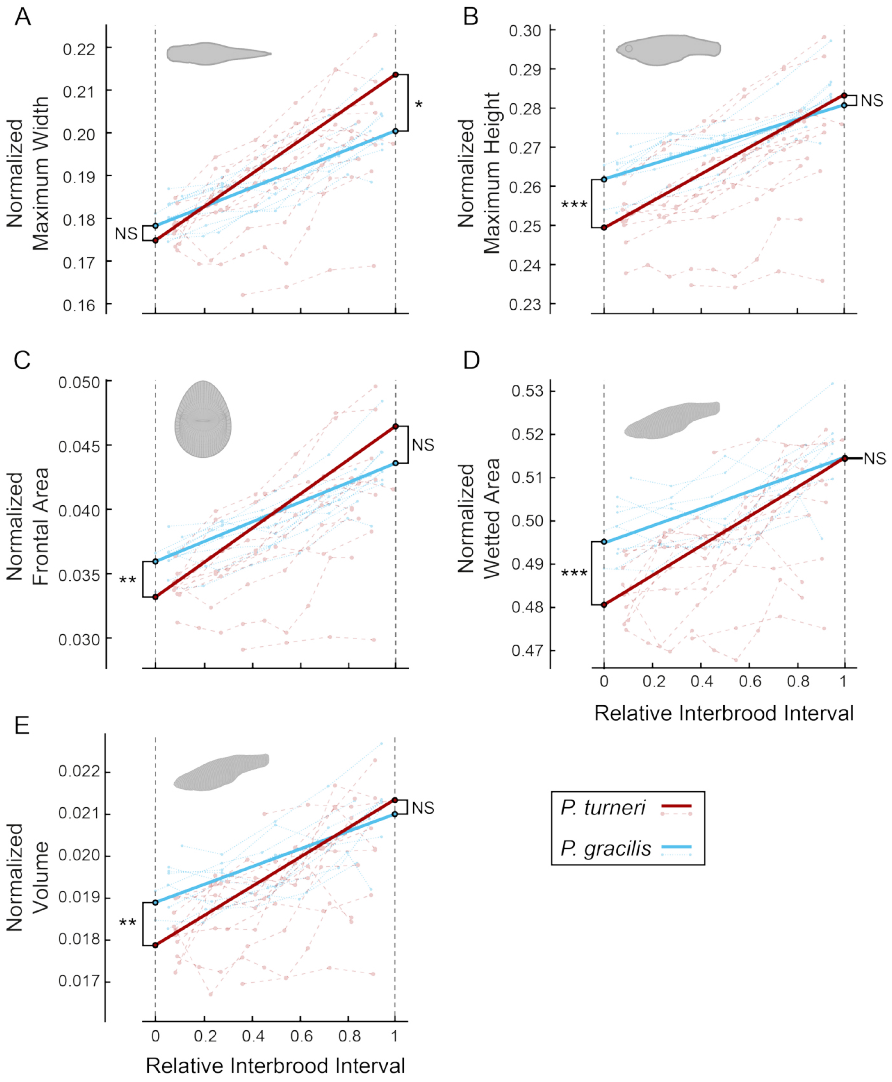



**Figure 3.** Shape parameters of pregnant *P. turneri* (with placenta) and *P. gracilis* (without placenta) from $N$ = 122 three-dimensional models. The multi-level longitudinal growth models (MLM) indicate changes in normalized (A) maximum width, (B) maximum height, (C) frontal surface area, (D) wetted surface area and (E) volume during one interbrood interval for pregnant *P. turneri* (red, $N$ = 14) and *P. gracilis* (blue, $N$ = 10). To account for individual variation in body size, one-dimensional parameters (A and B) were normalized by dividing the values by standard length ($L_{SL}$), the surface areas (C and D) by dividing by $L^2_{SL}$ and volume (E) by dividing by $L^3_{SL}$. Connected circles represent individual female growth trajectories, solid lines are plotted from the MLM estimates for intercept and slope with equal litter wet mass (NS = $P > 0.05$, * = $0.01 < P < 0.05$, ** = $0.001 < P < 0.01$, *** = $P < 0.001$). Projections show examples of the respective model projections (A–C) or the complete model (D,E).

of slopes: $F_{1,23} = 11.42$, $P = 0.0026$; figure 3A), maximum height ($F_{1,17.9} = 13.01$, $P = 0.0020$; figure 3B), frontal surface area ($F_{1,21.6} = 11.84$, $P = 0.0024$; figure 3C), wetted surface area ($F_{1,21.1} = 6.76$, $P = 0.0167$; figure 3D) and volume ($F_{1,22.2} = 5.92$, $P = 0.0235$; figure 3E). As a consequence, the measured differences at IB = 0 diminished towards the end of pregnancy (IB = 1) (post hoc comparison, maximum height: $P = 0.6531$; frontal surface area: $P = 0.0866$; wetted surface area: $P = 0.9541$; volume: $P = 0.4696$; figure 3B–E respectively), while at this point in time females of *P. turneri* have a larger maximum width ($P = 0.0127$; figure 3A). The steeper slopes in *P. turneri* are also reflected in the relative increase for maximum width (for *P. gracilis* maximum width at IB = 1 is 112 % of its value at IB = 0 compared to *P. turneri* whose maximum width at IB = 1 is 122 % of the value at IB = 0), maximum height (*P. gracilis*: 107 %, *P. turneri*: 113 %), frontal surface area (*P. gracilis*: 121 %, *P. turneri*: 140 %), wetted surface area (*P. gracilis*: 104 %, *P. turneri*: 107 %), and volume (*P. gracilis*: 111 %, *P. turneri*: 120 %).

*Morphological differences between virgins* – In line with the measured differences at IB = 0 for their pregnant conspecifics, virgin fish of *P. gracilis* have a larger overall normalized body size than virgins of *P. turneri*. We found significant effects of species on maximum width (MLM contrast: $F_{1,20.9} = 39.62$, $P < 0.0001$; MLM estimate ± SE: *P.g.* 0.1800 ± 0.0021, *P.t.* 0.1624 ± 0.0018), maximum height ($F_{1,21.2} = 84.02$, $P < 0.0001$; *P.g.* 0.2652 ± 0.0025, *P.t.* 0.2354 ± 0.0021), frontal surface area ($F_{1,20.6} = 77.98$, $P < 0.0001$; *P.g.* 0.0367 ± 0.0006, *P.t.* 0.0298 ± 0.0005), wetted surface area ($F_{1,19.5} = 35.28$, $P < 0.0001$; *P.g.* 0.4972 ± 0.0032, *P.t.* 0.4726 ± 0.0027), and volume ($F_{1,19.3} = 32.62$, $P < 0.0001$; *P.g.* 0.0191 ± 0.0003, *P.t.* 0.0171 ± 0.0002).

## 4. Discussion

A key aspect of this study is that we compare two different reproductive states (pregnant and virgin fish) in two phylogenetically closely related 'sister' species that differ in the way that they provision their embryos [7]: *Poeciliopsis gracilis* is a lecithotrophic species that lacks a placenta, while *Poeciliopsis turneri* represents one of three independent origins of extensive placentation in the genus *Poeciliopsis* [7,10]. *Poeciliopsis gracilis* and *P. turneri* have moderate levels of superfetation [23,36]. Due to smaller litters per parturition, higher levels of superfetation could result in reduced litter wet mass (Table S2). Since the level of superfetation also affects the length of the interbrood interval [9,23,42], time was normalized in our MLM models to account for variation in interbrood interval both within and between species. In absolute terms, the difference in growth rate between the two species would be even more pronounced than is currently shown by our results. Finally, we measured the morphology of virgin controls, because they offer a morphological 'base-line' similar to the start of the first pregnancy: all virgin females

carried unfertilized, fully yolk-provisioned eggs but were not yet affected by having to carry overlapping broods (superfetation). We showed that virgins of *P. turneri* have a more slender body shape than those of *P. gracilis*, in line with the observed differences at the beginning of the interbrood interval of pregnant females. At IB = 0, pregnant *P. gracilis* are morphologically more alike their virgin conspecifics than pregnant *P. turneri*, probably because the subsequent brood is already further developed in the latter species due to its higher level of superfetation (Table S3).

Together these findings provide the first evidence in support of two key predictions of the locomotor cost hypothesis that the evolution of post-fertilization maternal provisioning by means of a placenta leads to a more slender body shape at the beginning of the pregnancy, and that this 'morphological benefit' diminishes over the course of the pregnancy (figure 1; [9,36,37]). Our results further show that maximum width is higher in females of *P. turneri* during late pregnancy, which could lead to a reduction in abdominal flexibility during this period, more than that experienced by *P. gracilis*.

Whether, and to what extent, the measured morphological differences translate directly into differences in swimming performance requires further investigation, as other parameters that determine performance (e.g. physiology, flexural stiffness) could also be affected by pregnancy. Body shape, however, is known to affect the drag forces a fish experiences during swimming, such that more slender animals have a better continuous swimming performance and lower metabolic costs of swimming (e.g. [46–50]). There are two main types of drag that act on swimming fish of this size: pressure drag and friction drag; the former is related to the frontal surface area and the latter to the wetted surface area [51,52]. In *P. turneri*, both surface areas are lower at the beginning of pregnancy, and increase more rapidly over time than in *P. gracilis* (figure 3C–D), implying an absolute benefit during the early stages and a mean benefit over the whole interbrood interval. During undulatory swimming, the experienced drag is highly complex due to the changing pressure and shear stress distribution on the deforming body [53]. It is, however, apparent that the survival value of optimizing swimming speed and cost of transport (and thus reducing drag forces on the body) could be a driving force behind the evolution of fish morphology [54].

To study female morphology in three dimensions, we used a novel method for collecting longitudinal data, adapted from an in-house developed program originally designed to create 3D-models for zebrafish (*Danio rerio*) larvae [43]. Our method offers two advantages over conventional two-dimensional geometric morphometric approaches. First, we took pictures while the female is in the water, thereby avoiding the need to kill, anesthetize and/or handle the fish with a paintbrush or tweezers [26,55–59]. Minimizing stress, potential physical damage and risk of death is particularly important in longitudinal studies where a single individual is measured repeatedly over a period of time. Second, we use information from 251 equidistant cross-sections and two

orthogonal planes to reconstruct 3D body models. Even in a single 2D-plane (e.g. lateral view), our method produces a more accurate approximation of female body shape than landmark-based geometric morphometric approaches, which are often based on a limited number of (semi-)landmarks (typically between 12 to 17) and are hampered by a lack of clear landmarks in the abdominal region of pregnant females [55–59]. Perhaps more importantly, we show that different planes can yield different patterns of shape change through time (e.g. compare the effects in maximum width and height; figure 3A–B), suggesting that information from one plane cannot be readily used to make inferences about temporal changes during the pregnancy in the other planes nor overall streamlining (e.g. volume or frontal surface area).

We studied only one of eight independent origins of the evolution of the placenta in the family Poeciliidae [8,10]. To test the generality of our results, a wider comparative survey is required that includes other independent evolutionary origins of the placenta. Recent studies in the family Poeciliidae have revealed three independent origins of placentation in the genus *Poeciliopsis* (of which this study examined one; [7]) and two independent origins of placentation in the genus *Poecilia* (in the subgenera *Micropoecilia* and *Pamphorichthys*, respectively; [60,61]). These (sub)genera contain closely related species that differ in whether they have a placenta and are eminently suitable for further comparative experimental studies [13,14,23,24,37] to test whether the morphological benefits we found in this study are repeated in other placental lineages.

Moreover, it is possible that drag reduction is one of the driving forces behind the evolution of a placenta in other families of live-bearing bony fish (e.g. Anablepidae, Goodeidae, Zenarchopteridae) [62–64] and of a placenta or other ways of post-fertilization nutrient allocation (matrotrophy) in live-bearing cartilaginous fishes [5,65,66]. Furthermore, it would be worthwhile to study the effects of a placenta on morphology and relevant performance parameters in mobile animals that otherwise try to maximize slenderness, for instance live-bearing Squamate reptiles with a predominantly burrowing or 'sand-swimming' mode of locomotion (e.g. viviparous skinks) [67] or animals that are girth-restricted, for instance by the crevices they inhabit [68]. Finally, the placenta evolved many times independently throughout the animal kingdom, in livebearing animal lineages with a large diversity of lifestyles [2–6]; the broader applicability of the locomotor cost hypothesis requires further study.

To conclude, in this study we compared changes in volume and frontal surface area during gestation between a lecithotrophic and a placental fish species using a new 3D-modelling approach. Our results provide the first empirical evidence in support of the locomotor cost hypothesis, which states that the evolution of a placenta can lead to a more slender body shape at the start of the pregnancy and that this effect disappears towards the end of the pregnancy (figure 1, 3). To test the generality of our findings, future research should focus on additional independent placental lineages. The biomechanical

importance of drag reduction for locomotion, however, suggests that the locomotor cost hypothesis could potentially be applicable to the evolution of placentas in other swimming live-bearing lineages.

**Acknowledgements**

We thank David Reznick (UC Riverside) for supplying the original stocks of our study species and Cees J. Voesenek for his help with the custom-made MATLAB program. We further thank David Reznick, Andrew Furness (UC Irvine) and the Experimental Zoology Journal Club for commenting on an earlier version of the manuscript.

## References

1. Mossman H. 1937 Comparative morphogenesis of the fetal membranes and accessory uterine structures. *Carnegie Contrib. to Embryol.* **26**, 129–246.
2. Wourms JP. 1981 Viviparity: the maternal-fetal relationships in fishes. *Am. Zool.* **21**, 473–515.
3. Wourms JP, Grove BD, Lombardi J. 1988 The maternal-embryonic relationship in viviparous fishes. In *Fish Physiology* (eds WS Hoar, DJ Randal), pp. 1–134. New York: Academic Press.
4. Wake MH. 1992 Evolutionary scenarios, homology and convergence of structural specializations for vertebrate viviparity. *Am. Zool.* **32**, 256–263.
5. Blackburn DG. 2015 Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *J. Morphol.* **276**, 961–990. (doi:10.1002/jmor.20272)
6. Ostrovsky AN, Lidgard S, Gordon DP, Schwaha T, Genikhovich G, Ereskovsky AV. 2016 Matrotrophy and placentation in invertebrates: a new paradigm. *Biol. Rev.* **91**, 673–711. (doi:10.1111/brv.12189)
7. Reznick DN, Mateos M, Springer MS. 2002 Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298**, 1018–1020. (doi:10.1126/science.1076018)
8. Hrbek T, Seckinger J, Meyer A. 2007 A phylogenetic and biogeographic perspective on the evolution of Poeciliid fishes. *Mol. Phylogenet. Evol.* **43**, 986–998. (doi: 10.1016/j.ympev.2006.06.009)
9. Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009 Evolution of placentas in the fish family Poeciliidae: an empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **40**, 271–289. (doi:10.1146/annurev.ecolsys.110308.120209)
10. Pollux BJA, Meredith RW, Springer MS, Garland TJ, Reznick DN. 2014 The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature* **513**, 233–236. (doi:10.1038/nature13451)
11. Trexler JC, DeAngelis DL. 2003 Resource allocation in offspring provisioning: an evaluation of the conditions favoring the evolution of matrotrophy. *Am. Nat.* **162**, 574–585. (doi:10.1086/378822)
12. Reznick DN, Yang AP. 1993 The influence of fluctuating resources on life history: patterns of allocation and plasticity in female guppies. *Ecology* **74**, 2011–2019. (doi:

10.2307/1940844)
13. Banet AI, Reznick DN. 2008 Do placental species abort offspring? Testing an assumption of the Trexler-DeAngelis model. *Funct. Ecol.* **22**, 323–331. (doi:10.1111/j.1365-2435.2007.01367.x)
14. Banet AI, Au AG, Reznick DN. 2010 Is mom in charge? Implications of resource provisioning on the evolution of the placenta. *Evolution* **64**, 3172–3182. (doi:10.1111/j.1558-5646.2010.01059.x)
15. Pollux BJA, Reznick DN. 2011 Matrotrophy limits a female's ability to adaptively adjust offspring size and fecundity in fluctuating environments. *Funct. Ecol.* **25**, 747–756. (doi:10.1111/j.1365-2435.2011.01831.x)
16. Blackburn DG, Vitt LJ, Beuchat CA. 1984 Eutherian-like reproductive specializations in a viviparous reptile. *Proc. Natl. Acad. Sci.* **81**, 4860–4863. (doi:10.1073/pnas.81.15.4860)
17. Wourms JP, Lombardi J. 1992 Reflections on the evolution of piscine viviparity. *Am. Zool.* **32**, 276–293.
18. Trexler JC. 1997 Resource availability and plasticity in offspring provisioning: embryo nourishment in sailfin mollies. *Ecology* **78**, 1370–1381. (doi: 10.1890/0012-9658(1997)078[1370:RAAPIO]2.0.CO;2)
19. Holbrook GL, Schal C. 2004 Maternal investment affects offspring phenotypic plasticity in a viviparous cockroach. *Proc. Natl. Acad. Sci.* **101**, 5595–5597. (doi:10.1073/pnas.0400209101)
20. Schrader M, Travis J. 2005 Population differences in pre- and post-fertilization offspring provisioning in the least killifish, *Heterandria formosa*. *Copeia* **2005**, 649–656. (doi: 10.1643/CE-04-230R)
21. Wildman DE, Chen C, Erez O, Grossman LI, Goodman M, Romero R. 2006 Evolution of the mammalian placenta revealed by phylogenetic analysis. *Proc. Natl. Acad. Sci.* **103**, 3203–3208. (doi:10.1073/pnas.0511344103)
22. Reznick DN, Meredith RW, Collette BB. 2007 Independent evolution of complex life history adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae, Beloniformes) and Poeciliidae (Cyprinodontiformes). *Evolution* **61**, 2570–2583. (doi:10.1111/j.1558-5646.2007.00207.x)
23. Pires MN, Bassar RD, McBride KE, Regus JU, Garland TJ, Reznick DN. 2011 Why do placentas evolve? An evaluation of the life-history facilitation hypothesis in the fish genus *Poeciliopsis*. *Funct. Ecol.* **25**, 757–768. (doi:10.1111/j.1365-2435.2011.01842.x)
24. Bassar RD, Auer SK, Reznick DN. 2014 Why do placentas evolve? A test of the life-history facilitation hypothesis in two clades in the genus *Poeciliopsis* representing two independent origins of placentas. *Funct. Ecol.* **28**, 999–1010. (doi:10.1111/1365-2435.12233)
25. Shaffer LR, Formanowicz DR. 1996 A cost of viviparity and parental care in scorpions: reduced sprint speed and behavioural compensation. *Anim. Behav.* **51**, 1017–1024. (doi:10.1006/anbe.1996.0104)
26. Plaut I. 2002 Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
27. Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* **164**, 38–50. (doi:10.1086/421412)

28. Belk MC, Tuckfield RC. 2010 Changing costs of reproduction: age-based differences in reproductive allocation and escape performance in a livebearing fish. *Oikos* **119**, 163–169. (doi:10.1111/j.1600-0706.2009.17742.x)
29. Banet AI, Svendsen JC, Eng KJ, Reznick DN. 2016 Linking reproduction, locomotion, and habitat use in the Trinidadian guppy (*Poecilia reticulata*). *Oecologia* **181**, 87–96. (doi:10.1007/s00442-015-3542-9)
30. Shine R. 1980 Costs of reproduction in reptiles. *Oecologia* **46**, 92–100. (doi:10.1007/BF00346972)
31. Winne CT, Hopkins WA. 2006 Influence of sex and reproductive condition on terrestrial and aquatic locomotor performance in the semi-aquatic snake *Seminatrix pygaea*. *Funct. Ecol.* **20**, 1054–1061. (doi:10.1111/j.1365-2435.2006.01180.x)
32. Wu W, Meijer OG, Lamoth CJC, Uegaki K, van Dieën JH, Wuisman PIJM, de Vries JIP, Beek PJ. 2004 Gait coordination in pregnancy: transverse pelvic and thoracic rotations and their relative phase. *Clin. Biomech.* **19**, 480–488. (doi:10.1016/j.clinbiomech.2004.02.003)
33. Noren SR, Redfern JV, Edwards EF. 2011 Pregnancy is a drag: hydrodynamics, kinematics and performance in pre- and post-parturition bottlenose dolphins (*Tursiops truncatus*). *J. Exp. Biol.* **214**, 4151–4159. (doi:10.1242/jeb.059121)
34. Langerhans RB, Reznick DN. 2010 Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics. In *Fish Locomotion*, pp. 200–248. (doi:10.1201/b10190-8)
35. James RS, Johnston IA. 1998 Influence of spawning on swimming performance and muscle contractile properties in the short-horn sculpin. *J. Fish Biol.* **53**, 485–501. (doi:10.1006/jfbi.1998.0722)
36. Thibault RE, Schultz RJ. 1978 Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **32**, 320. (doi:10.2307/2407600)
37. Pires M, Arendt J, Reznick D. 2010 The evolution of placentas and superfetation in the fish genus ***Poecilia*** (Cyprinodontiformes: Poeciliidae: subgenera *Micropoecilia* and *Acanthophacelus*). *Biol. J. Linn. Soc.* **99**, 784–796. (doi:10.1111/j.1095-8312.2010.01391.x)
38. Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN. 2005 Do faster starts increase the probability of evading predators? *Funct. Ecol.* **19**, 808–815. (doi:10.1111/j.1365-2435.2005.01033.x)
39. Turner CL. 1937 Reproductive cycles and superfetation in Pœciliïd fishes. *Biol. Bull.* **72**, 145–164.
40. Turner CL. 1940 Superfetation in viviparous Cyprinodont fishes. *Copeia*, 88–91.
41. Scrimshaw NS. 1944 Superfetation in Poeciliid fishes. *Copeia* **1944**, 180–183. (doi:10.2307/1437814)
42. Reznick DN, Miles DB. 1989 Review of life history patterns in Poeciliid fishes. In *Ecology and Evolution of Livebearing Fishes (Poeciliidae)* (eds GK Meffe, FF Snelson), pp. 125–148. Prentice Hall, Englewood Cliffs, NJ.
43. Voesenek CJ, Pieters RPM, van Leeuwen JL. 2016 Automated reconstruction of three-dimensional fish motion, forces, and torques. *PLoS One* **11**, 8–10. (doi:10.1371/journal.pone.0146682)
44. Singer JD. 1998 Using SAS PROC MIXED to fit multilevel models, hierarchical models, and individual growth models. *J. Educ. Behav. Stat.* **23**, 323–355. (doi:10.3102/

10769986023004323)
45. Singer JD, Willett JB. 2003 *Applied longitudinal data analysis: Modeling change and event occurrence*. New York: Oxford University Press, Inc.
46. Pettersson LB, Hedenström A. 2000 Energetics, cost reduction and functional consequences of fish morphology. *Proc. R. Soc. B Biol. Sci.* **267**, 759–764. (doi:10.1098/rspb.2000.1068)
47. Weihs D. 2002 Stability versus maneuverability in aquatic locomotion. *Integr. Comp. Biol.* **42**, 127–134. (doi: 10.1093/icb/42.1.127 PMID: 21708701)
48. Law TC, Blake RW. 2005 Comparison of the fast-start performances of closely related, morphologically distinct threespine sticklebacks (*Gasterosteus spp.*). *J. Fish Biol.* **67**, 834–848. (doi:10.1111/j.1095-8649.2005.00788.x)
49. Langerhans RB. 2009 Morphology, performance, fitness: functional insight into a post-Pleistocene radiation of mosquitofish. *Biol. Lett.* **5**, 488–491. (doi:10.1098/rsbl.2009.0179)
50. van Wassenbergh S, Potes NZ, Adriaens D. 2015 Hydrodynamic drag constrains head enlargement for mouthbrooding in cichlids. *J. R. Soc. Interface* **12**. (doi:10.1098/rsif.2015.0461)
51. Sfakiotakis M, Lane DM, Davies JBC. 1999 Review of fish swimming modes for aquatic locomotion. *IEEE J. Ocean. Eng.* **24**, 237–252. (doi:10.1109/48.757275)
52. Videler JJ. 1993 *Fish swimming*. Dordrecht: Springer Netherlands. (doi:10.1007/978-94-011-1580-3)
53. Tytell ED, Borazjani I, Sotiropoulos F, Baker TV, Anderson EJ, Lauder GV. 2010 Disentangling the functional roles of morphology and motion in the swimming of fish. *Integr. Comp. Biol.* **50**, 1140–1154. (doi:10.1093/icb/icq057)
54. Tokic G, Yue DKP. 2012 Optimal shape and motion of undulatory swimming organisms. *Proc. R. Soc. London, B Biol. Sci.* **279**, 3065–3074. (doi:10.1098/rspb.2012.0057)
55. Zúñiga-Vega JJ, Reznick DN, Johnson JB. 2007 Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. *Oikos* **116**, 995–1005. (doi:10.1111/j.2007.0030-1299.15763.x)
56. Zúñiga-Vega JJ, Suárez-Rodríguez M, Espinosa-Pérez H, Johnson JB. 2011 Morphological and reproductive variation among populations of the Pacific molly *Poecilia butleri*. *J. Fish Biol.* **79**, 1029–1046. (doi:10.1111/j.1095-8649.2011.03081.x)
57. Wesner JS, Billman EJ, Meier A, Belk MC. 2011 Morphological convergence during pregnancy among predator and nonpredator populations of the livebearing fish *Brachyrhaphis rhabdophora* (Teleostei: Poeciliidae). *Biol. J. Linn. Soc.* **104**, 386–392. (doi:10.1111/j.1095-8312.2011.01715.x)
58. Ingley SJ, Billman EJ, Belk MC, Johnson JB. 2014 Morphological divergence driven by predation environment within and between species of *Brachyrhaphis* fishes. *PLoS One* **9**, 31–36. (doi:10.1371/journal.pone.0090274)
59. Frías-Alvarez P, Zúñiga-Vega JJ. 2016 Superfetation in live-bearing fishes is not always the result of a morphological constraint. *Oecologia* **181**, 645–658. (doi:10.1007/s00442-015-3477-1)
60. Meredith RW, Pires MN, Reznick DN, Springer MS. 2011 Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in *Poecilia* (Poeciliidae: Cyprinodontiformes). *Mol. Phylogenet. Evol.* **59**, 148–57. (doi:10.1016/j.

ympev.2011.01.014)
61. Meredith RW, Pires MN, Reznick DN, Springer MS. 2010 Molecular phylogenetic relationships and the evolution of the placenta in *Poecilia* (*Micropoecilia*) (Poeciliidae: Cyprinodontiformes). *Mol. Phylogenet. Evol.* **55**, 631–639. (doi:10.1016/j.ympev.2009.11.006)
62. Uribe MC, Aguilar-Morales M, De la Rosa-Cruz G, García-Alarcón A, Campuzano-Caballero JC, Guerrero-Estévez SM. 2010 Ovarian structure and embryonic traits associated with viviparity in Poeciliids and Goodeids. In *Viviparous fishes II* (eds MC Uribe, HJ Grier), pp. 211–229. New Life Publications, Homestead, Florida.
63. Uribe MC, De la Rosa-Cruz G, García-Alarcón A. 2014 Branchial placenta in the viviparous teleost *Ilyodon whitei* (Goodeidae). *J. Morphol.* **275**, 1406–1417. (doi:10.1002/jmor.20315)
64. Schindler JF. 2015 Structure and function of placental exchange surfaces in goodeid fishes (Teleostei: Atheriniformes). *J. Morphol.* **276**, 991–1003. (doi:10.1002/jmor.20292)
65. Dulvy NK, Reynolds JD. 1997 Evolutionary transitions among egg-laying, live-bearing and maternal inputs in sharks and rays. *Proc. R. Soc. London. Ser. B Biol. Sci.* **264**, 1309–1315.
66. Hamlett WC, Kormanik G, Storrie M, Stevens BS, Walker TI. 2005 Chondrichthyan parity, lecithotrophy and matrotrophy. In *Reproductive Biology and Phylogeny of Chondrichthyes* (ed WC Hamlett), pp. 395–434. CRC Press.
67. Stewart JR, Blackburn DG. 2014 Viviparity and placentation in lizards. In *Reproductive Biology and Phylogeny of Lizards and Tuatara* (eds JL Rheubert, DS Siegel, SE Trauth), pp. 448–563. CRC Press.
68. Schwarzkopf L, Barnes M, Goodman BA. 2010 Belly up: Reduced crevice accessibility as a cost of reproduction caused by increased girth in a rock-using lizard. *Austral Ecol.* **35**, 82–86. (doi:10.1111/j.1442-9993.2009.02015.x)

**Table S1.** Multi-level modelling output for fixed effects in the pregnant and virgin control models, for all measured morphological parameters. LWM: Litter wet mass

| Type 3 Test of Fixed Effects | Species | Species × Time | Species × Time × LWM |
|---|---|---|---|
| | | | |
| **Pregnant model** | | | |
| **Maximum width** | $F_{2,21.3} = 11879.6$, $P < 0.0001$ | $F_{2,23.3} = 94.60$, $P < 0.0001$ | $F_{2,19.5} = 10.11$, $P = 0.0010$ |
| **Maximum height** | $F_{2,21.5} = 12600.0$, $P < 0.0001$ | $F_{2,18.0} = 95.08$, $P < 0.0001$ | $F_{2,17.5} = 9.95$, $P = 0.0013$ |
| **Frontal surface area** | $F_{2,21.7} = 3978.58$, $P < 0.0001$ | $F_{2,21.9} = 99.70$, $P < 0.0001$ | $F_{2,19.7} = 12.44$, $P = 0.0003$ |
| **Wetted surface area** | $F_{2,25.0} = 44623.4$, $P < 0.0001$ | $F_{2,21.4} = 59.97$, $P < 0.0001$ | $F_{2,18.3} = 6.45$, $P = 0.0076$ |
| **Volume** | $F_{2,24.5} = 8327.83$, $P < 0.0001$ | $F_{2,22.5} = 60.12$, $P < 0.0001$ | $F_{2,18.3} = 7.76$, $P = 0.0036$ |
| | | | |
| **Virgin model** | | | |
| **Maximum width** | $F_{2,20.9} = 7554.59$, $P < 0.0001$ | | |
| **Maximum height** | $F_{2,21.3} = 11952.4$, $P < 0.0001$ | | |
| **Frontal surface area** | $F_{2,20.6} = 3620.88$, $P < 0.0001$ | | |
| **Wetted surface area** | $F_{2,19.5} = 28014.9$, $P < 0.0001$ | | |
| **Volume** | $F_{2,19.3} = 5224.14$, $P < 0.0001$ | | |

**Table S2.** General and reproductive parameters of the experimental fish used in this study.

| Species | Treatment | $N_{females}$ | Mean (± SE) SL (m) | Mean (± SE) IB interval (days) | Mean (± SE) $N_{models\ per\ individual}$ | Mean (± SE) Litter wet mass (g) |
|---|---|---|---|---|---|---|
| ***P. gracilis*** | Pregnant | 10 | 0.047 (0.001) [a] | 18.3 (0.26) | 4.9 (0.2) | 0.1794 (0.0143) † |
| | Virgin | 10 | 0.048 (0.001) [b] | | | |
| ***P. turneri*** | Pregnant | 14 | 0.047 (0.001) [a] | 11.9 (0.90) | 5.6 (0.4) | 0.1073 (0.0090) ‡ |
| | Virgin | 14 | 0.048 (0.001) [b] | | | |

[a,b] Values with the same superscript not significantly different (MLM, Tukey-Kramer adjusted p-values, alpha < 0.05)

†,‡ Values with the same symbol not significantly different (t-test, t-value = 4.48, df = 22, $P = 0.0002$)

**Table S3.** Relative values of morphological parameters of *Poeciliopsis gracilis* and *Poeciliopsis turneri*, compared to their virgin conspecifics. Absolute values can be found in the main text. IB: interbrood interval.

| Morphological parameter | *Poeciliopsis gracilis* | | | | *Poeciliopsis turneri* | | |
|---|---|---|---|---|---|---|---|
| | **Virgin** | **Pregnant** (IB = 0) | **Pregnant** (IB = 1) | | **Virgin** | **Pregnant** (IB = 0) | **Pregnant** (IB = 1) |
| **Maximum width** | 100 % | 99 % | 111 % | | 100 % | 108 % | 131 % |
| **Maximum height** | 100 % | 99 % | 106 % | | 100 % | 106 % | 120 % |
| **Frontal surface area** | 100 % | 98 % | 119 % | | 100 % | 112 % | 157 % |
| **Wetted surface area** | 100 % | 100 % | 104 % | | 100 % | 102 % | 109 % |
| **Volume** | 100 % | 99 % | 110 % | | 100 % | 105 % | 125 % |

## Supporting Material S4. Fish rearing, feeding and husbandry

*Study species – Poeciliopsis gracilis* originated from a small tributary of Rio Motagua, near the village Jones in Zacapa (Guatemala), and *Poeciliopsis turneri* were collected in Rio Purificacion, near Casimiro Castillo, Jalisco (Mexico). These fish stocks were originally housed at the Reznick lab (University of California Riverside, USA). The fish used in the experiments were bred from laboratory stocks that originate from the Reznick lab and are currently held at the Aquatic Research Facilities (ARF) at Wageningen University & Research (The Netherlands).

*Poeciliopsis gracilis* and *P. turneri* are closely related 'sister' species that differ markedly in when and how they provision their developing embryos [7]. The matrotrophy index (MI), a dimensionless number defined as the dry mass of the neonate at birth divided by the dry mass of the egg at fertilization, can be used as a proxy for the level of post-fertilization maternal provisioning: *P. gracilis* has an MI of 0.69 (lecithotrophic) and *P. turneri* an MI of 41.4 (placentotrophic, [7]). Both exhibit a moderate degree of superfetation, the presence of multiple broods that differ in the developmental stage of the embryos [24,42], the differences in degree of superfetation are reflected in interbrood interval (mean (± SE) *P. gracilis* 18.3 (± 0.26) days vs. *P. turneri* 11.9 (± 0.90) days; Table S2).

*Fish rearing and pre-experimental husbandry* – Breeding stocks were kept in 40 L tanks. From these tanks, new-born juveniles were moved to other stock tanks based on age cohort (age differences within cohorts spanning 2–4 weeks). Sexual maturity was

monitored daily and males were removed when sexual characteristics started to develop. Female fish were allowed to grow to a length of approximately 4 cm (± 4–6 months after parturition), after which they were isolated in 9 L isolation tanks (Tecniplast, Bugugiatte, Italy) enriched with gravel and a plastic plant. The water in the isolation tanks was maintained at 24–25°C and refreshed (~18 L $h^{-1}$). To ensure homogenisation of water quality among the isolation tanks, all tanks were supplied with water coming from the same biological filtering system. A juvenile conspecific was added to the tanks with virgin *P. gracilis* to reduce potential stress of isolation. The fish were randomly assigned to either a pregnant- or a virgin-treatment group, with one individual of each combined into a 'measurement block'. One to three males were added to tanks of the pregnant group, and switched around regularly. All companion fish were removed before the start of the experiments. Before the experiments started, the pregnant fish were allowed to go through at least three parturition events, as the first pregnancies are often smaller in brood size and number (Pollux & Reznick, unpublished data). Tanks were checked for new-borns at least once a day and parturition history was meticulously documented. Because of an initial high mortality in the isolated *P. turneri*, 7 additional pregnant females were randomly taken from the stock tanks and included in our study.

*Fish feeding protocol* – Stock fish were fed recently hatched brine shrimp (*Artemia nauplii*) in the morning (around 8 AM), and either flake paste (TetraMin; Tetra GmbH, Melle, Germany; on Tuesday, Thursday, Saturday and Sunday) or liver paste (Monday, Wednesday and Friday) in the afternoon (around 4 PM). The diet of isolated females consisted of flake- and liver paste during both the mornings and afternoons (days similar to stock), except during the weekend when the females received *Artemia nauplii*. In addition, at the end of each day each isolated female was fed adult brine shrimp. Sixteen to 24 hours prior to the measurements, females were deprived of food to avoid an effect of feeding on body shape (i.e. abdominal extension).

# Chapter 3

## Coasting in live-bearing fish: the drag penalty of being pregnant



Elsa M. Quicazan-Rubio[1], Johan L. van Leeuwen[1], Klaas can Manen[2], Mike Fleuren[1], Bart Pollux[1], and Eize J. Stamhuis[2]

[1] *Experimental Zoology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, The Netherlands*

[2] *Department of Ocean Ecosystems, Faculty of Science & Engineering, University of Groningen, Groningen, The Netherlands*

Published in *J. R. Soc. Interface* **16**: 20180714. (2019)

**Abstract**

Swimming performance of pregnant live-bearing fish is presumably constrained by the additional drag associated with the reproductive burden. Yet, it is still unclear how and to what extent the reproductive investment affects body drag of the females. We examined the effect of different levels of reproductive investment on body drag. The biggest measured increase in body volume due to pregnancy was about 43%, linked to a wetted area increase of about 16% and 69% for frontal area. We printed 3D models of live-bearing fish in a straight body posture representing different reproductive allocation levels. We measured the drag and visualized the flow around these models in a flow tunnel at different speeds. Drag grew in a power fashion with speed and exponentially with the increase of reproductive allocation, thus drag penalty for becoming thicker was relatively low for low speeds compared to high ones. We show that the drag increase with increasing reproductive allocation was most likely due to bigger regions of flow separation behind the enlarged belly. We suggest that the rising drag penalty with an increasing reproductive allocation, possibly together with pregnancy related negative effects on muscle- and abdominal bending performance, will reduce the maximum swimming speed.

## 1. Introduction

Oviparous (egg-laying) and viviparous (live-bearing) females exhibit considerable body shape changes during egg-carrying and pregnancy, respectively, due to the increase in reproductive allocation (RA, the proportion of body mass dedicated to reproduction). Both egg-carrying and pregnancy have been shown to negatively affect the locomotor performance of females in a number of different taxa (e.g., invertebrates [1,2], reptiles [3–8], fish [9,10], birds [11,12], and humans [13]).

The causes for the lower locomotor performance can be divided into two non-mutually exclusive categories: physiological and physical. First, the extra energy required to provision eggs before fertilization (in oviparous animals) or nourish developing embryos during gestation (in viviparous animals) might lead to lower energetic investments towards the locomotor muscles, negatively affecting their contractile properties and power output [14]. Secondly, the additional reproductive mass may negatively affect a female's ability to accelerate, while an increase in body volume could increase body drag and limit axial bending, negatively influencing a female's general swimming performance [10,14].

The mode of locomotion (e.g. flying, running, crawling, swimming, etc.) and the medium through which animals move (e.g. air, water, sand) determines how reproduction affects female locomotion. In swimming animals, both egg-carrying and pregnancy have

been shown to lead to lower fast-start escape and sustained swimming performances, which has been attributed (at least partly) to an increase in drag on the body caused by an increase in frontal (projection of the fish area on the transversal plane, FA) and wetted area (WA, area exposed to the fluid) [9,10,15–17].

Despite the presumed relevance of drag on the swimming performance of gravid and pregnant females, the effect of reproductive investment on drag production and the understanding of how extra drag originates remains poorly understood. So far, the effect of pregnancy on drag production has been examined only for the bottlenose dolphin, *Tursiops truncatus* [18], where drag was derived from videos of freely coasting pre- and post-parturition females. Pregnancy altered the morphology and kinematics of the females, and increased their drag during coasting, diminishing their locomotor performance. However, these observations were limited to only two dolphins and the effect of their reproductive investment was not taken into account in the analyses. These two constraints, the low sample size and the difficulty of correlating female body shape change with litter size, can be circumvented by working with Poeciliid live-bearing fish. Thanks to the small body sizes and short life-cycles, this family is a convenient model for the study of the effect of reproduction on swimming performance.

Poeciliid fish use a wide range of swimming motions [19], including the burst-and-coast swimming style, alternating undulations of the body and caudal fin (BCF) with unpowered coasting movements. During BCF swimming, some parts of the body surface contribute to drag, whereas others contribute to propulsion and these contributions vary over the swimming cycle. In this case, drag can potentially be computed (using computational fluid mechanics) as the sum of all the forces on the body surface that counteract forward motion, while those forces on the body surface that add to the forward motion add up to the net propulsion. General application of this approach in adult fish is, however, prohibited by the considerable computational efforts and the unavoidable inaccuracies imposed by the turbulent nature of the flow. On the other hand, in a coasting fish (with a constant body shape), drag is the net fluid force on the body which decelerates the fish, which in turn reduces the drag because drag depends approximately quadratically to coasting speed [20]. A coasting fish can be simulated reasonably well by a rigid body in a flow tunnel [21,22], and this setup facilitates the direct measurement of drag. And although measurements on rigid bodies at constant speeds is a simplification of the decelerating coasting in real fish, this represents a first estimate of the drag produced by pregnant swimming fish.

We aim to fill the knowledge gap on the effects of reproductive allocation (RA) on the drag production of aquatic gravid and pregnant fish, using *Poeciliopsis gracilis* (Heckel, 1848). This species is a member of the Poeciliidae, a family of live-bearing fishes with considerable increases in RA during gestation, ranging from 4.1% to over 35% of their body weight (brood sizes ranges from 1 to over 200 new-borns [23]). We obtained

morphological information of females throughout their pregnancy to construct a series of representative three-dimensional body models of females with different RA. We then measured the drag of each fish model in a flow tunnel at a series of speeds and visualized the flow around the body to identify if and how the expected differences in drag were reflected in differences in the associated flow patterns. Thus, we tested how the increase in RA affects drag and the flow separation along the body.

## 2. Material and methods

### 2.1. Biological aspects

For detailed description about the fish rearing and husbandry see S1 Text. *Poeciliopsis gracilis* has a high RA at the start of its pregnancy because almost all the nutrients for the embryo are stored in the egg yolk prior to fertilization [24]. The females usually carry simultaneously about two litters that differ in the stage of embryonic development, a reproductive strategy referred to as superfetation [23,25,26]. At delivery, a second litter is at about half-way its internal developmental time and a third one is about to start.

We used a total of 20 females to measure brood sizes over a period of 6–12 months. A subset of those females ($N_{fish}$ = 10) were used to quantify RA and changes in 3D body shape during one inter-brood interval (IB, the period between two parturition events). Females were not fed for 16 h prior to measurements to minimize possible confounding effects on body shape from food in the gastrointestinal tract. We focused on 3 stages of the inter-brood interval: (i) the start of the inter-brood interval (mean ± SE: 0.8 ± 0.1 days after delivery of a brood; $N_{fish}$ = 10), hereafter referred to as IB = 0, (ii) the midpoint of the inter-brood interval (IB = 0.5) and (iii) the end of the inter-brood interval (mean ± SE: 1.6 ± 0.3 days before the delivery of the next brood; IB = 1). We measured the body volume before and shortly after the female delivered the offspring and expressed the differences between these volumes as a percentage of the female volume after delivery. We called this percentage the *Reproductive Allocation Increase* (RAI).

A positive linear relationship between RAI and the number of new-borns of these 10 females during one IB (figure 1*a*, grey bars) appeared to be significant (figure 1*b*, $p < 0.05$, $R^2 = 0.516$), indicating that the most developed litter has a dominant influence on the volume growth of the female. This correlation also allows a (rough) estimate of the effect of number of embryos in the oldest litter on body drag (see below). We also documented the clutch sizes of all the experimental population ($N_{fish}$ = 20) for at least 6 months up to maximally one year and they produced clutch sizes varying between 1 and 48 new-borns (figure 1*a*, black bars), a range positioned within the natural one (1–70 newborns, [27], Pollux & Reznick, unpublished data).

### 2.2. Three-dimensional model construction

We quantified body shape ($N_{fish}$ = 10) by taking photographs from three perpendicular

views (lateral, ventral, and frontal; figure S2*a*), following the method used by [17,28]. The outlines obtained (figure 2*a* and S2*b*), were subsequently fitted with a series of 100 evenly spaced cross-sectional area ellipses along the body, whose major and minor axes were assigned as local height and width of the outline respectively. We excluded the fins to avoid confounding effects to our data due to their variable shape during swimming (e.g. due to folding and unfolding or bending of the fins).

Body posture appeared to be different during coasting than when at rest, namely in opercular abduction and orientation of the caudal peduncle (figure S2*c,d,e*). Detailed series of pictures from the body could only be made when a fish was at rest. Thus, we corrected for the opercular abduction and caudal peduncle downward bending to get the closer representation of the body shape during coasting (see S2 Text for detailed information regarding body posture corrections).



The model representing IB = 0 was built by averaging width and height of the 100 sections ($N_{fish}$ = 10). We then calculated the average of the differences in width and height along the body between segments IB = 0 and IB = 0.5, and between IB = 0 and IB = 1 (figure 2*b*). During pregnancy, the changes in height and width of the ellipses representing the head and peduncle were below 0.023% of the standard length (SL) of the fish. Because these two regions are not expected to change in size during pregnancy, we set this value as the threshold and any changes below it were considered non-significant and set to zero. We smoothed the resulting curves using a simple moving average filter with a window of 5 data points (using the MATLAB function 'moving' by Aslak Grinsted, figure S3). We added the corresponding filtered data to the IB = 0 model to build the IB = 0.5 and IB = 1 (figure 2*c*). The external shape of the eyes could not be captured with the longitudinal series of ellipses that were used to quantify body shape. For each fish, the outer shape of both eyes was digitized from the images at IB = 0 and were modelled with a short series of super ellipses (for details see S3 Text). The averaged eye model for all females was merged with the head of each of the new models (one example in figure 2*d*).

The three average body models (IB = 0, IB = 0.5 and IB = 1) were derived from the subsample of our lab population ($N_{fish}$ = 10). The brood sizes and RAs in that subsample were rather small (ranging from 14 to 31), smaller than those observed for the full population over several inter-brood intervals (ranging up to 48 babies). To account for this variation, we linearly extrapolated the relationship between brood size and body reproductive allocation increase (brood size fit = 0.8662 RAI + 9.254) and built an additional 6 body models representing the range of brood sizes and body volumes of the laboratory population (figures 1*b* and 2*e*). These models were obtained by adding to the IB = 0 model the difference in width and height between IB = 0 and IB = 1, multiplied by 1.5, 2, 2.5, 3, 3.5, and 4 (figure 2*e*). Because the dorsal outline shows only minor changes throughout pregnancy, we kept its shape constant among models (figure 2*e*). We used the same eye model for all fish models.

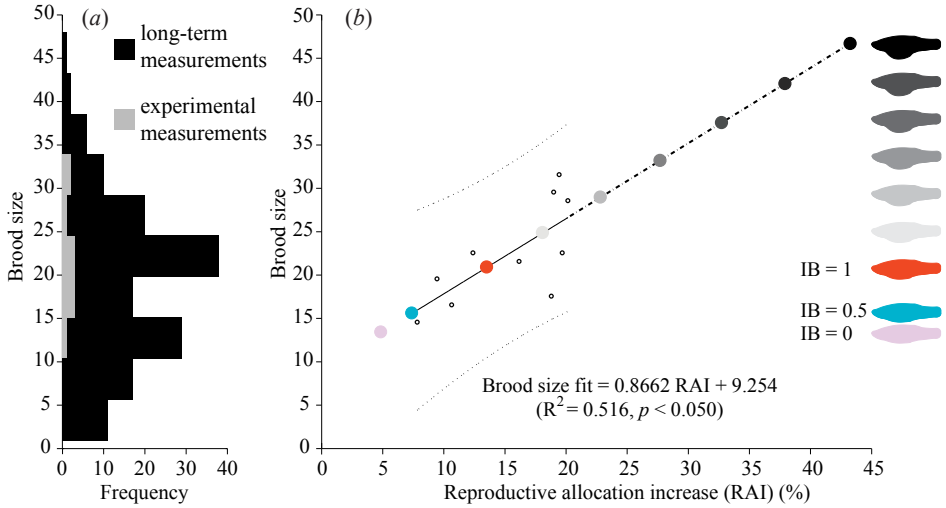



**Figure 1.** Brood size and RAI relationship. (*a*) Brood sizes frequency. Black squares: frequencies for $N_{fish}$ = 20 examined through multiple inter-brood intervals. Grey squares: frequencies for $N_{fish}$ = 10 in the experimental interbrood interval. (*b*) Linear regression of brood size and RAI from beginning to end of one interbrood interval of $N_{fish}$ = 10 (small open circles). Dotted lines represent 95% confidence intervals. Fish profiles represent the full range of population's brood sizes, built by extrapolating the linear regression. The bottom three models represent IB = 0, 0.5 and 1, from the bottom up. The remaining fish models represent bigger broods modelled by adding 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 times the averaged change in width and height seen between IB = 0 and IB = 1 (see paragraph 2.2).

We constructed a total of 9 fish models (RAI = 4.8, 7.4, 13.5, 18.1, 22.8, 27.7, 32.7, 37.9, 43.2) by means of 3D printing. The trunk was shaped by generating ellipses with the heights and widths for each model. The ellipses along the body were implemented in stereolithography (STL) meshes. We resized the models to optimize them for the flow and force measurements, given the tunnel dimensions and specifications of the force transducer. All meshes were scaled at 1:3.2 using the averaged standard length of the fish (mean ± SD: 0.0474 ± 0.0029 m), resulting in models of 15 cm length. A hole was modelled on the dorsal side on top of the centre of mass of the fish model to fit a carbon-fibre rod (diam. 5 mm). Physical models were 3D printed with Polyamide PA 2200 on a Selective Laser Sintering printer (EOS Formiga P 100), with 0.1 mm resolution.

To measure the effect that surface texture and the shape from a single pregnant fish would have on the drag production, we photographed an additional (eleventh) female in the same way as described above, and 1–2 days before anticipated delivery we sacrificed it using an overdose of anaesthetic (Tricaine-S, MS-222) and weighed it. We then mounted the female by hanging it straight from the tail, froze it in liquid nitrogen, and scanned it

with a micro-CT scanner (Phoenix v[tome]x m of General Electric; voxel size 28.8 µm$^3$). From the CT images, a mesh of the fish was reconstructed using AVIZO® Fire software. Cleaning and further processing of the mesh was performed in Blender 2.72b. We digitally removed the fins, restored the eyes and took the lateral half that was least affected by the mounting and scanning processes and duplicated it to build a symmetrical model that represented the body shape during coasting. The mesh was treated as the ones of the other models to obtain a printed 3D model.

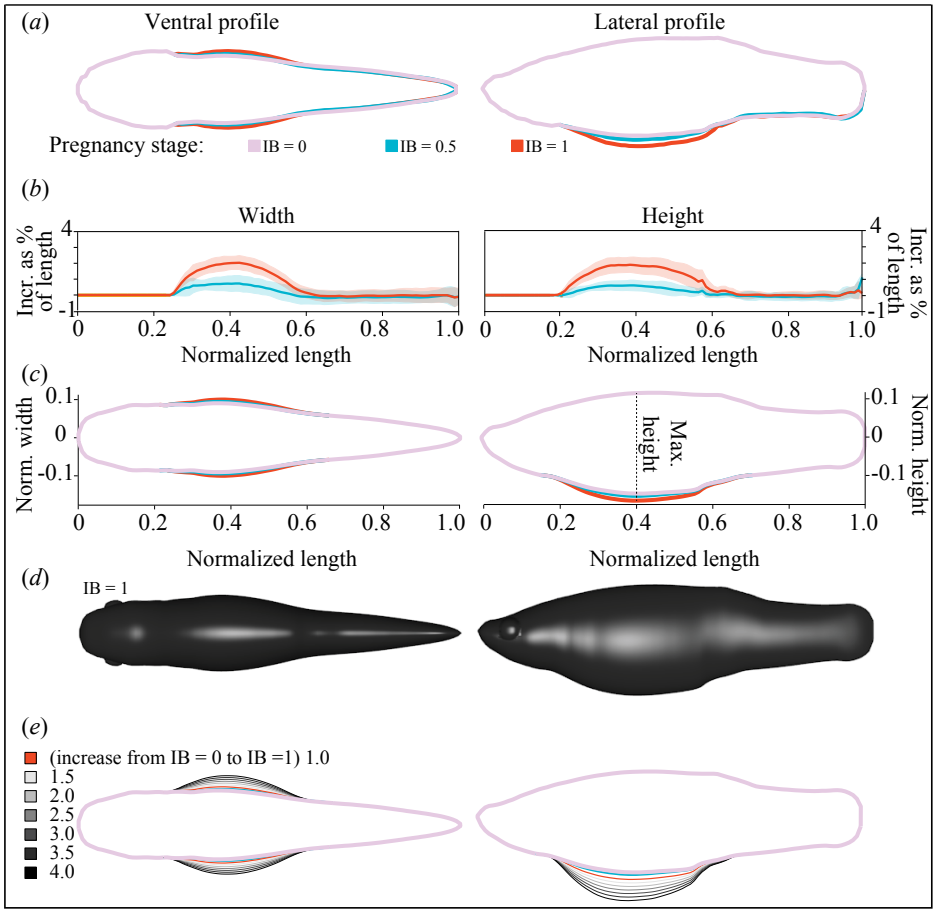



**Figure 2.** Construction of models based on body shapes of $N_{fish}$ = 10 followed through 1 interbrood interval: IB = 0 (1 – 2 days after delivery, innermost light curve), IB = 0.5 (mid time of the interval, middle curve), IB = 1 (1–2 days before delivery, external dark curve). (*a*) Width (left) and height (right) increase of one individual. (*b*) Along the body, mean and standard error of width and height increases with width and height ($N_{fish}$ = 10). (*c*) Models IB = 0, IB = 0.5 and IB = 1. The latter two models were built by adding the mean width and height increases to the IB = 0 model. (*d*) Example of tested 3D model, representing IB = 1. (*e*) Lateral and ventral view of extrapolated models built after adding changes in width and height to the IB = 0 model (specified in main text).

To describe the body shape of the resulting eleven models, we computed the WA and maximum FA (figures S4*a* and 5*b*) from the mesh information in Blender 2.72b. To refer to the body's streamlining, we used fineness ratio (FR = standard length/max. height, von Mises 1945, figure S4*b*). The fineness ratios of our fish models (FR = 2.97–3.80) overlapped well with the range found in natural *P. gracilis* populations (FR = 2.92–4.28, derived from data in [27]). Standard length and maximum height were obtained from the mesh information in Blender. None of these variables were used in statistical analyses due to their correlation with volume increase. They were used to explain possible causes of the differences in drag ($F_D$), drag coefficient ($C_D$, i.e. drag normalized to reference area and the square of flow speed with respect to the object times 0.5 [22]), and flow behaviour.

### 2.3. Force measurements

We used the 300 L flow tunnel described in [22] (see S4 Text for details). Models were submerged in the middle of the cross-section of the tank and attached (by a rod) to a force-measuring platform above the tank. On top of the platform, a force sensor (Honigmann GmbH RFS® 150-XY, Nr.705052, x: 5 N, y: 5 N, 1 mV/V) was mounted which was connected to a computer using an electronic precision measuring amplifier Tensiotron® TS 621. Data were recorded with a sample frequency of 1000 Hz during 5 s per test using HCC-Easy data transfer, an analysis program for Honigmann tension sensors.

The 10 *P. gracilis* females in our study reached maximum speeds of ~35 SL $s^{-1}$ in fast-start escape responses (Quicazan-Rubio *et al.*, unpublished data). Because we cannot exclude that the species could reach higher speeds in nature, we included also some higher speeds (up to 50 SL $s^{-1}$, table S1). To ensure the same flow patterns for the coasting live fish and the 3D printed models, we kept the body shape and Reynolds number (*Re*) the same: i.e. we increased the size of the fish models, while decreasing the flow velocity in the tunnel to keep *Re* constant ([29], see S4 Text for details). *Re* represents inertial versus viscous effects in the flow and is described by the equation:

$$Re = \frac{UL}{v}, \tag{1}$$

where $U$ is the 'free flow' speed, $L$ is the characteristic length of the object, and $v$ is the kinematic viscosity of the water. For $L$, we used the standard length (SL) of the fish (unit: m). The relatively large size of the fish model with respect to the actual fish allowed us to improve the accuracy of our drag and flow measurements. The experimental drag forces were expected to comply with the Rayleigh drag equation [20,22,30]:

$$F_D = \frac{1}{2}\rho A U^2 C_D, \tag{2}$$

where ρ is the density of water (1000 kg $m^{-3}$), $A$ is in this case the WA, and $C_D$ is the drag

coefficient dependent on shape. $F_D$ measurements were converted to $C_D$ using eq. 2.

$$C_D = \frac{2F_D}{\rho U^2 A}, \quad (3)$$

**2.4. Statistical analysis**

The effects of the pregnancy stages and increased reproductive investment on the drag forces and coefficients of drag were analysed with General Linear Models (GLM), using the proc MIXED procedure (SAS version 9.2; SAS Institute Inc., Cary, North Carolina, USA. 2007). Changes in drag over the different speeds were assessed with repeated measures analyses of variance (RMANOVA) using fish model (9 levels, corresponding to all models except the CT-scanned: IB = 0, IB = 0.5, and IB = 1, as well as 6 levels of higher reproductive allocation increases, RAI) and water speed (15 levels, table S1) as fixed effects, and the repetitions per measurement (5) as the subject effect [31]. Changes in $C_D$ over the examined *Re* range were also assessed using RMANOVA, with fish model (9 levels: the same ones as for the $F_D$), and *Re* (15 levels, table S1) included as fixed effects, and measurement repetitions (5) as the subject effect.

To measure the effect that surface texture and the shape from a single pregnant fish would have on the drag production, we examined the similarity of results between the CT-scanned model and the IB = 1 model since they have similar reproductive allocation. We applied GLM as above. Changes in drag ($F_D$) over the different speeds were assessed with RMANOVA including fish model (2 levels: IB = 1 and CT-scan), and water speed (15 levels, table S1) and the interaction between both as fixed effects, and the repetitions per measurement (5) as the subject effect [31]. Changes in drag coefficient ($C_D$) over the different Reynolds numbers were also assessed using RMANOVA, with fish model (2 levels: the same as for $F_D$), and *Re* (15 levels, table S1) included as fixed effects, and the times each measurement was taken (5) as the subject effect.

For all analyses, we selected a Toeplitz covariance structure based on Akaike's Information Criteria (AIC). All the multiple comparisons were done using a Bonferroni adjusted comparison-wise error rate of $p$ ($0.05/n$, with $n$ being the number of comparisons). The analyses were performed on the raw data.

**2.5. Particle Image Velocimetry**

To visualize the flow behaviour around the abdomen, we performed Particle Image Velocimetry (PIV; [32]) recordings at three (tunnel) speeds: 0.32 m $s^{-1}$, 0.56 m $s^{-1}$ and 0.64 m $s^{-1}$ over a selection of models that covered the sizes of the lab fish, RAI of 4.8, 13.5, 18.1, 27.7, and 43.2% and the CT-scanned model. We analysed the images with PIVlab [33], averaged the vector fields of 50 frames, and visualized the local velocities and the streamlines (see S5 Text for details).

## 3. Results

### 3.1. Body shape changes due to reproductive allocation increase

The body volume of pregnant females increased due to the growing embryos by nearly 13% (the increase in volume is equivalent to RAI) for the interbrood interval photographed on 10 females, and when extrapolated for the biggest brood documented in the 20 females population (48 new-borns), it corresponded to about 43.2% increase (figure 1*b*). The increase in reproductive allocation corresponded with the increase in WA and FA (figure S4*a*). Wetted area increased faster (WA = 5 x $10^{-05}$ RAI + 0.0113, $p < 0.0001$) but proportionally less (~16% for the biggest RAI) than the FA (FA = 2 x $10^{-05}$ RAI + 0.0009, $p < 0.0001$, ~69% for the biggest RAI). The streamlining of the females decreased linearly with increased volume (FR = –0.0215 RAI + 3.8565, $p < 0.0001$, figure S4*b*).

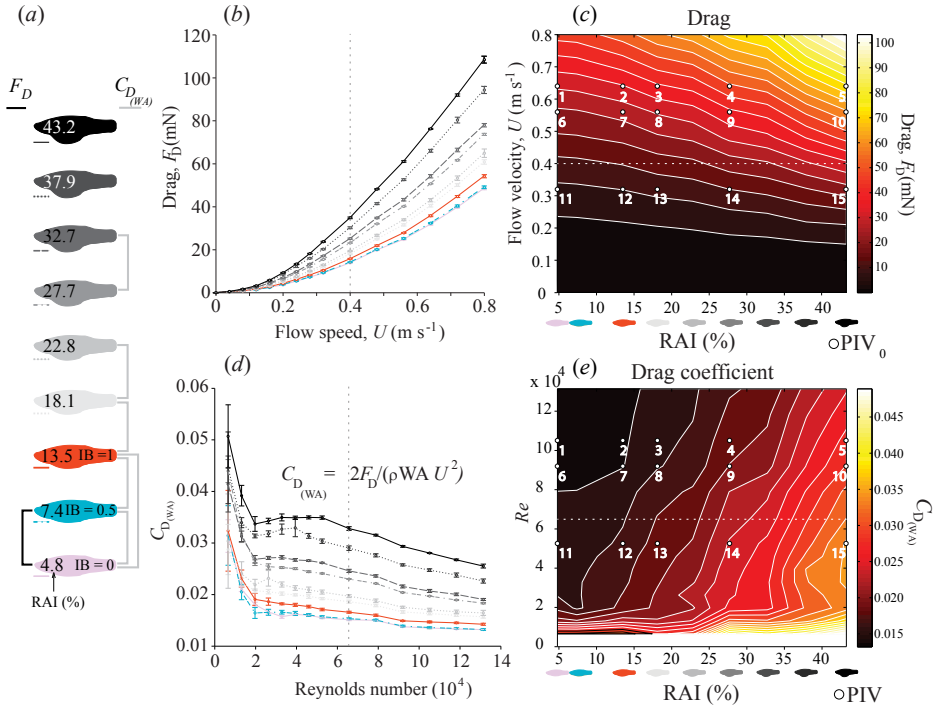



**Figure 3.** Raw data of $F_D$ and $C_D$ from models representing different RAI and tested at different $U$. (*a*) GLM analysis results of comparisons among models for $F_D$ and $C_D$. The brackets represent the non-significant differences (Bonferroni adjusted comparison-wise error rate of p(0.05/36) < 0.0014). Brackets at the left show results for $F_D$, and at the right for $C_D$. (*b*) $F_D$ results from the flow-tank experiments. (*c*) Contour plot of $F_D$ as a function of $U$ and RAI (obtained with cubic interpolation of the data set). (*d*) $C_D$ calculated using eq. 2 with WA. (*b,d*) Error bars represent the between-sampling standard error of the mean. Vertical dotted line represents equivalent speed and *Re*. (*e*) Contour plot of $C_D$ as a function of *Re* and RAI. (*c,e*) White dots represent the RAI and the $U$ at which PIV data were taken, corresponding to figure 4. Horizontal dotted line represents equivalent speed and *Re*.

### 3.2. Drag

Both RAI and flow speed significantly affected drag production (table 1). Drag was significantly different among all pairwise comparisons of model fish along the tested velocities (figure 3*a*, $p < 0.0014$), except for the models with lower RAI, 4.8 and 7.4% (IB = 0 and IB = 0.5) that showed similar drag ($p = 0.0922$; non-significant after Bonferroni correction). This was probably due to the small change in abdominal size in the first half of the interbrood interval (figure 2*b*). We observed that for the same force there was a significant reduction in speed when RAI increased (figure 3*b,c*), and at high speeds drag increased more rapidly which is represented by closer isolines with steeper slopes in figure 3*c*.

### 3.3. Drag coefficient

There was a significant effect of RAI and of *Re* on the drag coefficient (table 1). For almost all the data points, $C_D$ lowered with *Re*, following the expected tendency, and the drop became deeper with higher RAI (figure 3*c,e* and S6*b,d*). Comparing among models, the higher RAI became, the quicker the $C_D$ increased. This was more easily observed at a constant *Re* (e.g. dotted straight line in figure 3*d,e*), where the space between lines was smaller at low RAI at high RAI (figure 3*d*), which corresponds in figure 3*e* to the bigger space between the contour lines at low RAI than at high RAI. Thus, in general thicker body shapes presented bigger differences in $C_D$ than with thinner models.

**Table 1.** Type 3 test of fixed effects of $F_D$ and $C_D$ for RAI (RAI: Reproductive allocation increase, *U*: Speed, *Re*: Reynolds number, Num DF: numerator degrees of freedom, Den DF: denominator degrees of freedom).

| Dependent variable | Effect | Num DF | Den DF | *F - Value* | *Pr > F* |
|---|---|---|---|---|---|
| $F_D$ | RAI | 8 | 540 | 4736.67 | <.0001 |
| | *U* | 14 | 540 | 33470.2 | <.0001 |
| $C_D$ | RAI | 8 | 540 | 218.89 | <.0001 |
| | *Re* | 14 | 540 | $2.71 \times 10^{11}$ | <.0001 |

### 3.4. Drag expressed in terms of RAI and *U*

To explain the relationship between drag and both *U* and RAI, we performed a curve fit of $F_D$ from all models (except the CT-scanned model), RAI and *U*, running the least square method (LSM) in Gnuplot 4.0. We found that drag grows exponentially with respect to RAI and in a power fashion with *U*:

$$F_D(\mathrm{RAI}, U) = 0.0606\ e^{0.0230\ \mathrm{RAI}}\ U^{1.8012}\ , \tag{4}$$

To calculate the fitted $C_D$ we transformed eq. 3 following eq. 2:

$$C_D(\mathrm{RAI}, U) = \frac{2 \times 0.0606\ e^{0.0230\ \mathrm{RAI}}\ U^{1.8012}}{\rho\,(4.6558 \times 10^{-5}\,\mathrm{RAI} + 0.0113)U^2}\ , \tag{5}$$

These equations allowed us to describe what happens beyond the limits tested, using just the experimental data (figure S6).

### 3.5. Flow behaviour

All models showed separation of the boundary layer and the separation point was close to the anal region on the low RAI models and moved anteriorly when the RAI increased (figure 4). The size of the separation region increased with RAI, which corresponds to higher $C_D$, and for each model this region became smaller at higher speeds, which corresponded to lower $C_D$, as expected [34]. We also observed that the region of separated flow presented the highest differences in local speeds within the region of interest (figures 4 and 5*d*).

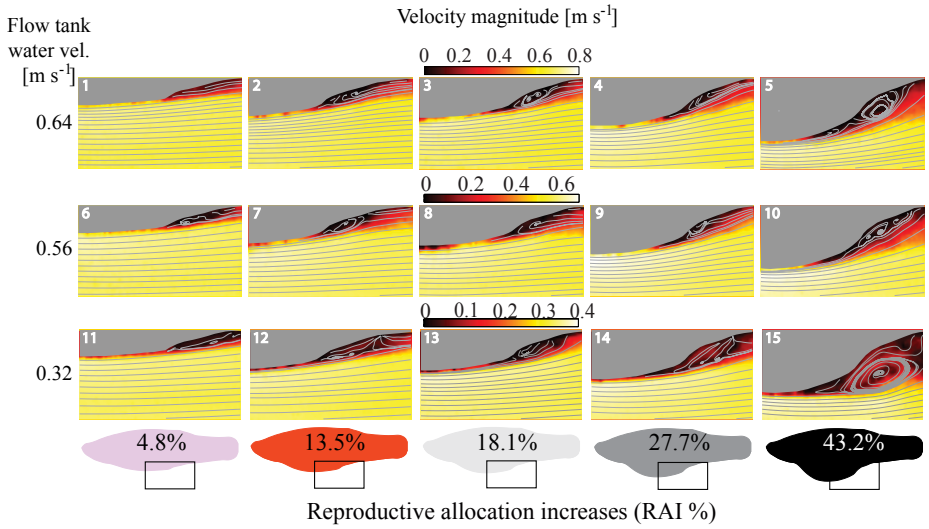



**Figure 4.** Flow fields recorded in flow tank from models representing different increases in reproductive allocation, tested at 0.32, 0.56, and at 0.64 m/s, *Re* ~ 50000, 90000 and 105000 respectively. The white numbers correspond to the locations in figure 3*c,e*.

### 3.6. Comparison between CT-scan model and averaged model with similar reproductive allocation

The CT-scan of an actual fish had a 5.6% larger FA, a 9.4% larger WA than the IB = 1 model (figure 5*b*1) possibly due to the replica of the scales and some differences in overall shape: e.g. it was less streamlined (FR of CT-scan model was 3.39, and of the IB = 1 model was 3.55, figure 5*b*2). There were no significant differences in drag for speeds up to ~0.6 m $s^{-1}$, which were the ones used by our population. Beyond that speed, the drag from the CT-scan model was significantly lower (at tunnel speed of 0.64 m $s^{-1}$, $p$ = 4.42 x $10^{-5}$; 0.72 m $s^{-1}$, $p$ = 8.30 x $10^{-10}$; and 0.80 m $s^{-1}$, $p$ = 1.08 x $10^{-6}$, all significant after Bonferroni correction. Figure 5*c*1). The drag coefficients of both the CT-scan and IB = 1

model decreased with increased Reynolds number (figure 5*c*2) and were not significantly different over the examined speed range (table S2, figure 5*c*2). The flow visualization in figure 5*d*1–3 suggested that a large portion of the drag was coming from the region behind the abdomen, where the higher differences in local speeds and therefore in pressures were expected. The flow follows the contour for most of the length of the fish, but the boundary layer separates around the anus on both models (figure 5*d*). The region of separated flow has a similar size among the speeds tested and between both models, but it has a laminar tendency on the CT-scan model whereas it presents whirls adjacent to model IB = 1.

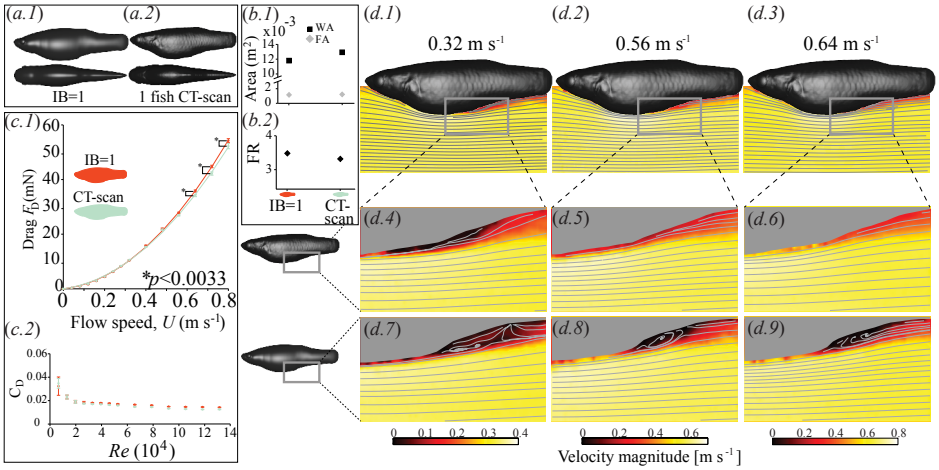



**Figure 5.** Comparison between models representing similar reproductive allocation. (*a*) Lateral and ventral view of models, (*a*.1) model IB = 1, (*a*.2) model from the CT-scan of 1 fish. (*b*) Models shape, (*b*.1) cross-sectional (FA) and wetted area (WA), (*b*.2) fineness ratio. (*c*) $F_D$, $C_D$ and statistical results from the flow-tank experiments. Error bars represent the between-sampling standard error of the mean. (*c*.1) $F_D$, * indicates significant differences using GLM analysis, comparing $F_D$ within the same speed, between the two fish models. Bonferroni adjusted comparison-wise error rate of $p$ (0.05/15) < 0.0033. Drag equation after polynomial regression for IB = 1: $F_{d\_fit} = 0.0744U^2 + 0.0085U$, $R^2 = 0.9997$, for CT-scan: $F_{d\_fit} = 0.0682U^2 + 0.0103U$, $R^2 = 0.9993$ (*c*.2) $C_D$. No significant differences were found between the two models. (*d*) Flow fields recorded in flow tank at 0.32, 0.56 and 0.64 m $s^{-1}$, equivalent to $Re$ ~50000, 90000, 105000 respectively. (*d*.1,2,3) CT-scan model from snout to tail. (*d*.4,5,6) Detail of abdomen and beginning of peduncle region of CT-scan model. (*d*.7,8,9) Detail of abdomen and beginning of peduncle region of model IB = 1.

## 4. Discussion

During pregnancy, aquatic animals are expected to experience greater drag forces due to an increased frontal area and surface-to-volume ratio [9,10,30]. Our study is the first to quantify the variations in drag due to changes in body shape in live-bearing fish. Specifically, we show that females 'pay a price' for (i) increasing their reproductive allocation and/or

(ii) swimming at higher speeds: drag grows exponentially with reproductive allocation and in a power fashion with speed. We furthermore identified the increment in size of the region of flow separation as the cause of the extra drag when the reproductive allocation grows bigger.

### 4.1. Body shape changed during pregnancy

The biggest brood observed in our lab population caused an increase of about 43.2% in volume (RAI), 69% in FA, 16% in WA, 28% in maximum height (girth) and a decrease in 22% in FR (figure S4). This roughly corresponds with values found in other pregnant live-bearing fish and aquatic mammals. The two pregnant bottlenose dolphins studied by [18] experienced comparable increases in FA (43 and 69%, respectively) and girth (16 and 26%), and *Gambusia affinis* experienced an increase in FA of about 52% [10]. The findings in this study might therefore also be relevant to other live-bearing and gravid animals (fish, reptiles, mammals). They might also be pertinent to aquatic animals with disturbances in their body shape due to tags or entangled fishing gear, both of which increase the drag production and animals exhibit a lower swimming speed, circumventing the extra energetic costs associated with the extra drag [35,36].

### 4.2. $F_D$ and $C_D$ changed with RAI and $U$

Females pay a price for increasing reproductive allocation and for increasing speed. Drag changed exponentially with RAI and in a power fashion with $U$ (eq. 3, figure 3*a,d* and S6*a,c*). It was proportional to $U^{1.8}$, close to the expected value of $U^2$, reflecting the inertial flow regime in which *P. gracilis* swims ($Re > 103.5$). We observed that for the same drag there was a significant reduction in speed when RAI increased and that this effect was more acute at higher speeds and higher RAI (figure 3*a,d*). The same tendency was observed for the $C_D$, it increased more sharply with increasing RAI, as it is seen in figure 3*e*, where the contour lines are closer together at higher RAI. For bottlenose dolphins, drag increased faster with speed ($U^{2.13}$ pre-parturition and $U^{2.17}$ post-parturition [18]), than for *P. gracilis*, which might be related to higher *Re* at which they swim.

### 4.3. Origin of the extra drag

We suggest that the differences in $F_D$ between models are mainly due to the increase of flow separation with increasing RAI. And the differences in $F_D$ between speeds are mainly due to the characteristics of the flow within the region of separated flow. When RAI increased, $F_D$ and $C_D$ increased too and the region of flow separation became bigger (figures 3 and 4), which indicates a higher rate of momentum transfer to the water and consequently less kinetic energy available, so the animal would be slowed down [37]. On the other hand, at a constant RAI and increased speed, $F_D$ increased, and $C_D$ decreased, which was reflected by a sharp decrease in the wake width of the separated flow from 0.32 to 0.56 m $s^{-1}$ and a similar wake width between 0.56 and 0.64 m $s^{-1}$ (figures 3*c,e* and 4). This difference in the

wake width may be due to the transition from laminar to turbulent flow that happens at the *Re* analysed with PIV (*Re* ~ 50,000-105,000). At around *Re* = 100,000, the flow close to the surface becomes turbulent, and so the fluid momentum increases allowing the flow to follow further the surface before separating. This also abruptly decreases the wake width [29].



### 4.4. Consequences of higher drag

Because pregnancy alters body shape, drag has been proposed to cause a considerable decrease in swimming performance of gravid and pregnant aquatic females such as bottlenose dolphins [18], short-horn sculpin [14], and aquatic snakes [5,38]. Poeciliid females at advanced stages of pregnancy also showed impaired locomotor performance [9,10] and we have demonstrated how drag augments considerably with reproductive allocation, supporting the hypothesis that drag might be one of the main causes of poorer swimming performance of pregnant females, presumably together with lower muscle performance and limited axial bending, although the last two have not been proven yet.

### 4.5. Other aspects of pregnancy and fish swimming

In this study, we quantified drag forces on stiff, 3D-printed fish models placed in a flow tunnel. However, other biomechanical, physiological or behavioural aspects of fish swimming under natural conditions are likely to influence the production of drag.

First, *P. gracilis* has a burst and coast swimming style, making alternating undulations of its BCF, with unpowered coasting movements [39]. We studied the coasting phase of swimming by measuring the drag forces produced by rigid bodies and found significant differences with increasing reproductive allocation. It has been computed that the drag forces for undulatory swimming are about 3 times higher than for rigid bodies [20], for two reasons: first, the undulating body causes a thinner boundary layer, increasing friction drag; second, the transverse recoil of the body caused by the lateral movement of the tail, produces extra drag when the tail resists the recoil movement [40]. Bending would be limited in pregnant fish by both the decrease of muscle/body ratio and the increase in FA in the abdominal region. It is difficult to predict the exact behaviour of the drag production for complete burst-and-coast cycles on live untethered fish that might also change in flexural stiffness throughout pregnancy [9]. Nevertheless, we expect that the scaling of drag, depending on RAI and speed that we found, may be indicative for the undulatory swimming as well.

Second, the presence of non-active fins in coasting live fish might cause small alterations to the production of drag. Furthermore, during coasting in the inertial regime the changes in body shape have a marked effect on drag that overtakes the effect of fins posture [41]. In the case of the anal fin, which is located in the region where flow separation takes place, this may affect the flow pattern in this region. The fin is, however, abducted and oriented flat in the sagittal plane when in neutral position and will therefore

hardly interact with the separated flow. In such a posture it theoretically may dampen or redirect 3D (out-of-plane) flow components in the separation region but this will be similar between models and have a small to probably negligible effect on the overall drag compared to the differences caused by changes in RAI, similar to what has been observed in zebrafish for differences in body shape [41].

Third, the characteristics of the skin surface such as compliance, and the presence of mucus and scales, might alter the production of drag on live fish [21]. Mucus could reduce drag by increasing the thickness of the boundary layer [42] and in some fish it can reduce $C_D$ by up to 10% [43]. We observed that the model built after the CT-scanned images (figure 5) produced significantly lower drag at speeds above the ones used by our population of *P. gracilis* (figure 5*c*1). We could attribute this difference to the surface roughness and the body shape of the CT-scan model. Although we cannot discriminate the influence of each individual characteristic, it is possible that at relatively high speeds, the surface roughness facilitates keeping the boundary layer attached further against an adverse pressure gradient before separating [44]. However, at the speeds used by our population, it seems that neither the surface texture, nor the slight difference in body shape between the two models had a significant influence on the production of drag, but more detailed tests on the interaction of surface texture and body shape effects on drag are needed. Nevertheless, models like the ones used in this study represent the production of drag based on RAI and *U* avoiding the technical difficulties of measuring on untethered live individuals at the right reproductive stages.

Fourth, muscles power locomotion, and their output limits the swimming performance of a wide variety of fish [45]. During pregnancy there is an excess allocation of energy to eggs and developing embryos, due to the mobilization of energy reserves and proteins from muscles and organs such as the liver to the gonads [46], and reflected on a significant increase in oxygen consumption [47,48] (except during BCF swimming in *P. reticulata*, where fin movement is a strong determinant of the oxygen consumed [19]). There might be also a decreased ratio of skeletal muscle mass to body mass, muscle disuse, and physical stretch [14]. All of the above may negatively influence the contractile properties of the muscles and decrease power output throughout the cycle as have been found for gravid fish where the maximum isometric force and maximum power output decrease by 35 and 36%, respectively [14]. The power required to overcome drag and produce thrust at higher velocities increases sharply since power is proportional to $U^3$ [44,49]. While we did not study muscle performance, a restricted allocation of energy to the muscles may limit the power females can produce, further limiting the speed they can potentially attain at a given RAI during their pregnancy.

Finally, higher drag might decrease fast-start escape performance, possibly increasing a female's risk of predation [50], and it could also reduce the sustained swimming performance, limiting their ability to inhabit fast-flowing parts of the river

[51]. Gravid and pregnant females may adjust their behaviour to avoid situations in which a high locomotor performance is demanded. For example, some snakes and lizards have been shown to respond to a decrease in escape performance during gestation by relying more on crypsis to avoid predation (e.g. garter snake, *Thamnophis ordinoides* and common lizard *Zootoca vivipara*) [3,38], while pregnant guppies (*P. reticulata*) respond to a lower sustained swimming performance by displaying a micro-habitat shift, preferentially inhabiting shallow areas in the river that are characterized by low water flow velocities [51].

In conclusion, our results showed that the increase in females' volume due to the developing embryos, and/or the increase in swimming speeds, significantly rise the production of drag. This effect was consistent with a bigger region of flow separation located behind the abdomen. Drag grew in a power fashion with speed and exponentially with the increase of reproductive allocation, thus drag penalty for becoming thicker was relatively low for low speeds compared to high ones. We suggest that this will cause the females' maximum swimming speed to decrease with reproductive allocation. The effects of higher drag might be even worsened by a negative effect of pregnancy on muscles and abdominal bending performance.

**Ethics.** The experiments were approved by the Wageningen University Animal Experiments Committee. Permit number 2013103.
**Data accessibility.** Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.4c039m4

**Author contributions**
E.M.Q.R, J.L.V.L., E.J.S., K.V.M., and B.J.A.P. conceptualized the experiment, E.M.Q.R, E.J.S. K.V.M., J.L.V.L., M.F., designed the experimental methodology (hardware and software). E.M.Q.R. performed the experiments and analysed the data, E.M.Q.R. wrote the first draft of the manuscript with input from J.L.V.L., B.J.A.P., M.F. and E.J.S. All authors provided feedback on the manuscript.

**Competing interests:** We declare we have no competing interests.

**Funding:** This study was supported by Doctorados en el Exterior de COLCIENCIAS, Convocatoria grant (No. 512 de 2010) to E.M.Q.R., NWO-ALW Open Programme grant (821.02.024) to J.L.V.L. and VIDI grant (864.14.008) of the Netherlands Organisation for Scientific Research (NWO) to B.J.A.P.

## Acknowledgements

We thank David Reznick for providing stocks of *Poeciliopsis gracilis*. We also thank T. Wiegers-Van der Wal, S. Visser, W. Nusselder, E. Roux, J. Lengkeek, and M. ter Veld

for their efforts on the fish care, Dennis Worst for his help testing the experimental setup, and Cees J. Voesenek for his help with the custom-made body model MATLAB routine. We appreciate the insightful feedback on the methodology from members of the Experimental Zoology Group at Wageningen University and of the Ocean Ecosystem group at the University of Groningen and from Luis García Rueda, Jan Wouter Kruyt and Daniel Quicazan. We also thank Mauricio Torres Mejía for his comments on a previous version of the manuscript.

## References

1. Winfield IJ, Townsend CR. 1983 The cost of copepod reproduction: increased susceptibility to fish predation. *Oecologia* **60**, 406–411. (doi:10.1007/BF00376860)
2. Isaacs R, Byrne DN. 1998 Aerial distribution, flight behaviour and eggload: their inter-relationship during dispersal by the sweetpotato whitefly. *J. Anim. Ecol.* **67**, 741–750. (doi:10.1046/j.1365-2656.1998.00236.x)
3. Bauwens D, Thoen C. 1981 Escape tactics and vulnerability to predation associated with reproduction in the lizard *Lacerta vivipara*. *J. Anim. Ecol.* **50**, 733–743. (doi:10.2307/4133)
4. Garland TJ, Else PL. 1987 Seasonal, sexual, and individual variation in endurance and activity metabolism in lizards. *Am. J. Physiol.* **252**, R439–R449.
5. Seigel RA, Huggins MM, Ford NB. 1987 Reduction in locomotor ability as a cost of reproduction in gravid snakes. *Oecologia* **73**, 481–485. (doi:10.1007/BF00379404)
6. Shine R. 2003 Effects of pregnancy on locomotor performance: an experimental study on lizards. *Oecologia* **136**, 450–6. (doi:10.1007/s00442-003-1281-9)
7. van Damme R, Bauwens D, Verheyen RF. 1989 Effect of relative clutch mass on sprint speed in the lizard Lacerta vivipara. *J. Herpetol.* **23**, 459. (doi:10.2307/1564069)
8. Lourdais O, Brischoux F, DeNardo DF, Shine R. 2004 Protein catabolism in pregnant snakes (*Epicrates cenchria maurus* Boidae) compromises musculature and performance after reproduction. *J. Comp. Physiol. B* 174, 383–391. (doi:10.1007/s00360-004-0424-6)
9. Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* 164, 38–50. (doi:10.1086/421412)
10. Plaut I. 2002 Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
11. Veasey JS, Houston DC, Metcalfe NB. 2001 A hidden cost of reproduction: the trade-off between clutch size and escape take-off speed in female zebra finches. *J. Anim. Ecol.* **70**, 20–24. (doi:10.1111/j.1365-2656.2001.00476.x)
12. Lee SJ, Witter MS, Cuthill IC, Goldsmith AR. 1996 Reduction in escape performance as a cost of reproduction in gravid starlings, *Sturnus vulgaris*. *Proc. R. Soc. London, B Biol. Sci.* **263**, 619–623. (doi:10.1098/rspb.1996.0093)
13. Wu W, Meijer OG, Lamoth CJC, Uegaki K, van Dieën JH, Wuisman PIJM, de Vries JIP, Beek PJ. 2004 Gait coordination in pregnancy: transverse pelvic and thoracic rotations and their relative phase. *Clin. Biomech.* **19**, 480–488. (doi:10.1016/j.

clinbiomech.2004.02.003)
14. James RS, Johnston IA. 1998 Influence of spawning on swimming performance and muscle contractile properties in the short-horn sculpin. *J. Fish Biol.* **53**, 485–501. (doi:10.1006/jfbi.1998.0722)
15. Booth RK. 1998 Swimming performance of Anadromous Atlantic salmon, *Salmo salar* L., during their spawning migration in the Exploits River, Newfoundland, Canada. PhD dissertation. University of Waterloo, Waterloo, ON, Canada.
16. Belk MC, Tuckfield RC. 2010 Changing costs of reproduction: age-based differences in reproductive allocation and escape performance in a livebearing fish. *Oikos* **119**, 163–169. (doi:10.1111/j.1600-0706.2009.17742.x)
17. Fleuren M, Quicazan-Rubio EM, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS One* **13**, e0195976. (doi:10.1371/journal.pone.0195976)
18. Noren SR, Redfern JV, Edwards EF. 2011 Pregnancy is a drag: hydrodynamics, kinematics and performance in pre- and post-parturition bottlenose dolphins (*Tursiops truncatus*). *J. Exp. Biol.* **214**, 4151–4159. (doi:10.1242/jeb.059121)
19. Svendsen JC, Banet AI, Christensen RHB, Steffensen JF, Aarestrup K. 2013 Effects of intraspecific variation in reproductive traits, pectoral fin use and burst swimming on metabolic rates and swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *J. Exp. Biol.* **216**, 3564–74. (doi:10.1242/jeb.083089)
20. Alexander RM. 2003 *Principles of animal locomotion*. New Jersey: Princeton University Press.
21. McHenry MJ, Lauder GV. 2005 The mechanical scaling of coasting in zebrafish (*Danio rerio*). *J. Exp. Biol.* **208**, 2289–301. (doi:10.1242/jeb.01642)
22. van Wassenbergh S, van Manen K, Marcroft TA, Alfaro ME, Stamhuis EJ. 2014 Boxfish swimming paradox resolved: forces by the flow of water around the body promote manoeuvrability. *J. R. Soc. Interface* **12**, 20141146–20141146. (doi:10.1098/rsif.2014.1146)
23. Pires MN, Banet AI, Pollux BJA, Reznick DN. 2011 Variation and evolution of reproductive strategies. In *Ecology and Evolution of Poeciliid Fishes* (eds JP Evans, A Pilastro, I Schlupp), pp. 28–37. Chicago: The University of Chicago Press.
24. Reznick DN, Mateos M, Springer MS. 2002 Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298**, 1018–1020. (doi:10.1126/science.1076018)
25. Turner CL. 1937 Reproductive cycles and superfetation in Poeciliid fishes. *Biol. Bull.* **72**, 145–164. (doi:10.2307/1537249)
26. Scrimshaw NS. 1944 Superfetation in Poeciliid fishes. *Copeia* **1944**, 180–183. (doi:10.2307/1437814)
27. Frías-Alvarez P, Zúñiga-Vega JJ. 2016 Superfetation in live-bearing fishes is not always the result of a morphological constraint. *Oecologia* **181**, 645–658. (doi:10.1007/s00442-015-3477-1)
28. Voesenek CJ, Pieters RPM, van Leeuwen JL. 2016 Automated reconstruction of three-dimensional fish motion, forces, and torques. *PLoS One* **11**, 8–10. (doi:10.1371/journal.pone.0146682)
29. Vogel S. 1994 *Life in moving fluids*. 2nd edn. New Jersey: Princeton University Press.
30. Webb PW. 1975 Hydrodynamics and energetics of fish propulsion. *Bull. Fish. Res.*

*Board Canada* **190**, 1–158.
31. Littell RC, Henry PR, Ammerman CB. 1998 Statistical analysis of repeated measures data using SAS procedures. *J. Anim. Sci.* **76**, 1216. (doi:10.2527/1998.7641216x)
32. Stamhuis EJ. 2006 Basics and principles of particle image velocimetry (PIV) for mapping biogenic and biologically relevant flows. *Aquat. Ecol.* **40**, 463–479. (doi:10.1007/s10452-005-6567-z)
33. Thielicke W, Stamhuis EJ. 2014 PIVlab - Towards user-friendly, affordable and accurate digital particle image velocimetry in MATLAB. *J. Open Res. Softw.* **2**, e30. (doi:10.5334/jors.bl)
34. Anderson JD. 2001 *Fundamentals of aerodynamics*. 3rd edn. New York: McGraw-Hill Companies.
35. van der Hoop JM. 2017 Effects of added drag on cetaceans: fishing gear entanglement and external tag attachment. PhD dissertation. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution. USA.
36. Suzuki I, Sato K, Fahlman A, Naito Y, Miyazaki N, Trites AW. 2014 Drag, but not buoyancy, affects swim speed in captive Steller sea lions. *Biol. Open* **3**, 379–386. (doi:10.1242/bio.20146130)
37. Feldkamp SD. 1987 Swimming in the California sea lion: morphometrics, drag and energetics. *J. Exp. Biol.* **131**, 117–35.
38. Brodie III ED. 1989 Behavioral modification as a means of reducing the cost of reproduction. *Am. Nat.* **134**, 225. (doi:10.1086/284977)
39. Videler JJ, Weihs D. 1982 Energetic advantages of burst-and-coast swimming of fish at high speeds. *J. Exp. Biol.* **97**, 169–178.
40. Lighthill MJ. 1971 Large-amplitude elongated-body theory of fish locomotion. *Proc. R. Soc. London, B Biol. Sci.* **179**, 125–138.
41. McHenry MJ, Lauder GV. 2006 Ontogeny of form and function: Locomotor morphology and drag in zebrafish (*Danio rerio*). *J. Morphol.* **267**, 1099–1109. (doi:10.1002/jmor.10462)
42. Daniel TL. 1981 Fish mucus: *in situ* measurements of polymer drag reduction. *Biol. Bull.* **160**, 376–382. (doi:10.2307/1540846)
43. Sagnes P, Champagne J-Y, Morel R. 2000 Shifts in drag and swimming potential during grayling ontogenesis: relations with habitat use. *J. Fish Biol.* **57**, 52–68. (doi:10.1006/jfbi.2000.1288)
44. Fish FE, Rohr JJ. 1999 Review of dolphin hydrodynamics and swimming performance. San Diego (CA): SPAWARS System Center Technical Report 1801. (doi:10.21236/ADA369158)
45. Wakeling JM, Johnston IA. 1998 Muscle power output limits fast-start performance in fish. *J. Exp. Biol.* **201**, 1505–26.
46. Love RM. 1980 *The chemical biology of fishes*. London: Academic Press.
47. Karamushko LI, Christiansen JS. 2002 Aerobic scaling and resting metabolism in oviferous and post-spawning Barents Sea capelin *Mallotus villosus villosus* (Müller, 1776). *J. Exp. Mar. Bio. Ecol.* **269**, 1–8. (doi:10.1016/S0022-0981(01)00392-6)
48. Timmerman CM, Chapman LJ. 2003 The effect of gestational state on oxygen consumption and response to hypoxia in the sailfin molly, *Poecilia latipinna*. *Environ. Biol. Fishes* **68**, 293–299. (doi:10.1023/A:1027300701599)
49. Randall D, Burggren W, French K. 2002 *Eckert. Animal physiology. Mechanisms and*

*adaptations*. 5th edn. New York: W. H. Freeman and Company.
50. Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN. 2005 Do faster starts increase the probability of evading predators? *Funct. Ecol.* **19**, 808–815. (doi:10.1111/j.1365-2435.2005.01033.x)
51. Banet AI, Svendsen JC, Eng KJ, Reznick DN. 2016 Linking reproduction, locomotion, and habitat use in the Trinidadian guppy (*Poecilia reticulata*). *Oecologia* **181**, 87–96. (doi:10.1007/s00442-015-3542-9)

## Supplementary material S1. Fish rearing, feeding, husbandry and record of reproductive characteristics.

*Fish rearing & husbandry* – *Poeciliopsis gracilis* occurs in southern North America to Central America [1,2]. The fish stock was originally obtained in 2004 from a small tributary of Rio Motagua, near the village of Zacapa (Guatemala) and brought to the Reznick lab to establish a laboratory population (University of California Riverside, USA). Part of this laboratory population was subsequently transferred to the Pollux lab at the Aquatic Research Facilities (ARF) of Wageningen University (The Netherlands). The fish used in our experiments were bred from this laboratory population (assuming a generation time of 6 months, these fish may approximate the 20th generation in captivity).

We reared males and females from birth, sexes were separated upon discrimination of male sexual characteristics. Once fish reached sexual maturity (≈ 4–6 months after birth), virgin females were isolated in 10 L tanks together with a male to ensure a continuous availability of sperm. A common filtering system was used to assure equal water quality for all tanks. Water temperature was 24–25°C and photoperiod followed a 12:12 h light:dark-cycle. The fishes were fed once in the morning (around 8 am) and once in the afternoon (around 4 pm).

*Fish record of reproductive characteristics* – The mean interbrood interval (IB), the period between two parturition events, was 18.3 days (SD: ± 0.78) in our lab population (based on $N_{fish}$ = 20 females). Each interbrood interval thus started when a fully developed brood was delivered and ended when the subsequent brood was born.

To obtain information about brood sizes (i.e. number of offspring/brood) in our laboratory population, we recorded all brood sizes delivered by the 20 females for at least 6 months up to maximally one year (see figure S1 for brood sizes per female through time). Brood sizes varied between 1 and 48 new-borns (mean ± SD = 19.2 ± 9.4, figure 1*a*, black bins). On the day of delivery, we euthanized the new-borns with an overdose of MS-222 (Tricaine-S; Western Chemical Inc., Ferndale, WA, United States), carefully removed the excess liquid with a paper towel and weighed them (Mettler AE200 analytic balance; scale accuracy 0.0001 g; Mettler-Toledo B.V., Tiel, The Netherlands). Brood size was not significantly related with the individual wet mass of the new-borns (Linear regression: $N_{broods}$ = 10, $R^2$ = 0.2397, $p$ = 0.1509).

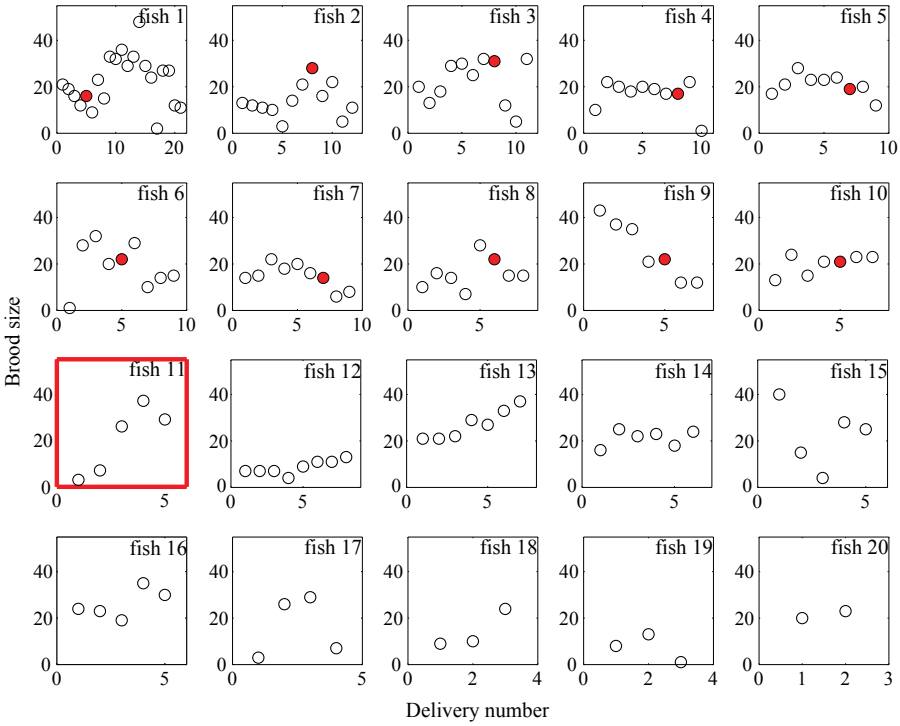



**Figure S1.** Documented broods sizes per delivery event ($N_{fish}$ = 20). Filled red circles are the broods used for model creation. In the red square, are the broods from the CT-scanned fish (fish 11).

**Supplementary material S2. Body posture corrections.**

The body posture of the fish appeared to be different between coasting and at rest, namely in opercular abduction and orientation of the caudal peduncle (figure S2*c,d,e*). Detailed series of pictures from the body could only be made when the fish was at rest. Thus, we applied the following corrections for the opercular abduction and caudal peduncle downward bending to get the closer representation of the body shape during coasting: (i) During rest, the opercula move in and out during the breathing cycle, and the photographed opercular abduction is not related to pregnancy stage. During coasting, the opercula are usually adducted, which could reduce body drag. Therefore, the head shape of each fish at coasting was obtained from the photograph showing the strongest opercular adduction (figure 2*a*, and S2*b,c*). (ii) The caudal peduncle bends downwards at rest, whereas it is aligned with the longitudinal axis of the body during coasting. To correct for such bending, we followed the central line of the trunk and peduncle following pictures of coasting events recorded on other fish from the laboratory population (figure S2*d*) when they moved freely in the isolation tanks, to align the peduncle with the rest of the trunk using a MATLAB routine. After visual examination, we set the beginning of the peduncle at 62% of the standard length of the fish starting from the snout. The maximum applied correction angles of the body axis at the posterior end of the caudal peduncle were between 0 and 20°, which had only minor effects on the height of the sections at the caudal peduncle (figure S2*e,f*).

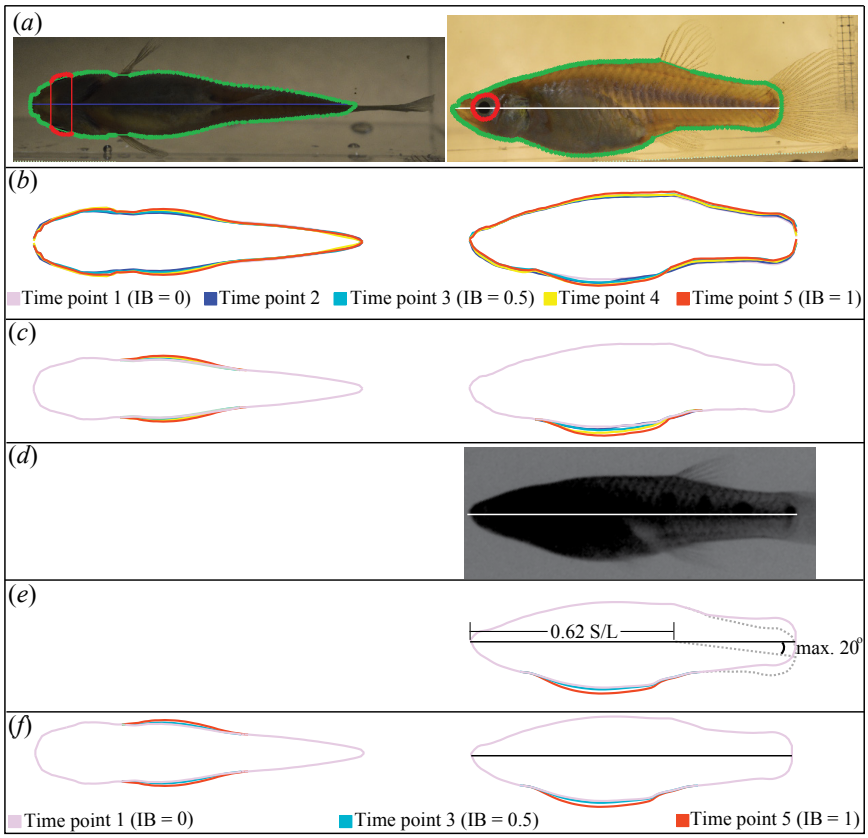



**Figure S2.** Transformation of body at opercula and peduncle for one fish. (*a*) Manual digitization done in Matlab of the ventral and lateral profiles of 1 fish at 1 time point. (*b*) Digitized profiles of 1 fish at 5 time points during an interbrood interval. (*c*) Correction for opercular abduction and alignment of dorsal line. The head shape was obtained from the day showing the strongest opercular adduction. The caudal peduncle from the first time point was applied to the other time points. Lateral profiles from all days were aligned to the dorsal line of the first time point. (*d*) Picture of gliding event where the caudal peduncle was aligned to the rest of the trunk. (*e*) Peduncle correction. Dotted line represents peduncle resting position, and solid line represents peduncle transformed position. (*f*) Ventral and lateral profiles averaged from 10 females of the 3 moments in time chosen for analysis: IB = 0, 0.5, and 1. Following pictures of gliding events, the caudal peduncle inclination was aligned to the rest of the trunk using a Matlab routine. The width and height of the profiles at time points 1, 3 and 5, where averaged among the 10 fish to calculate the width and height increase from the beginning of the interbrood interval to the middle and to the end of the interbrood interval.

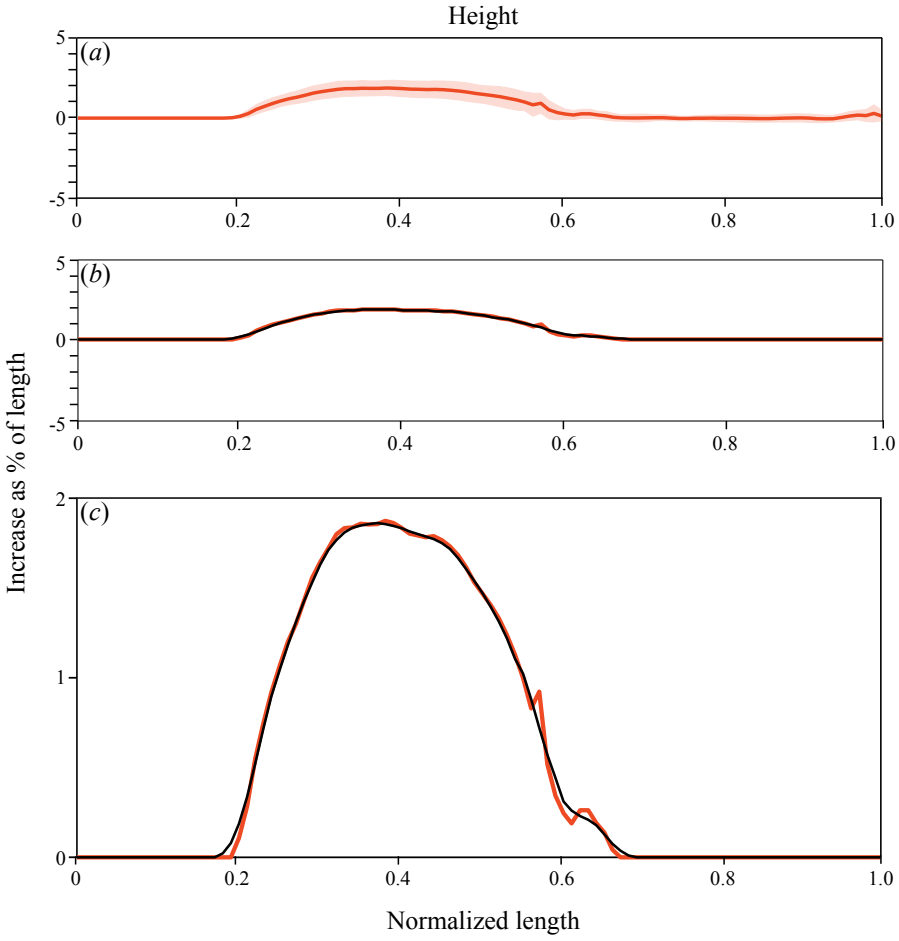



**Figure S3.** Application of simple moving average with a window of 5 data points to smooth curves. Example showing the smoothing of the height differences between IB = 0 and IB = 1. (*a*) raw data. (*b*) Moving average applied. Black line shows the smoothed data. (*c*) Moving average applied. Y axis was magnified for clarity.

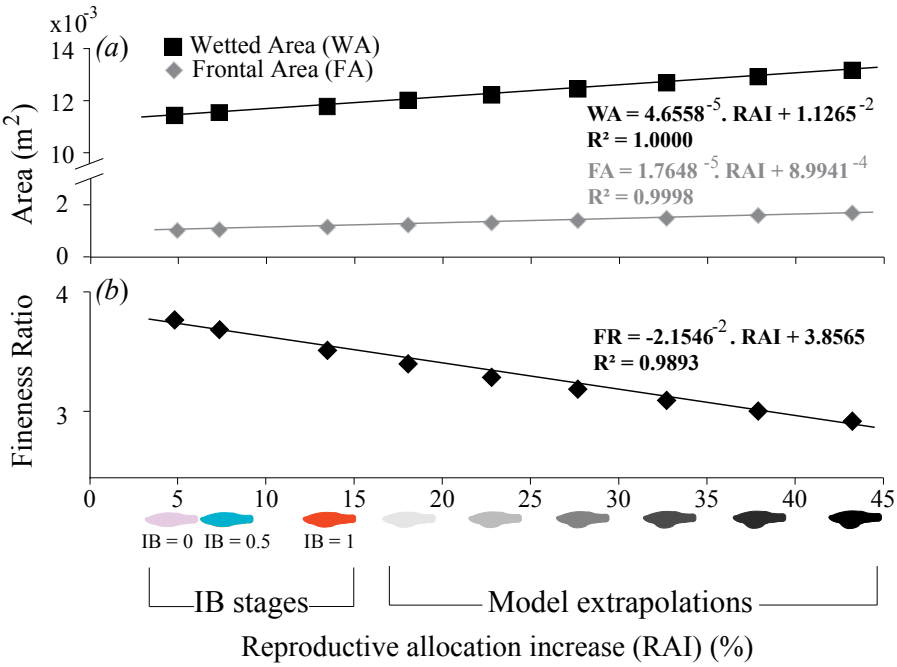



**Figure S4.** Frontal area (FA), wetted area (WA), and Fineness ratio (FR) for the used 3D models. (*a*) Frontal and wetted area. (*b*) Fineness ratio. The models represent different level of reproductive allocation increase (RAI, it is the body volume increase due to the offspring. The first three models at the left represent 3 stages within an interbrood interval: IB = 0, IB = 0.5, IB = 1, and the rest of the models represent the increase in volume due to bigger broods.

**Supplementary material S3. Super ellipse equation used to model the eyes.**

The super ellipse that we used for the outer shape of both eyes is described by the following equation:

$$\left|\frac{x}{a}\right|^{2.3}+\left|\frac{y}{b}\right|^{2.3}=1$$

where *a* and *b* are the lenghts of the major and minor axes respectivelly. The exponent value of 2.3 makes the superellipse more rounded than a regular ellipse whose exponent is 2.

**Supplementary material S4. Force measurement set up**

Force measurements setup – We used the 300 L flow tunnel described in [3], with dimensions 50 x 25 x 25 cm (length x height x width) of the test section. To prevent wave formation, a Perspex plate was placed on top of the test section during experiments. Models were submerged in the middle of the cross-section of the tank and attached (by the rod) to a force-measuring platform above the tank. The model was placed such that the longitudinal axis was parallel to the overall flow (figure S5*a*). To minimize the fluid forces on the rod and get a nearly undistorted signal of the drag forces of the models, a fairing was attached to the lower side of the platform that enveloped the rod without touching it (figure S5*b,c*). The cross-section of the fairing had a NACA-0012 profile and its lower tip was sharpened to 45 degrees with respect to the fairing main axis (figure S5*b,c*). This kept the boundary layer of the fairing as far away from the model as possible and minimized the influence of the fairing on the measurements. Furthermore, it almost completely removed the drag on the rod, which otherwise would be part of the measured drag. On top of the platform there was a force sensor (Honigmann GmbH RFS® 150-XY, Nr.705052, x: 5 N, y: 5 N, 1 mV/V) which was connected to a computer using an electronic precision measuring amplifier Tensiotron® TS 621 per channel/axis. Data were recorded with a sample frequency of 1000 Hz during 5 s per test using HCC-Easy data transfer, an analysis program for Honigmann tension sensors. Force sensor accuracy was validated using a sphere (diameter 60 mm). At the appropriate Reynolds number, its drag coefficient ($C_D$) based on frontal area was 0.4857 ± 0.0244 (mean ± SD), close to the literature value of 0.47 [4]. A non-dynamic calibration of the system was done three times before each measurement, by hanging known weights to the sensor system. Drag measurements were repeated 5 times.

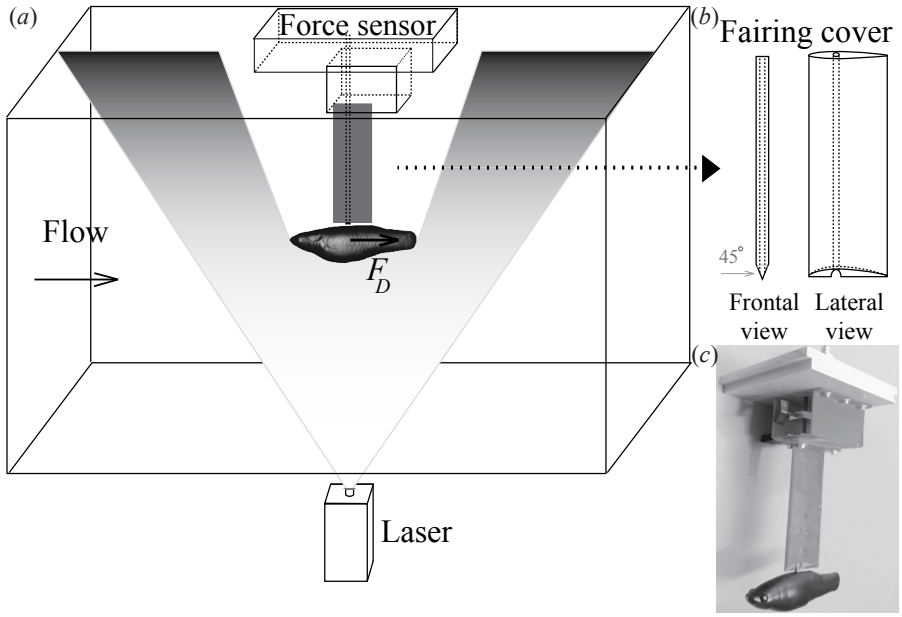



**Figure S5.** (*a*) Flow tank measurement section for force and flow (PIV) recordings. (*b*) The fish rod is covered by a fairing with cross-section of NACA-0012 profile. (*c*) Detail of force sensor, fairing and printed model.

**Table S1**. Tested speeds and their equivalent Reynolds numbers (*Re*).

| **Fish speed (SL s$^{-1}$)** | 0 | 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 17.5 | 20.0 | 25.0 | 30.0 | 35.0 | 40.0 | 45.0 | 50.0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Tunnel speed (m s$^{-1}$)** | 0 | 0.04 | 0.08 | 0.12 | 0.16 | 0.20 | 0.24 | 0.28 | 0.32 | 0.40 | 0.48 | 0.56 | 0.64 | 0.72 | 0.80 |
| ***Re* (x10$^4$)** | 0 | 0.66 | 1.31 | 1.97 | 2.63 | 3.28 | 3.94 | 4.60 | 5.26 | 6.57 | 7.88 | 9.20 | 10.51 | 11.83 | 13.14 |

## Supplementary material S5. Particle Image Velocimetry

To visualize the flow behaviour around the abdomen, we performed Particle Image Velocimetry (PIV; [5]) recordings at 3 (tunnel) speeds: 0.32 m $s^{-1}$, 0.56 m $s^{-1}$ and 0.64 m $s^{-1}$ over a selection of models that covered the sizes of the lab fish, RAI of 4.8, 13.5, 18.1, 27.7, and 43.2% and the CT-scanned model. We added neutrally buoyant polyamid beads (57 µm diameter, ILA GmbH, prod # 2157) to the flow and illuminated them with a laser sheet (SNOC Lasers Inc., DPSS Laser, Pmax = 2W CW, λ = 532 nm, Thorlabs sheet optics) in the sagittal plane (figure S5*a*). We recorded images with a high-speed camera FASTCAM-APX RS, Photron, Tokyo, Japan, placed perpendicular to the laser sheet, using a macro lens (55 mm AF Micro-NIKKOR, Nikon, Tokyo, Japan).

We analysed the images with PIVlab [6], using a Fast Fourier Transformation with window deformation, an interrogation area of 16 x 8 pixels and a Gaussian subpixel estimator of 2 x 3 points and we interpolated the missing data using a cubic spline. We averaged the vector fields of 50 frames and visualized the local velocities and the streamlines of the region covering the lowest abdominal point.

**Table S2.** Type 3 test of fixed effects of $F_D$ and $C_D$ for model representing IB = 1 and CT-scan. (Num DF: numerator degrees of freedom, Den DF: denominator degrees of freedom)

| Dependent variable | Effect | Num DF | Den DF | *F - Value* | *Pr > F* |
|---|---|---|---|---|---|
| $F_D$ | Fish model | 1 | 120 | 21.73 | <.0001 |
| | Speed | 14 | 120 | 7801.52 | <.0001 |
| | Fish model*Speed | 14 | 120 | 5.37 | <.0001 |
| $C_D$ | Fish model | 1 | 120 | 3.05 | 0.0831 |
| | Reynolds num. (Re) | 14 | 120 | $5.67 \times 10^{10}$ | <.0001 |
| | Fish model*Re | 14 | 120 | 0.28 | 0.9954 |

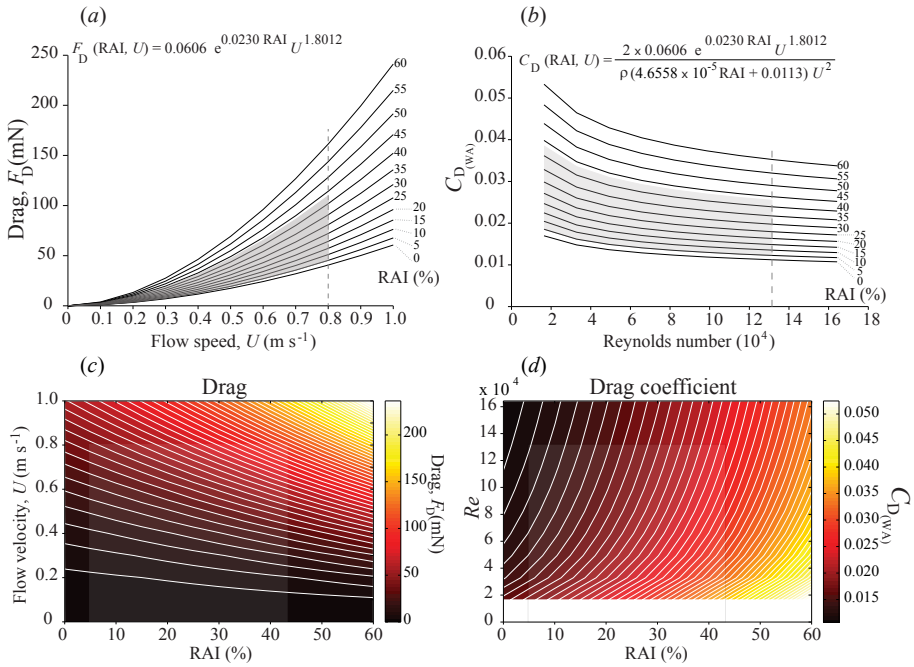



**Figure S6.** Values calculated from equation 3. The shaded areas represent the range of RAI and $U$ tested in the flow tunnel. (*a*) $F_D$ calculated for a wider range of the tested RAI and $U$. The following are the asymptotic standard errors of each parameter of the equation: a = 0.0606320, error of a = 0.0008032, b = 0.02301000, error of b = 0.0004037, and c = 1.80121, error of c = 0.006009. (*b*) $C_D$ calculated for the same range as for $F_D$. (*c*) Contour plot of $F_D$ as a function of $U$ and RAI for a wider range of the tested RAI and $U$. (*d*) Contour plot of $C_D$ as a function of $Re$ and RAI.

## References. Supplementary material


1. Huidobro-Campos L. 2000 Filogenia del complejo *Poeciliopsis gracilis* Regan (Pisces: Poeciliidae) y su biogeografía. Unpublished Master in Science Dissertation, Facultad de Ciencias, U.N.A.M., México, 93p.
2. Miller RR, Mincley WL, Norris SM. 2005 *Freshwater fishes of Mexico.* Illinois: University of Chicago Press.
3. van Wassenbergh S, van Manen K, Marcroft TA, Alfaro ME, Stamhuis EJ. 2014 Boxfish swimming paradox resolved: forces by the flow of water around the body promote manoeuvrability. *J. R. Soc. Interface* **12**, 20141146–20141146. (doi:10.1098/rsif.2014.1146)
4. Hoerner SF. 1965 *Fluid dynamic Drag*. Minland Park, NJ: Published by the author.
5. Stamhuis EJ. 2006 Basics and principles of particle image velocimetry (PIV) for mapping biogenic and biologically relevant flows. *Aquat. Ecol.* **40**, 463–479. (doi:10.1007/s10452-005-6567-z)
6. Thielicke W, Stamhuis EJ. 2014 PIVlab - Towards user-friendly, affordable and accurate digital particle image velocimetry in MATLAB. *J. Open Res. Softw.* **2**, e30. (doi:10.5334/jors.bl)

# Chapter 4

## Effects of pregnancy on the escape response of a live-bearing fish

Elsa M. Quicazan-Rubio, Mike Fleuren, Cees J. Voesenek, Bart Pollux, and Johan L. van Leeuwen

*Experimental Zoology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, The Netherlands*

**Abstract**

Predation is a strong selective pressure that may particularly affect pregnant females, because they are a relatively large source of nutrients and their changes in body shape might lower their locomotor performance making them more appealing and easier targets to predators. Here, we use the livebearing fish species *Poeciliopsis gracilis* to test the prediction that (1) females have a lower escape performance before delivery than when they just give birth and (2) pregnant females have lower escape performances than virgin ones. We elicited escape responses of pairs of virgin and pregnant females, before and after giving birth, and tracked their movement in a three-dimensional space. We found no significant differences in the escape performance of pregnant females before and after giving birth. This was presumably caused by the relatively small increase in female body volume over the course of pregnancy in our study, which was most likely related to (i) the relatively slender body of the gravid females throughout pregnancy due to superfetation (simultaneously occurrence of several litters that differ in the stage of the embryonic development) and (ii) the small litters produced by the pregnant females. Furthermore, we found no significant differences in escape performance between virgin and pregnant females. This was presumably caused by an unexpected high reproductive allocation of the virgin females due to the presence of fully provisioned, unfertilized eggs in the ovary. Finally, we discuss a number of technical limitations of our study that may have contributed to our inability to detect significant effects of pregnancy on female locomotor performance and provide suggestions for future studies.

## 1. Introduction

Predation is a strong selective pressure which can influence the physiology, morphology, and behaviour of prey species [1]. Predators may preferentially select pregnant and gravid females, because they are large and, due their eggs and embryos a high quality resource that is rich in energy and nutrition [2]. Furthermore, gravidity and pregnancy may negatively affect the locomotor performance of females making them slower or less agile than con-specific non-pregnant females and males, further increasing their susceptibility to predation [3]. A locomotor cost of reproduction in females has been reported for a wide range of clades. For instance, gravidity reduces the running speed of scorpions [4], the acceleration and manoeuvrability in copepods [5] and correlates negatively with the height of flying in the sweetpotato whitefly [6]. In reptiles, the gravidity related reduction in locomotor performance may be a combination of the physical burden and physiological changes of females during late pregnancy [7].

In fish, gravidity may have negative effects on escape response and sustained swimming [8–11]. Maximum and average velocity and the cumulative distance travelled during a fast start decline as gravidity progresses [8,10]. Possible causes of the lower swimming performance of gravid females are changes in the contractile properties of muscles during reproduction, as observed in the shorthorn sculpin [10], an increase in body drag due to an extension of the abdominal region as suggested by [8] and tested by [12 (**Chapter 3**)], limited axial bending due to the growing abdomen [10] and aerobic constrains [9].

The live-bearing fish *Poeciliopsis gracilis* (Poeciliidae) is an ecologically versatile species that inhabits a wide range of water bodies, such as streams, lagoons, dams, and pools in creeks and rivers [13,14], and occupies lotic as well as lentic environments [15]. Furthermore, it survives in highly perturbed waters, including those with urban and industrial pollutants [15,16]. Moreover, *P. gracilis* has an interesting reproductive strategy known as superfetation: females carry on average two simultaneous litters that are in different stages of development [17,18]. Superfetation is often correlated with relatively small broods and frequent parturitions [19]. It has been argued that superfetation may reduce the changes in body shape due to reproduction [20], lowering locomotor costs and enhancing performance throughout reproduction.

In the Poeciliidae, escape responses of pregnant females have only been tested on non-superfetatious species. In *Gambusia affinis* [9,21], pregnancy had negative effects on the escape velocity of old females, which had relatively larger broods, but did not significantly affect the younger females with lower reproductive allocations (RA, the proportion of body mass utilized in reproduction). In populations of *Poecilia reticulata* adapted to environments with different predation levels, the individuals from high predation locations outperformed those from the low predation ones, but their performance declined faster throughout pregnancy [8]. Furthermore, females with a high RA had lower escape performances. Finally, a field study showed that females from high predation environments often displayed temporary micro-habitat shifts during pregnancy to shallower areas within the river that were less accessible to predators [22].

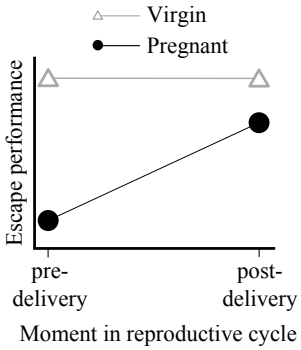



**Figure 1.** Predicted change in female maximum escape performance between 2 days before delivery and right after delivery in a pregnant live-bearing fish, compared to the performance of a virgin fish in those same time points. (1) The performance of the pregnant fish (black line) will improve after giving birth. (2) It will have a lower escape performance during its entire pregnancy than a virgin specimen (grey line) of the same species and equal body length. The virgin will maintain a constant escape performance during the same period.

To shed light on the effect of pregnancy on the escape response of *P. gracilis*, we measured the escape responses of females before and after parturition by quantifying their movements with an automated 3D tracking method that calculates the position of the centre of mass (CoM) of the body and the body orientation during the manoeuvre [23,24]. The CoM is a relevant point as predators may usually aim for a region of the body that is close to the CoM [25]. We judged the escape performance based on the distance covered, maximum speed and mean acceleration. Furthermore, to help explaining the cause of any differences in the aforementioned variables, we evaluated the differences in curvature along the body as it correlates to the efficacy of the escape performance [24]. We expect that pregnancy negatively affects escape performance as shown in other studies, and we also expect that females that are close to delivery have lower escape performances than the ones that just gave birth (figure 1). Furthermore, we expect that an increase in clutch size leads to a lower escape performance.

## 2. Material and methods

### 2.1. Experimental animals

Our laboratory stocks of *Poeciliopsis gracilis* (Heckel 1848) were kept at the CARUS Aquatic Research Facilities of Wageningen University and Research. The newborns from these stocks were removed at one-week intervals and kept in age cohorts in 40 L tanks. Males were moved to a different tank once they developed the secondary sexual characteristics. Once the females had reached sexual maturity (approximately 4–6 months after birth), they were randomly placed individually in tanks of 10 L (Tecniplast, Bugugiatte, Italy). In half of the tanks, one adult male was assigned, to ensure a continuous availability of sperm. The females from the other half of the tanks were accompanied by a juvenile of *Poecilia wingei* to encourage them to swim. Each tank was enriched with gravel and a plastic plant, and it received water from a common filtering system to assure equal water quality for all tanks. Water temperature was kept at 24–25°C and photoperiod followed a 12 h light: 12 h dark-cycle. They were fed twice daily with liver paste or flake paste (both feeds were alternated daily); respectively around 8 am and 4 pm. When parturition was detected in the tanks with the pregnant females, the fries were removed. All experimental procedures were approved by the Wageningen University Animal Experiments Committee, permit number 2013103.

To evaluate the effect of pregnancy at the pre- and post-parturition stages, we followed the pregnant females during at least 3 pregnancy cycles to determine the length of the interbrood interval (i.e. the period between parturitions). The average interbrood interval was 18.3 days (SD: ± 0.78, based on $N_{fish}$ = 20 females, 10 of these females were used in the current experiment and the remaining 10 pregnant females were enrolled in other experiments).

Females were grouped in 10 blocks of one pregnant and one virgin female for a total of 20 females. The fast-start performance of the pregnant and virgin female from each block was measured the same day. We sampled at the end of the interbrood interval (mean ± SE: 1.6 ± 0.3 days before the delivery of the next brood) and on the delivery day. These moments are hereafter referred to as 'pre-delivery' and 'post-delivery' stages, respectively. All new-borns from the litter were sacrificed on the day of parturition with an overdose of anaesthetic (Tricaine-S, MS-222). After carefully removing the excess liquid with a paper towel, we weighed them (Mettler AE200 analytic balance; scale accuracy 0.0001 g; Mettler-Toledo B.V., Tiel, The Netherlands). Brood size (i.e. number of newborns) was not significantly related with the individual wet mass of the newborns (Linear regression: $N_{broods} = 10$, $R^2 = 0.2397$, $p = 0.1509$).



### 2.2. 3D body model

Prior to the fast-start experiment, we took multiple sets of photographs from 3 views, lateral, ventral and frontal, using 3 cameras Nikon D3200, with Micro Nikkor 55 mm lenses, at perpendicular angles with respect to the glass walls of a 0.1 x 0.1 x 0.1 m fish tank. To construct the body model, we chose a set of 3 pictures where the fish was straight and with minimal rotation [26 (**Chapter 2**)]. Using an in-house MATLAB routine, a model of the fish shape was built by manually tracing points along the ventral, lateral and frontal outlines of the body, excluding the fins. Then, the fish shape described by the traced points was fit with a series of 51 cross sectional area ellipses along the length of the fish. This model was used as an input for the tracking algorithm. More detail on the construction of the body model and the tracking algorithm is found in [23,24,26 (**Chapter 2**)]. We derived the body mass from the calculations of body volume made in the 3D body model, assuming a density of the fish of 1000 kg/m$^3$ (based on observed densities of adult zebrafish: 1000 +/- 40 kg/m$^3$ [27]).

### 2.3. Calculation of the reproductive allocation increase

We estimated how much the reproductive allocation increased during the current pregnancy by assessing the volume change between 2–3 days before delivery and shortly after delivery with the 3D body model calculations. We expressed the difference as a percentage of the female volume after delivery and called it Reproductive Allocation Increase (RAI). The RAI and the number of newborns had a positive linear relationship ($p < 0.05$, $R^2 = 0.516$), indicating that the litter number determined most of the volume growth.

### 2.4. Escape response measurements

*Set up* – We used a setup and followed a protocol similar to the ones described in [24]. The fast-starts were recorded in an aquarium of 0.30 x 0.30 x 0.30 m (length x width x height). Given the dimensions of the aquarium, the ground effect was negligible when the fish was

in the field of view of the camera, which was more than one fish length away from any wall [28]. To lower the stress on the tested females caused by the new environment, companion juvenile fish were placed in a small tank along one of the vertical corners of the aquaria, out of the field of view. This tank was a ¼ cylinder of 0.06 m radius and 0.25 m high, and wholes of about 3 mm were drilled through it to allow the interchange of water between the two tanks.

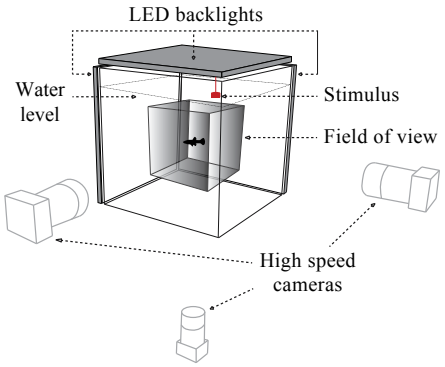



**Figure 2.** Outline of the experimental set up. The assembly allowed the fish to swim freely in any direction. The field of view located in the center of the tank (grey cube) was recorded by three orthogonal high-speed cameras. The LED lights, opposite to each camera allowed the recording of the dark silhouette of the fish against a white background. The fish was stimulated by a weight that fell freely in one of the corners of the tank.

*Cameras* – Three synchronized high-speed cameras, Mikroton EoSens CL MC1362 high-speed video cameras (Mikrotron, Unterschleissheim, Germany; resolution 1040 × 1020 pixels; 1/1000 s shutter speed), with lenses Voigtländer Ultron F = 40 mm 1:2 aspherical compact pancake lenses (RINGFOTO, Fürth, Germany), and with Epix PIXCI E8 frame grabbers (EPIX, Buffalo Grove, IL, USA), were placed with the respective optical axes oriented orthogonally to the bottom and two lateral sides of the tank. The three cameras were synchronized using a Quantum Composers 9214 digital delay pulse generator (Quantum Composers, Bozeman, MT, USA). We recorded at 470 frames per second, with a shutter time of 0.505 ms. The field of view was located at the center of the aquaria and covered 0.15 x 0.15 x 0.15 m (figure 2). The fish length was covered with at least 200 pixels when the longitudinal axis of the fish was orthogonal to either of the three cameras, which provides enough resolution to successfully track the fish with the images from the three cameras. We used back-light by placing light emitting diode (LED) panels (Model PL 3030-36, power of 36 W) (figure 2).

We followed a pinhole camera model calibration using the software developed by [29] where the change in size is linear with distance. We placed a calibration frame in the centre of the tank, occupying the field of view of the central volume. Three pictures were taken, one per camera view. We digitized 48 points over the calibration frame from the three views and the resultant coefficients were used in our in-house tracking software to calculate the position of the fish in the swimming arena.

*Stimulus* – The escape response was elicited by dropping into the tank a 120 g metallic

cylinder (0.02 m diameter, 0.02 m high). The height was fixed by attaching the cylinder to a string that allowed it to drop just below the surface of the water. To prevent visual stimulation before the stimulus touched the water, the stimulus was located inside a vertical opaque PVC tube that was placed just above the water surface [30]. The stimulus produced a pressure wave which has proven to elicit an escape response in other species [31,32], without substantially disturbing the flow in the region of the fish before the last video frame that we analysed of each fast start. The stimulus induced escape responses in all females.

*Measurements* – After placing the fish in the test aquarium it was allowed to acclimate for 15 min. The light intensity was set to the minimum when the fish was introduced in the tank. Two increments of light were made over the 15 minutes acclimation, one at 5 minutes where light was set to 50% of the maximum intensity and the other one at 10 minutes where the light intensity was set to 100%. After acclimation, the stimulus was released once the fish was within the field of view and videos from the 3 views were taken. The fish was left to rest at least 5 minutes in between tests.

To assure the repeatability of the performance, we chose consistent psychological factors, such as motivation and physiological factors, such as age, and minimum number of pregnancies before the current recorded one [33].



### 2.5. Fish tracking

The body model and the videos from the 3 cameras were imported into a 3D tracking routine [23] which projects the body model into the fish silhouettes on the 3 camera views. At each frame, an optimization routine was performed to find the fit with the least difference between the body model projection and the silhouette. This stage resulted in a time series of the position of each of the 51 points along the centre line of the fish. Next, we assumed a uniform tissue density (1000 kg $m^{-3}$) and calculated the position of the centre of mass (COM) and moment of inertia following [23]. The resulting time series of COM positions, body orientations and curvatures along the body were smoothed with Whittaker smoothing ($\lambda = 4$, order = 0; [34]).

### 2.6. Data analysis

For *Poecilia reticulata*, another species of the same fish family, the predator-prey interaction takes about 22 ms (J. A. Walker, unpublished data, in [8]). To determine the escape performance of the females within this critical time and at double this time, we analyzed the movement within two subsequent intervals of 22 ms, the first one starting at the onset of the escape response. And we also analyzed the movement within the 44 ms interval which included the two subsequent interval of 22 ms. The onset of the response was determined as the first frame just before the head made a side-ways movement. We discarded videos where the fish was (partially or completely) out of view on at least one of

the 3 cameras within 22 ms after the onset of the response.

Most of our analysis was focused on the motion of the centre of mass (CoM) of the fish, which moves with respect to the various parts of the body owing to the changes in body shape and it is the point where the net external force acts [25]. Predators usually aim for a region of the body that is close to the CoM. To quantify the escape performance, we calculated the distance covered, the maximum speed, and the mean acceleration of the CoM. Especially in short predation encounters, the speed and distance achieved in the first moments of the escape response are relevant indicators of its success [35–37]. The mean acceleration is computed as the gain in speed over the first and second 22 ms and the 44 ms interval divided by the duration of the interval. These time intervals are large enough to avoid accuracy problems owing to the double differentiation procedure. Finally, to explore whether changes in body shape affect any differences in performance, we calculated the curvature along the body as explained in [23].

### 2.7. Statistical analysis

To compare changes in body mass that occurred in pregnant and virgin females between the pre-delivery and the post-delivery day, we performed a one-way ANOVA. By means of a repeated measures analyses of variance (RMANOVA), we assessed the differences in the kinematic and curvature variables due to the reproductive status (virgin or pregnant) of the fish and the moment in the reproductive cycle (just before or just after the pregnant female gave birth). Reproductive status and moment in the reproductive cycle were used as fixed factors. Block was included as a random factor. All tests were performed in SAS (SAS version 9.2; SAS Institute Inc., Cary, North Carolina, USA. 2007). We selected the autoregressive (AR) covariance structure based on Akaike's information criteria (AIC). Linear regressions were used to test for an effect of distance to the stimulus and maximum velocity and of RAI on the kinematic and curvatures variables.

## 3. Results

To evaluate the effect of pregnancy, the stage of pregnancy, and the level of reproductive allocation on the escape response, we compared the swimming performance of pregnant and virgin females before and after the parturition of the pregnant individuals. Furthermore, we also compared the performance among the pregnant females, before delivery, depending on their level of reproductive allocation.

*Videos* – We recorded a total of 104 videos, for the 10 pregnant and 10 virgin individuals. After tracking them and doing an initial analysis, we omitted 18 videos that resulted in either bad tracking or where the entire fish was visible in each of the three synchronised videos less than 22 ms of the escape response. For the 22 ms instant, we included in the final analysis 23 events for the pre-delivery stage of the 10 pregnant fish and 24 events

for the post-delivery stage. Similarly, for the 10 virgins, we included 21 events on the pre-delivery day of the pregnant fish and 18 events on the post-delivery day. For the 44 ms time point, we removed from the analysis 18 events where the fish swam out of view. Consequently, the total recorded events for the pregnant females were 20, and 19 events for the pre- and post-delivery days respectively, and for the virgin females 15 and 14 events for the pre- and post-delivery days respectively.

*Reproductive allocation increase* – The reproductive allocation increase ranged between 7.08% and 19.30% in the pregnant females. As shown before it linearly relates to the number of new-borns delivered at the end of the current reproductive cycle [12 (**Chapter 3**)].



*Body mass* – As expected, the body mass and normalized body mass (body mass/$SL^3$) of the virgin females varied relatively little between the pre- and the post-delivery days of the pregnant females, whereas, both metrics decreased significantly for the pregnant females once they gave birth (mean body mass change for pregnant fish (mean ± SD) = 0.2626 ± 0.0613 g, for virgin = 0.0157 ± 0.0846 g, $F = 55.82$, $p < 0.0001$. Mean change in body mass normalized by $SL^3$ for pregnant (mean ± SD) = 2.704 ± 0.62 μg $mm^{-3}$, for virgin = 0.1446 ± 0.8804 μg $mm^{-3}$, $F = 56.48$, $p < 0.0001$; see also figure 3).

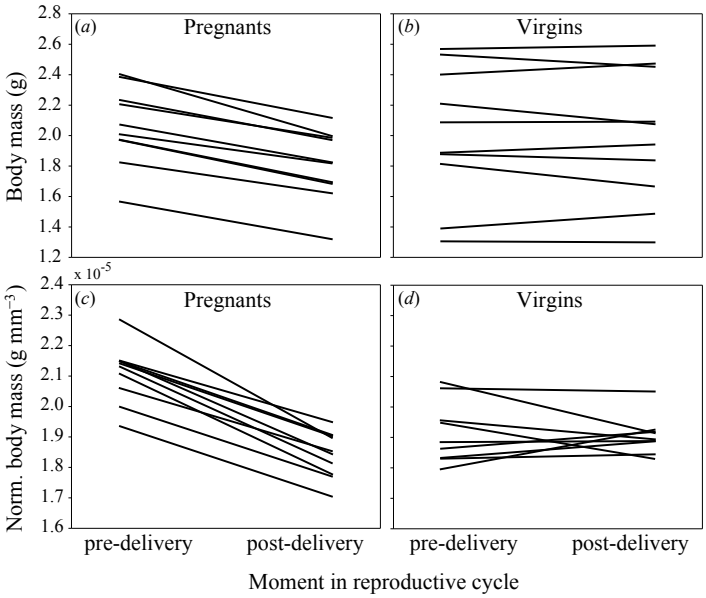



**Figure 3.** Body mass for pregnant and virgin females, at the pre- and post-delivery moments of the cycle. (*a*) Body mass of pregnant females. (*b*) Body mass of virgin females. (*c*) Idem to panel *a*, but for normalized body mass (mass $SL^{-3}$). (*d*) Idem to panel *b*, but for normalized body mass (mass $SL^{-3}$). $N_{pregnant} = 10$ females, $N_{virgin} = 10$ females.

*Maximum velocity vs initial distance to the stimulus* – There was not a significant effect of the initial distance to the stimulus on the maximum velocity that the individuals reached during the first and the second 22 ms intervals (figure 4, table 1).

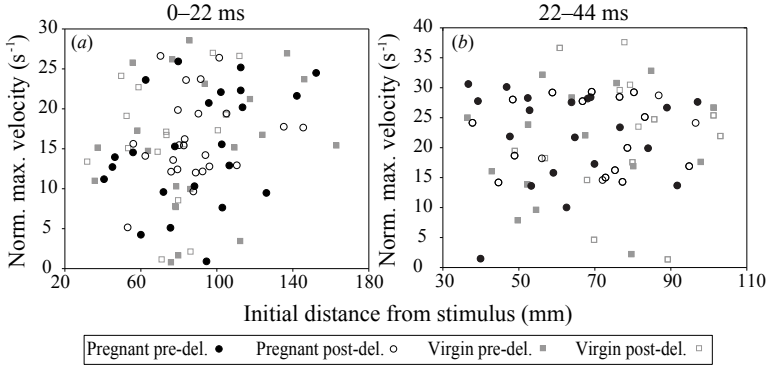



**Figure 4.** Maximum speed normalized by SL vs distance to the stimulus at the beginning of the manoeuvre. (*a*) Normalized maximum velocity during the first 22 ms. (*b*) Normalized maximum velocity during the second 22 ms. Pregnant individuals: pre-delivery (black filled circles), and post-delivery (open circles). Virgin individuals: pre-delivery time (closed gray squares), and post-delivery time (open gray squares). This is the graphical representation of the data shown for 0–22 ms and 22–44 ms in Table 1.

**Table 1.** Linear regressions of maximum velocity (normalized by SL) over the initial distance to the stimulus. Figure 4 visually presents the data corresponding to the first and second 22 ms interval.

| Dependent variable | 0 – 22 ms | | | | | |
|---|---|---|---|---|---|---|
| | Mean (mm $s^{-1}$) | SD | DF | $R^2$ | *F-value* | *Pr > F* |
| **Max. velocity pregnant pre-delivery** | 15.20 | 7.28 | 21 | 0.03 | 0.58 | 0.46 |
| **Max. velocity pregnant post-delivery** | 16.18 | 5.24 | 22 | 0.00 | 0.08 | 0.78 |
| **Max. velocity virgin pre-delivery** | 15.40 | 8.48 | 19 | 0.01 | 0.16 | 0.69 |
| **Max. velocity virgin post-delivery** | 16.39 | 7.10 | 16 | 0.07 | 1.15 | 0.30 |
| | **22 – 44 ms** | | | | | |
| **Max. velocity pregnant pre-delivery** | 22.02 | 7.84 | 18 | 0.00 | 0.01 | 0.91 |
| **Max. velocity pregnant post-delivery** | 22.23 | 5.99 | 17 | 0.00 | 0.03 | 0.86 |
| **Max. velocity virgin pre-delivery** | 20.40 | 9.35 | 13 | 0.02 | 0.29 | 0.60 |
| **Max. velocity virgin post-delivery** | 21.84 | 10.49 | 12 | 0.00 | 0.00 | 0.97 |

*Effects of reproductive status and moment in the reproductive cycle on the kinematic variables* – There were no significant differences in the escape angle, distance covered, maximum speed and mean acceleration between pregnant and virgin females and between the pre- and the post-delivery days (table 2, figure 5). The highest changes in escape angle and the highest mean accelerations happened during the first 22 ms of the manoeuvre. The distances covered and the maximum speeds in the second 22 ms interval were greater than the ones during the first 22 ms.

*Effects of reproductive status and moment in the reproductive cycle over the curvature* – There were no differences in maximum curvature for corresponding anterior and posterior locations along the body between the two time instances studied and between virgin and pregnant females (table 2). The curvatures were similar during the first and second 22 ms intervals.

*Effects of RAI over the kinematic and curvature variables* – There was not a significant linear relationship between RAI and any of the kinematic (figure S1, table 3), or curvature variables (figure S2, table 3).



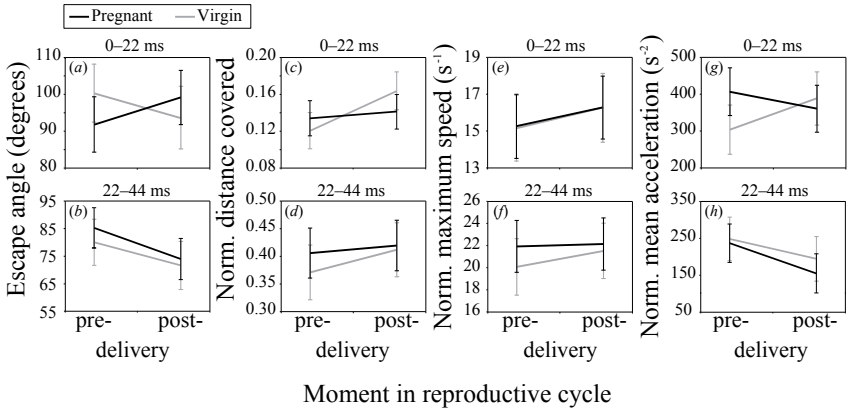



**Figure 5.** Kinematic variables vs reproductive status and reproductive stage. Black and gray lines represent pregnant and virgin females, respectively; vertical bars represent standard error of the mean. Escape angle during (*a*) the first 22 ms and (*b*) the second 22 ms. Distance covered (normalized by SL) during (*c*) the first 22 ms, and (*d*) the second 22 ms. Maximum speed normalized by SL during (*e*) the first 22 ms and (*f*) the second 22 ms. Mean acceleration normalized by SL during (*g*) the first 22 ms and (*h*) the second 22 ms. $N_{pregnant}$ = 10, $N_{virgin}$ = 10. Number of events for the first 22 ms (*a,c,e,g*): Pregnant females, 23 pre-delivery and 24 post-delivery events; virgin females, 21 events at pre-delivery instant of pregnant females, 18 at corresponding post-delivery instant. Number of analysed events for the second 22 ms (*b,d,f,h*): Pregnant females, 20 pre-delivery and 19 post-delivery events; and corresponding number of virgin females, 15, and 14 events.

**Table 2.** Type 3 test of fixed effects of kinematic and curvature variables (df, degrees of freedom).

| Dependent variable | Effect | 0 – 22 ms | | | | | 0 – 44 ms | | | | | 22 – 44 ms | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DF | *F* – *value* | *Pr > F* | Estimate | SE | DF | *F* – *value* | *Pr > F* | Estimate | SE | DF | *F* – *value* | *Pr > F* | Estimate | SE |
| Escape angle (degrees) | **Reproductive status** | 73 | 0.04 | 0.84 | | | 55 | 0.22 | 0.64 | | | 55 | 0.24 | 0.64 | | |
| | **Moment in rep. cycle** | 73 | 0.00 | 0.97 | | | 55 | 1.15 | 0.29 | | | 55 | 1.61 | 0.21 | | |
| | **Rep. status * Mom.rep. cycle** | 73 | 0.80 | 0.37 | | | 55 | 0.01 | 0.94 | | | 55 | 0.03 | 0.85 | | |
| | **Pregnant pre-delivery** | 73 | | | 91.8 | 7.51 | 55 | | | 83.2 | 7.02 | 55 | | | 85.2 | 7.28 |
| | **Pregnant post-delivery** | 73 | | | 99.1 | 7.35 | 55 | | | 74.3 | 7.18 | 55 | | | 74.0 | 7.44 |
| | **Virgin pre-delivery** | 73 | | | 100.3 | 7.86 | 55 | | | 79.0 | 8.09 | 55 | | | 80.0 | 8.36 |
| | **Virgin post-delivery** | 73 | | | 93.7 | 8.51 | 55 | | | 71.2 | 8.55 | 55 | | | 71.6 | 8.73 |
| Distance Covered (mm) | **Reproductive status** | 73 | 0.04 | 0.85 | | | 55 | 0.21 | 0.64 | | | 55 | 0.19 | 0.67 | | |
| | **Moment in rep. cycle** | 73 | 2.71 | 0.10 | | | 55 | 1.24 | 0.27 | | | 55 | 0.51 | 0.48 | | |
| | **Rep. status * Mom.rep. cycle** | 73 | 1.15 | 0.29 | | | 55 | 0.46 | 0.50 | | | 55 | 0.06 | 0.80 | | |
| | **Pregnant pre-delivery** | 73 | | | 6.1 | 0.81 | 55 | | | 24.1 | 2.65 | 55 | | | 18.5 | 2.02 |
| | **Pregnant post-delivery** | 73 | | | 6.5 | 0.79 | 55 | | | 25.0 | 2.68 | 55 | | | 19.3 | 2.06 |
| | **Virgin pre-delivery** | 73 | | | 5.5 | 0.83 | 55 | | | 21.6 | 2.90 | 55 | | | 17.4 | 2.22 |
| | **Reproductive status** | 73 | | | 7.4 | 0.88 | 55 | | | 25.4 | 2.89 | 55 | | | 19.0 | 2.19 |
| Dist. covered SL | **Reproductive status** | 73 | 0.07 | 0.79 | | | 55 | 0.35 | 0.56 | | | 55 | 0.34 | 0.56 | | |
| | **Moment in rep. cycle** | 73 | 2.25 | 0.14 | | | 55 | 1.37 | 0.25 | | | 55 | 0.58 | 0.45 | | |
| | **Rep. status * Mom.rep. cycle** | 73 | 1.16 | 0.28 | | | 55 | 0.67 | 0.42 | | | 55 | 0.14 | 0.71 | | |
| | **Pregnant pre-delivery** | 73 | | | 0.1 | 0.02 | 55 | | | 0.5 | 0.06 | 55 | | | 0.4 | 0.04 |
| | **Pregnant post-delivery** | 73 | | | 0.1 | 0.02 | 55 | | | 0.5 | 0.06 | 55 | | | 0.4 | 0.05 |
| | **Virgin pre-delivery** | 73 | | | 0.1 | 0.02 | 55 | | | 0.4 | 0.06 | 55 | | | 0.4 | 0.05 |
| | **Reproductive status** | 73 | | | 0.2 | 0.02 | 55 | | | 0.6 | 0.06 | 55 | | | 0.4 | 0.06 |

| Dependent variable | Effect | 0 – 22 ms | | | | | 0 – 44 ms | | | | | 22 – 44 ms | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DF | *F – value* | *Pr > F* | Estimate | SE | DF | *F – value* | *Pr > F* | Estimate | SE | DF | *F – value* | *Pr > F* | Estimate | SE |
| Maximum velocity (mm $s^{-1}$) | Reproductive status | 73 | 0.00 | 0.95 | | | 55 | 0.28 | 0.60 | | | 55 | 0.28 | 0.60 | | |
| | Moment in rep. cycle | 73 | 0.77 | 0.38 | | | 55 | 0.15 | 0.70 | | | 55 | 0.15 | 0.70 | | |
| | Rep. status * Mom.rep. cycle | 73 | 0.00 | 1.00 | | | 55 | 0.04 | 0.84 | | | 55 | 0.04 | 0.84 | | |
| | Pregnant pre-delivery | 73 | | | 698.2 | 77.58 | 55 | | | 1001.8 | 103.52 | 55 | | | 1001.8 | 103.52 |
| | Pregnant post-delivery | 73 | | | 751.4 | 81.64 | 55 | | | 1017.0 | 104.46 | 55 | | | 1017.1 | 104.47 |
| | Virgin pre-delivery | 73 | | | 694.1 | 75.68 | 55 | | | 943.7 | 112.69 | 55 | | | 943.7 | 112.69 |
| | Reproductive status | 73 | | | 748.1 | 74.51 | 55 | | | 990.3 | 111.04 | 55 | | | 990.2 | 111.04 |
| Max. velocity / SL ($s^{-1}$) | Reproductive status | 73 | 0.39 | 0.53 | | | 55 | 0.45 | 0.50 | | | 55 | 0.45 | 0.50 | | |
| | Moment in rep. cycle | 73 | 0.10 | 0.75 | | | 55 | 0.21 | 0.65 | | | 55 | 0.21 | 0.65 | | |
| | Rep. status * Mom.rep. cycle | 73 | 1.20 | 0.28 | | | 55 | 0.12 | 0.73 | | | 55 | 0.12 | 0.73 | | |
| | Pregnant pre-delivery | 73 | | | 15.2 | 1.74 | 55 | | | 21.9 | 2.34 | 55 | | | 21.9 | 2.34 |
| | Pregnant post-delivery | 73 | | | 16.3 | 1.71 | 55 | | | 22.1 | 22.14 | 55 | | | 22.1 | 2.36 |
| | Virgin pre-delivery | 73 | | | 15.2 | 1.78 | 55 | | | 20.1 | 2.55 | 55 | | | 20.1 | 2.56 |
| | Virgin post-delivery | 73 | | | 16.3 | 1.87 | 55 | | | 21.5 | 2.50 | 55 | | | 21.5 | 2.50 |
| Mean accel. (mm $s^{-2}$) | Reproductive status | 73 | 0.02 | 0.88 | | | 55 | 0.00 | 0.95 | | | 55 | 0.39 | 0.54 | | |
| | Moment in rep. cycle | 73 | 1.69 | 1.20 | | | 55 | 0.06 | 0.81 | | | 55 | 1.83 | 0.18 | | |
| | Rep.status * Mom.rep. cycle | 73 | 0.09 | 0.76 | | | 55 | 0.33 | 0.57 | | | 55 | 0.08 | 0.78 | | |
| | Pregnant pre-delivery | 73 | | | 26611.0 | 3111.40 | 55 | | | 19063.0 | 2053.16 | 55 | | | 10702.0 | 2360.03 |
| | Pregnant post-delivery | 73 | | | 29085.0 | 3065.64 | 55 | | | 18510.0 | 2076.82 | 55 | | | 6936.2 | 2405.22 |
| | Virgin pre-delivery | 73 | | | 25474.0 | 3186.41 | 55 | | | 17995.0 | 2254.98 | 55 | | | 11487.0 | 2673.19 |
| | Reproductive status | 73 | | | 29480.0 | 3360.86 | 55 | | | 19367.0 | 2246.35 | 55 | | | 9022.0 | 2743.37 |

| Dependent variable | Effect | 0 – 22 ms | | | | | 0 – 44 ms | | | | | 22 – 44 ms | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DF | *F – value* | *Pr > F* | Estimate | SE | DF | *F – value* | *Pr > F* | Estimate | SE | DF | *F – value* | *Pr > F* | Estimate | SE |
| Mean accel. / SL ($s^{-2}$) | Reproductive status | 73 | 0.39 | 0.53 | | | 55 | 0.05 | 0.83 | | | 55 | 0.26 | 0.61 | | |
| | Moment in rep. cycle | 73 | 0.10 | 0.75 | | | 55 | 0.09 | 0.76 | | | 55 | 1.80 | 0.18 | | |
| | Rep. status * Mom.rep. cycle | 73 | 1.20 | 0.28 | | | 55 | 0.45 | 0.50 | | | 55 | 0.08 | 0.78 | | |
| | Pregnant pre-delivery | 73 | | | 406.8 | 64.74 | 55 | | | 417.7 | 46.85 | 55 | | | 236.8 | 52.07 |
| | Pregnant post-delivery | 73 | | | 360.4 | 63.64 | 55 | | | 403.2 | 47.39 | 55 | | | 153.3 | 53.11 |
| | Virgin pre-delivery | 73 | | | 303.8 | 66.81 | 55 | | | 383.3 | 51.52 | 55 | | | 248.4 | 59.09 |
| | Reproductive status | 73 | | | 388.2 | 71.99 | 55 | | | 420.8 | 51.05 | 55 | | | 194.5 | 60.44 |
| Max. body curvature ($rad\ mm^{-2}$) | Reproductive status | 73 | 2.55 | 0.11 | | | 55 | 0.77 | 0.38 | | | 55 | 1.03 | 0.31 | | |
| | Moment in rep. cycle | 73 | 0.81 | 0.37 | | | 55 | 0.72 | 0.40 | | | 55 | 0.61 | 0.44 | | |
| | Rep. status * Mom.rep. cycle | 73 | 4.13 | 0.04 | | | 55 | 2.10 | 0.15 | | | 55 | 3.26 | 0.08 | | |
| | Pregnant pre-delivery | 73 | | | 91.9 | 5.73 | 55 | | | 93.2 | 5.28 | 55 | | | 90.5 | 5.63 |
| | Pregnant post-delivery | 73 | | | 96.3 | 5.67 | 55 | | | 95.3 | 5.31 | 55 | | | 95.1 | 5.69 |
| | Virgin pre-delivery | 73 | | | 93.5 | 5.82 | 55 | | | 95.2 | 5.63 | 55 | | | 94.0 | 6.16 |
| | Reproductive status | 73 | | | 82.2 | 6.06 | 55 | | | 87.0 | 5.70 | 55 | | | 82.4 | 6.12 |
| Max. body ant. / SL ($rad\ mm^{-3}$) | Reproductive status | 73 | 3.15 | 0.08 | | | 55 | 2.15 | 0.15 | | | 55 | 2.13 | 0.15 | | |
| | Moment in rep. cycle | 73 | 0.76 | 0.39 | | | 55 | 0.50 | 0.48 | | | 55 | 0.55 | 0.46 | | |
| | Rep. status * Mom.rep. cycle | 73 | 4.02 | 0.05 | | | 55 | 1.69 | 0.20 | | | 55 | 2.96 | 0.09 | | |
| | Pregnant pre-delivery | 73 | | | 2.0 | 0.15 | 55 | | | 2.0 | 0.14 | 55 | | | 2.0 | 0.14 |
| | Pregnant post-delivery | 73 | | | 2.1 | 0.15 | 55 | | | 2.1 | 0.14 | 55 | | | 2.1 | 0.14 |
| | Virgin pre-delivery | 73 | | | 2.0 | 0.15 | 55 | | | 2.0 | 0.15 | 55 | | | 2.0 | 0.15 |
| | Virgin post-delivery | 73 | | | 1.8 | 0.15 | 55 | | | 1.8 | 0.15 | 55 | | | 1.8 | 0.15 |

| Dependent variable | Effect | 0 – 22 ms | | | | | 0 – 44 ms | | | | | 22 – 44 ms | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DF | *F – value* | *Pr > F* | Estimate | SE | DF | *F – value* | *Pr > F* | Estimate | SE | DF | *F – value* | *Pr > F* | Estimate | SE |
| Max. curv. anterior (rad mm$^{-2}$) | Reproductive status | 73 | 0.88 | 0.35 | | | 55 | 0.11 | 0.74 | | | 55 | 0.16 | 0.69 | | |
| | Moment in rep. cycle | 73 | 0.44 | 0.51 | | | 55 | 0.41 | 0.52 | | | 55 | 1.09 | 0.30 | | |
| | Rep.status*Mom.rep.cycle | 73 | 3.77 | 0.06 | | | 55 | 2.54 | 0.12 | | | 55 | 3.65 | 0.06 | | |
| | Pregnant pre-delivery | 73 | | | 78.2 | 5.60 | 55 | | | 78.5 | 5.53 | 55 | | | 70.7 | 5.91 |
| | Pregnant post-delivery | 73 | | | 83.7 | 5.52 | 55 | | | 82.6 | 5.58 | 55 | | | 75.3 | 6.00 |
| | Virgin pre-delivery | 73 | | | 82.4 | 5.73 | 55 | | | 83.9 | 6.02 | 55 | | | 78.6 | 6.59 |
| | Reproductive status | 73 | | | 71.3 | 5.90 | 55 | | | 74.4 | 5.92 | 55 | | | 63.1 | 6.53 |
| Max. body curv. / SL (rad mm$^{-3}$) | Reproductive status | 73 | 0.25 | 0.62 | | | 55 | 0.46 | 0.50 | | | 55 | 0.48 | 0.49 | | |
| | Moment in rep. cycle | 73 | 0.83 | 0.36 | | | 55 | 0.35 | 0.56 | | | 55 | 1.18 | 0.28 | | |
| | Rep.status*Mom.rep.cycle | 73 | 0.36 | 0.55 | | | 55 | 2.20 | 0.14 | | | 55 | 3.40 | 0.07 | | |
| | Pregnant pre-delivery | 73 | | | 1.3 | 0.20 | 55 | | | 1.7 | 0.14 | 55 | | | 1.6 | 0.14 |
| | Pregnant post-delivery | 73 | | | 1.0 | 0.20 | 55 | | | 1.8 | 0.14 | 55 | | | 1.6 | 0.14 |
| | Virgin pre-delivery | 73 | | | 1.0 | 0.21 | 55 | | | 1.8 | 0.15 | 55 | | | 1.7 | 0.15 |
| | Reproductive status | 73 | | | 1.0 | 0.23 | 55 | | | 1.6 | 0.14 | 55 | | | 1.3 | 0.15 |
| Max. curv. posterior (rad mm$^{-2}$) | Reproductive status | 73 | 2.25 | 0.14 | | | 55 | 0.77 | 0.38 | | | 55 | 1.03 | 0.31 | | |
| | Moment in rep.cycle | 73 | 0.85 | 0.36 | | | 55 | 0.72 | 0.40 | | | 55 | 0.61 | 0.44 | | |
| | Rep.status*Mom.rep.cycle | 73 | 4.34 | 0.04 | | | 55 | 2.10 | 0.15 | | | 55 | 3.26 | 0.08 | | |
| | Pregnant pre-delivery | 73 | | | | | 55 | | | 93.2 | 5.28 | 55 | | | 90.5 | 5.63 |
| | Pregnant post-delivery | 73 | | | | | 55 | | | 95.3 | 5.31 | 55 | | | 95.1 | 5.69 |
| | Virgin pre-delivery | 73 | | | | | 55 | | | 95.2 | 5.63 | 55 | | | 94.0 | 6.16 |
| | Reproductive status | 73 | | | | | 55 | | | 87.0 | 5.070 | 55 | | | 82.4 | 6.12 |

| Dependent variable | Effect | 0 – 22 ms | | | | | 0 – 44 ms | | | | | 22 – 44 ms | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DF | *F* – *value* | *Pr* > *F* | Estimate | SE | DF | *F* – *value* | *Pr* > *F* | Estimate | SE | DF | *F* – *value* | *Pr* > *F* | Estimate | SE |
| $\frac{\text{Max. body post.}}{\text{SL}}$ (rad mm$^{-3}$) | Reproductive status | 73 | 0.51 | 0.48 | | | 55 | 2.15 | 0.15 | | | 55 | 2.13 | 0.15 | | |
| | Mom. in rep. cycle | 73 | 0.91 | 0.34 | | | 55 | 0.50 | 0.48 | | | 55 | 0.55 | 0.46 | | |
| | Rep. status * Mom.rep. cycle | 73 | 0.44 | 0.51 | | | 55 | 1.69 | 0.20 | | | 55 | 2.96 | 0.09 | | |
| | Pregnant pre-delivery | 73 | | | 1.5 | 0.23 | 55 | | | 2.0 | 0.14 | 55 | | | 2.0 | 0.14 |
| | Pregnant post-delivery | 73 | | | 1.1 | 0.23 | 55 | | | 2.1 | 0.14 | 55 | | | 2.1 | 0.15 |
| | Virgin pre-delivery | 73 | | | 1.2 | 0.24 | 55 | | | 2.0 | 0.15 | 55 | | | 2.0 | 0.16 |
| | Virgin post-delivery | 73 | | | 1.1 | 0.26 | 55 | | | 1.8 | 0.15 | 55 | | | 1.8 | 0.15 |

**Table 3.** Linear regressions of kinematic and curvature variables over RAI (reproductive allocation increase).

| Dependent variable | 0 – 22 ms | | | | | |
|---|---|---|---|---|---|---|
| | Mean | SD | DF | $R^2$ | *F-value* | *Pr > F* |
| Distance covered (mm) | 6.16 | 3.43 | 21 | 0.13 | 3.21 | 0.09 |
| $\frac{\text{Dist. covered}}{\text{SL}}$ | 0.14 | 0.08 | 21 | 0.13 | 3.23 | 0.09 |
| Maximum velocity (mm $s^{-1}$) | 687.24 | 323.41 | 21 | 0.11 | 2.63 | 0.12 |
| $\frac{\text{Max. velocity}}{\text{SL}}$ ($s^{-1}$) | 15.20 | 7.28 | 21 | 0.11 | 2.67 | 0.12 |
| Mean acceleration (mm $s^{-2}$) | 26144.00 | 13414.00 | 21 | 0.10 | 2.32 | 0.14 |
| $\frac{\text{Mean accel.}}{\text{SL}}$ ($s^{-2}$) | 403.82 | 382.78 | 21 | 0.00 | 0.01 | 0.91 |
| Max. body curvature (rad $mm^{-2}$) | 93.12 | 21.77 | 21 | 0.00 | 0.00 | -0.05 |
| $\frac{\text{Max. body curv.}}{\text{SL}}$ (rad $mm^{-3}$) | 2.06 | 0.51 | 21 | 0.00 | 0.00 | 0.97 |
| Max. curv. anterior region (rad $mm^{-2}$) | 79.55 | 20.38 | 21 | 0.00 | 0.01 | 0.91 |
| $\frac{\text{Max. body ant.}}{\text{SL}}$ (rad $mm^{-3}$) | 1.27 | 1.37 | 21 | 0.10 | 2.48 | 0.13 |
| Max. curv. posterior region (rad $mm^{-2}$) | 92.54 | 23.77 | 21 | 0.00 | 0.00 | 1.00 |
| $\frac{\text{Max. body post.}}{\text{SL}}$ (rad $mm^{-3}$) | 1.50 | 1.63 | 21 | 0.10 | 2.48 | 0.13 |
| | 0 – 44 ms | | | | | |
| Distance covered (mm) | 23.71 | 10.05 | 18 | 0.06 | 1.20 | 0.29 |
| $\frac{\text{Dist. covered}}{\text{SL}}$ | 0.52 | 0.22 | 18 | 0.06 | 1.19 | 0.29 |
| Maximum velocity (mm $s^{-1}$) | 997.36 | 345.20 | 18 | 0.03 | 0.63 | 0.44 |
| $\frac{\text{Max. velocity}}{\text{SL}}$ ($s^{-1}$) | 22.02 | 7.84 | 18 | 0.03 | 0.61 | 0.44 |
| Mean acceleration (mm $s^{-2}$) | 19060.00 | 7034.00 | 18 | 0.01 | 0.11 | 0.74 |
| $\frac{\text{Mean accel.}}{\text{SL}}$ ($s^{-2}$) | 420.82 | 158.34 | 18 | 0.01 | 0.11 | 0.74 |
| Max. body curvature (rad $mm^{-2}$) | 94.67 | 19.66 | 18 | 0.01 | 0.14 | 0.71 |
| $\frac{\text{Max. body curv.}}{\text{SL}}$ (rad $mm^{-3}$) | 2.08 | 0.47 | 18 | 0.01 | 0.12 | 0.74 |
| Max. curv. ant.region (rad $mm^{-2}$) | 79.95 | 19.76 | 18 | 0.00 | 0.02 | 0.90 |
| $\frac{\text{Max. body ant.}}{\text{SL}}$ (rad $mm^{-3}$) | 1.76 | 0.46 | 18 | 0.00 | 0.02 | 0.88 |
| Max. curv. post. region (rad $mm^{-2}$) | 94.67 | 19.66 | 18 | 0.01 | 0.14 | 0.71 |
| $\frac{\text{Max. body post.}}{\text{SL}}$ (rad $mm^{-3}$) | 2.08 | 0.47 | 18 | 0.01 | 0.12 | 0.74 |

**Table 3.** Continuation

| Dependent variable | 22 – 44 ms | | | | | |
|---|---|---|---|---|---|---|
| | Mean | SD | DF | $R^2$ | *F-value* | *Pr > F* |
| Distance covered (mm) | 18.40 | 7.19 | 18 | 0.04 | 0.67 | 0.42 |
| $\frac{\text{Dist. covered}}{\text{SL}}$ | 0.40 | 0.16 | 18 | 0.04 | 0.66 | 0.43 |
| Maximum velocity (mm $s^{-1}$) | 997.36 | 345.20 | 18 | 0.03 | 0.63 | 0.44 |
| $\frac{\text{Max. velocity}}{\text{SL}}$ ($s^{-1}$) | 22.02 | 7.84 | 18 | 0.03 | 0.61 | 0.44 |
| Mean acceleration (mm $s^{-2}$) | 10921.00 | 9665.00 | 18 | 0.10 | 2.05 | 0.17 |
| $\frac{\text{Mean accel.}}{\text{SL}}$ ($s^{-2}$) | 242.41 | 214.84 | 18 | 0.10 | 2.09 | 0.16 |
| Max. body curvature (rad $mm^{-2}$) | 92.21 | 20.60 | 18 | 0.03 | 0.64 | 0.43 |
| $\frac{\text{Max. body curv.}}{\text{SL}}$ (rad $mm^{-3}$) | 2.03 | 0.48 | 18 | 0.03 | 0.62 | 0.44 |
| Max. curv. ant. region (rad $mm^{-2}$) | 72.14 | 23.30 | 18 | 0.00 | 0.01 | 0.92 |
| $\frac{\text{Max. body ant.}}{\text{SL}}$ (rad $mm^{-3}$) | 1.59 | 0.53 | 18 | 0.00 | 0.01 | 0.91 |
| Max. curv. post. region (rad $mm^{-2}$) | 92.21 | 20.60 | 18 | 0.03 | 0.64 | 0.43 |
| $\frac{\text{Max. body post.}}{\text{SL}}$ (rad $mm^{-3}$) | 2.03 | 0.48 | 18 | 0.03 | 0.62 | 0.44 |

## 4. Discussion

We evaluated the escape response of pregnant females of *Poeciliopsis gracilis* in a controlled environment. We predicted: (i) pregnant females to have lower performances at the end of pregnancy (close to parturition) than at the beginning of pregnancy (just after parturition) and (ii) pregnant females to have lower locomotion performances than virgin individuals. Contrary to our predictions, we did not find any significant differences in the kinematic and body-curvature variables between treatments, suggesting that pregnancy does not have a strong negative effect on the escape performance of *P. gracilis*.

We compared the escape performance of pregnant females before and after giving birth and found no significant differences in escape performance over the course of a pregnancy. This was most likely related to the relatively small increase in female body volume over the course of pregnancy in our study. These small increases in body volume may have been caused in part by (i) superfetation and (ii) the small brood sizes of our lab-reared experimental females. Superfetation is a reproductive strategy that may allow females to maintain a relatively slender body throughout pregnancy [26 (**Chapter 2**)]. Prior studies have shown that pregnancy can have negative effects on the locomotor performance

of females [8–10]. In the non-superfetatious lecithothrophic species *Gambusia affinis* and *Poecilia reticulata*, critical swimming velocity ($U_{crit}$) and escape responses are negatively affected by reproduction, and pregnant females show lower performances, especially towards the end of the pregnancy [8,9,21]. Superfetation is thought to reduce the amplitude around the mean reproductive allocation, ultimately reducing the maximum reproductive burden of females compared to non-superfetatious species [19]. Indeed, *P. gracilis* appeared to display body shape changes during pregnancy that were smaller compared to those of non-superfetatious species (*P. gracilis* change in body mass: means ± SD: 14.7 ± 3.4 % vs *G. affinis* mean: 53% [9]). Here, we argue that the smaller increase in body volume associated with superfetation may partly explain our difficulty in uncovering potential decreases in fast-start escape response of females throughout their pregnancy. Interestingly, these findings appear to support one of the hypotheses for the evolution of superfetation, i.e. the *Locomotor cost hypothesis*, which proposes that superfetation may have evolved to reduce the locomotor costs of pregnancy [19]. The second reason why we may not have found any significant differences in escape performance over the course of a pregnancy are the relatively small brood sizes of our lab-reared experimental females. The broods delivered during our experiment (mean ± SD: 21.9 ± 5.78; range: 14–31) appear to be somewhat smaller than those observed in previous experiments (mean ± SD: 30.2 ± 12.9; range: 9–70 for Pollux *et al.*, in prep). The reason for the relatively smaller broods in our current experimental females is unclear, but could have been caused by sub *ad libitum* food conditions. The small broods in turn, imply small changes in reproductive allocation, and hence body volume, which makes it difficult to detect significant declines in locomotor differences over the course of a pregnancy. While in our study we did not find an effect of pregnancy on female locomotor performance, we do not exclude the possibility that significant declines in locomotor performance occur in females that carry much larger broods (e.g. up to 70 offspring/brood observed in previous experiments).

Furthermore, the absence of significant differences between virgin and pregnant female *P. gracilis* is likely related to the unexpected high reproductive allocation observed in the virgin females. *P. gracilis* is a lecithotrophic species that allocates all resources required for embryo development prior to fertilization by producing large, fully provisioned eggs. In reproducing (i.e. non-virgin) females, the allocation of resources to the unfertilized eggs typically occurs just before or shortly after the previous litter is born, within a very narrow time interval (< 24–48 hours). Because the virgin females in our study were not reproducing and had never been exposed to males in their life, we initially assumed that they would not carry any 'provisioned' eggs and would therefore have a very low reproductive allocation. The virgins in our study, however, were carrying many fully provisioned unfertilized eggs (see figure 1 in General Discussion), which meant that our virgins also had high reproductive allocations, almost comparable to those of pregnant females. The small differences in reproductive allocation between virgins and

pregnant females in our study likely caused the absence of significant differences in escape performance between them.

In addition to the biological reasons listed above, there were a number of technical limitations in our study that may have contributed to our inability to detect significant effects of pregnancy on female locomotor performance. These technical limitations included (1) a small field of view inside the swimming tank, (2) a stimulus that was not the most intense possible and (3) sub-optimal time resolution. First, the small field of view meant that the last part of several escape responses went out of the field of view and these videos had to be excluded from our analyses. The relatively low number of responses that we could analyse has limited the power of the statistical analysis. Second, not all females displayed a maximal performance in each of the analysed events, possibly because of an insufficient stimulus intensity or due to the fish habituating to it over time. This introduced a source of variation in female fast-start escape performance that might have made it more difficult to detect statistical differences. Third, the suboptimal temporal and spatial resolution of the high-speed videos did not allow the analysis in 3D of changes in instantaneous acceleration. Instantaneous acceleration is presumably negatively correlated with both reproductive allocation and swimming velocity. We would expect significant differences for those females with higher reproductive investment and swimming at high speeds.

Future studies on the effect of pregnancy on the escape response of live-bearing fish may benefit from an enhanced spatial and temporal resolution of the camera systems, which would also enable the usage of a larger field of view. Additionally, to prevent habituation to the stimulus and ensure a higher occurrence of maximum performance, we suggest combining different stimuli. Furthermore, a larger number of females should be included that have been reared under *ad libitum* food conditions, increasing the possibility of studying females with larger broods and providing better statistical power. Finally, it might be interesting to study a non-superfetatious placental species within the Poeciliidae. Placental females allocate most resources to the embryo after fertilization, meaning that they will have a very low reproductive allocation at the beginning of their pregnancy (much lower than in lecithotrophic species) and show a much more dramatic increase in body volume throughout their pregnancy [19]. These extreme changes in body volume are more likely to induce a decrease in escape performance towards the end of the pregnancy. Notably, these effects are intensified by the absence of superfetation [19]. Therefore, it would be interesting to compare placental species that differ in their level of superfetation (e.g. *Pamphorichthys hollandi* which lacks superfetation and *Phalloptychus januarius* which has the highest known level of superfetation of all vertebrates).

**Acknowledgements**

We thank Eric Karruppannan and his TD studio team, and Remco Pieters for their help with building and testing the experimental setups. We also thank T. Wiegers-Van der Wal, S. Visser, W. Nusselder, E. Roux, J. Lengkeek and M. ter Veld for their continuous efforts in fish care.

## References

1. Domenici P, Blagburn JM, Bacon JP. 2011 Animal escapology I: theoretical issues and emerging trends in escape trajectories. *J. Exp. Biol.* **214**, 2463–2473. (doi:10.1242/jeb.029652)
2. Berglund A, Rosenqvist G. 1986 Reproductive costs in the prawn *Palaemon adspersus*: effects on growth and predator vulnerability. *Oikos* **46**, 349–354. (doi:10.2307/3565833)
3. Magnhagen C. 1991 Predation risk as a cost of reproduction. *Trends Ecol. Evol.* **6**, 183–186. (doi:10.1016/0169-5347(91)90210-O)
4. Shaffer LR, Formanowicz DR. 1996 A cost of viviparity and parental care in scorpions: reduced sprint speed and behavioural compensation. *Anim. Behav.* **51**, 1017–1024. (doi:10.1006/anbe.1996.0104)
5. Winfield IJ, Townsend CR. 1983 The cost of copepod reproduction: increased susceptibility to fish predation. *Oecologia* **60**, 406–411. (doi:10.1007/BF00376860)
6. Isaacs R, Byrne DN. 1998 Aerial distribution, flight behaviour and eggload: their inter-relationship during dispersal by the sweetpotato whitefly. *J. Anim. Ecol.* **67**, 741–750. (doi:10.1046/j.1365-2656.1998.00236.x)
7. Webb JK. 2004 Pregnancy decreases swimming performance of female northern death adders (*Acanthophis praelongus*). *Copeia* **2004**, 357–363. (doi:10.1643/CH-03-171R1)
8. Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* **164**, 38–50. (doi:10.1086/421412)
9. Plaut I. 2002 Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
10. James RS, Johnston IA. 1998 Influence of spawning on swimming performance and muscle contractile properties in the short-horn sculpin. *J. Fish Biol.* **53**, 485–501. (doi:10.1006/jfbi.1998.0722)
11. Svendsen JC, Aarestrup K, Dolby J, Svendsen TC, Christensen RHB. 2009 The volitional travel speed varies with reproductive state in mature female brown trout *Salmo trutta*. *J. Fish Biol.* **75**, 901–907. (doi:10.1111/j.1095-8649.2009.02310.x)
12. Quicazan-Rubio EM, van Leeuwen JL, van Manen K, Fleuren M, Pollux BJA, Stamhuis EJ. 2019 Coasting in live-bearing fish: the drag penalty of being pregnant. *J. R. Soc. Interface* **16**. (doi: 10.1098/rsif.2018.0714)
13. Frías-Alvarez P, Zúñiga-Vega JJ. 2016 Superfetation in live-bearing fishes is not always the result of a morphological constraint. *Oecologia* **181**, 645–658. (doi:10.1007/s00442-015-3477-1)

14. Gómez-Márquez JL, Peña-Mendoza B, Salgado-Hugarte IH, Sánchez-Herrera AK, Sartré-Baez L. 2007 Reproduction of the fish *Poeciliopsis gracilis* (Cyprinodontiformes: Poeciliidae) in Coatetelco, a tropical shallow lake in Mexico. *Rev. Biol. Trop.* **56**, 1801–12. (doi:10.15517/rbt.v56i4.5760)
15. Contreras-Macbeath T, Gaspar-Dillanes MT, Huidobro-Campos L, Mejía-Mojica H. 2014 Peces invasores en el centro de México. In: Mendoza, R, Koledd, P. (Eds), Especies acuáticas invasoras de México. Comisión nacional para el conocimiento y uso de la biodiversidad, México City.
16. Gutiérrez Jiménez IM, Molina Arroyo HR, Mangas-Ramírez E, López Téllez MC, Fernández Crispin A, Campos Cabral V. 2018 Toxicidad del Zinc sobre indicadores biológicos y fisiológicos en hembras de *Poeciliopsis gracilis* (Cyprinodontiformes: Poeciliidae). *Rev. Latinoam. el Ambient. y las Ciencias* **9**, 90–109.
17. Turner CL. 1937 Reproductive cycles and superfetation in Poeciliid fishes. *Biol. Bull.* **72**, 145–164. (doi:10.2307/1537249)
18. Scrimshaw NS. 1944 Superfetation in Poeciliid fishes. *Copeia* **1944**, 180–183. (doi:10.2307/1437814)
19. Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009 Evolution of placentas in the fish family Poeciliidae: An empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **40**, 271–289. (doi:10.1146/annurev.ecolsys.110308.120209)
20. Fleuren M. 2017 Reproductive adaptations to reduce locomotor costs in viviparous fish (Poeciliidae). PhD thesis. Wageningen University and Research, Wageningen, The Netherlands. (doi:10.18174/424276)
21. Belk MC, Tuckfield RC. 2010 Changing costs of reproduction: age-based differences in reproductive allocation and escape performance in a livebearing fish. *Oikos* **119**, 163–169. (doi:10.1111/j.1600-0706.2009.17742.x)
22. Banet AI, Svendsen JC, Eng KJ, Reznick DN. 2016 Linking reproduction, locomotion, and habitat use in the Trinidadian guppy (*Poecilia reticulata*). *Oecologia* **181**, 87–96. (doi:10.1007/s00442-015-3542-9)
23. Voesenek CJ, Pieters RPM, van Leeuwen JL. 2016 Automated reconstruction of three-dimensional fish motion, forces, and torques. *PLoS One* **11**, 8–10. (doi:10.1371/journal.pone.0146682)
24. Fleuren M, van Leeuwen JL, Quicazan-Rubio EM, Pieters RPM, Pollux BJA, Voesenek CJ. 2018 Three-dimensional analysis of the fast-start escape response of the least killifish, *Heterandria formosa*. *J. Exp. Biol.*, jeb.168609. (doi:10.1242/jeb.168609)
25. Webb PW. 1978 Fast-start performances and body form in seven species of teleost fish. *J. Exp. Biol.* **74**, 211–226.
26. Fleuren M, Quicazan-Rubio EM, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS One* **13**, e0195976. (doi:10.1371/journal.pone.0195976)
27. Robertson GN, Lindsey BW, Dumbarton TC, Croll RP, Smith FM. 2008 The contribution of the swimbladder to buoyancy in the adult zebrafish (*Danio rerio*): A morphometric analysis. *J. Morphol.* **269**, 666–673. (doi:10.1002/jmor.10610)
28. Webb PW. 1988 Simple Physical principles and vertebrate aquatic locomotion. *Am. Zool.* **28**, 709–725. (doi:10.1093/icb/28.2.709)
29. Hedrick TL. 2008 Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspir. Biomim.* **3**, 034001.

(doi:10.1088/1748-3182/3/3/034001)
30. Marras S, Killen SS, Claireaux G, Domenici P, McKenzie DJ. 2011 Behavioural and kinematic components of the fast-start escape response in fish: individual variation and temporal repeatability. *J. Exp. Biol.* **214**, 3102–3110. (doi:10.1242/jeb.056648)
31. Eaton RC, Emberley DS. 1991 How stimulus direction determines the trajectory of the Mauthner-initiated escape response in a teleost fish. *J. Exp. Biol.* **161**, 469–487.
32. Tytell ED, Lauder GV. 2002 The C-start escape response of *Polypterus senegalus*: bilateral muscle activity and variation during stage 1 and 2. *J. Exp. Biol.* **205**, 2591–603.
33. Oufiero CE, Garland TJ. 2009 Repeatability and correlation of swimming performances and size over varying time-scales in the guppy (*Poecilia reticulata*). *Funct. Ecol.* **23**, 969–978. (doi:10.1111/j.1365-2435.2009.01571.x)
34. Eilers PHC. 2003 A perfect smoother. *Anal. Chem.* **75**, 3631–3636. (doi:10.1021/ac034173t)
35. Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN. 2005 Do faster starts increase the probability of evading predators? *Funct. Ecol.* **19**, 808–815. (doi:10.1111/j.1365-2435.2005.01033.x)
36. Webb PW. 1976 The effect of size on fast-start performance of rainbow trout *Salmo gairdneri* and a consideration of piscivorous predator–prey interaction. *J. Exp. Biol.* **65**, 157–177.
37. Domenici P, Blake RW. 1993 Escape trajectories in angelfish (*Pterophyllum eimekei*). *J. Exp. Biol.* **177**, 253–272.

## Supplementary Material

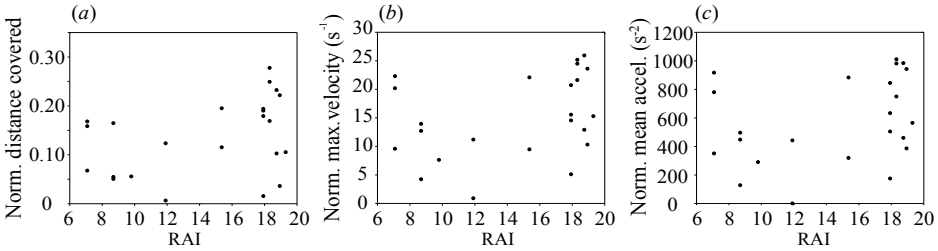



**Figure S1.** Distance covered, maximum velocity and mean acceleration vs RAI of pregnant females before delivery. (*a*) Maximum distance covered vs RAI. (*b*) Maximum distance covered vs RAI normalized by SL. (*c*) Maximum velocity. (*d*) Maximum velocity normalized by SL. (*e*) Mean acceleration. (*f*) Mean acceleration normalized by SL. The data points located at the same RAI correspond to one fish.

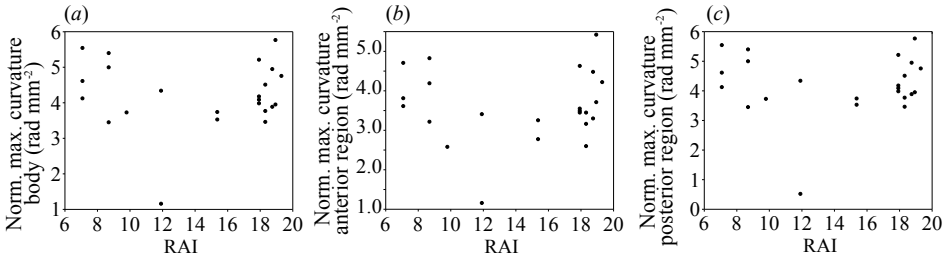



**Figure S2.** Maximum curvatures of pregnant females before delivery. (*a*) Maximum curvature along the body, normalized by SL. (*b*) Maximum curvature on the anterior region of the body, normalized by SL. (*c*) Maximum curvature on the posterior region of the body, normalized by SL. The data points located at the same RAI correspond to one fish.

# Chapter 5

## Does pregnancy affect muscle composition and expression of muscle-related genes in live-bearing fish? A route map to answer this question

Elsa M. Quicazan-Rubio, Jeffrey Verhoeff, Henk Schipper, Karen M. Leon-Kloosterziel, Mike Fleuren, Johan L. van Leeuwen, and Bart J.A. Pollux

*Experimental Zoology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, The Netherlands*

**Abstract**

A live-bearing mode of reproduction (viviparity) may negatively affect the locomotor performance of females. Pregnant females may respond to this performance decline by modifying their locomotor musculature in two opposing ways: by sacrificing muscle tissue and diverting this energy to reproduction or by increasing their energy investment in muscle tissue to help maintain their locomotor performance. Here, we test if and how a pregnancy induces changes in the muscle architecture and gene expression of muscle-related genes. To this end, we performed a series of pilot experiments using virgin and pregnant females from the live-bearing fish species *Poeciliopsis gracilis* (family Poeciliidae). We used various histological techniques to compare stained cross-sections of pregnant and virgin females and quantify the number and thickness of different fibre types in different muscle regions. With real-time quantitative PCR, we quantified transcription levels of genes involved in different processes related to muscle physiology and metabolism. Our results suggest that pregnancy does not affect muscle architecture and functioning in *P. gracilis*. This study herewith provides a first exploration of whether muscles in Poeciliid fishes change and adapt during the pregnancy. Finally, we elaborate on the limitations of our research and provide detailed suggestions for future studies.

## 1. Introduction

Reproduction negatively affects the locomotor performance of females in a variety of animal lineages, including fish [1–3], reptiles [4–7], birds [8,9], and mammals [10]. These detrimental effects on locomotor performance may have negative effects on survival, possibly by reducing the female's ability to obtain food and escape from predators [3,11–16].

Muscle performance is essential to escape behaviour, search for food, and any other movement of the gravid females. It has been found that gravidity (the carrying of eggs or developing young) causes changes in the muscles at different levels. For example, in several groups, such as fish [17,18], scallops [14], birds [16], some insects [19], and snakes [15] there is diversion of energy reserves and proteins from muscles and organs such as the liver, to the reproductive tissue, which can cause muscular loss, decrease of muscle strength and limitation of the aerobic capacity of muscles [1,15].

Furthermore, gravidity alters the proportion and diameter of the muscle fibres, as has been found for guinea pigs, rats, and rabbits [20–23]. Due to the higher production of oestrogen during reproduction, which might favour the increase of the slow muscle fibres because of their higher expression of oestrogen receptors [23,24], these fibres increase the area that they cover during pregnancy. This might affect the locomotor performance of females, but to our knowledge there are no studies yet on this specific subject.

Moreover, the energy invested in reproduction is usually reflected in belly growth, which causes stretching of the muscle fibres in and around that region. This might increase the number of sarcomeres in series in the affected muscle fibres [1,25], which affects abdominal muscle function. In humans, this reduces the ability to stabilize the pelvis against resistance [26] and might reduce the ability of the post-parturition females to flex their body [27]. The stretched abdomen might also cause the fibres to reorient to slightly different angles negatively affecting their contractile properties [26,28]. The growing abdomen might also result in a reduction in the ratio of skeletal muscles with respect to the body, accompanied by an increase in flexural stiffness, resulting in a lower axial bending [1,3,29]. The lower ratio of muscles also implies that relatively less power is available to propel a bigger and heavier body [1], resulting in a decline of the maximal acceleration [1,3,30].

In addition to the energy deviation from the muscles to the reproductive tissues, none of the other changes caused by pregnancy in the muscles has been studied in pregnant fish. It is known that the muscular activity of some females is altered during the gravidity. For scallops, two possible outcomes have been established after studying their muscle performance during reproduction. The longer recuperation time of clapping capacity after intense exercise and the reduced metabolic capacity might negatively affect the locomotory performance of reproductive scallops [14]. However, the force outputs of the reproductive scallops suggest that their escape performance might improve instead [31]. In fish, a decline in the escape response due to gravidity has been observed in the short-horn sculpin [1], for which gravid females show a decrease in muscle power output that might partially cause their less efficient escape response. However, the causes of these changes in gravid fish have only been partially covered.

The muscular changes presented above describe the effect of reproduction on gravid terrestrial animals or in egg-carrying aquatic animals. One could argue that these effects should be amplified in live-bearing aquatic animals that internally carry their developing embryos during their entire pregnancy. Their bodies present a thick girth that increases the body surface and cross-sectional area which increases the drag that they need to overcome to propel through water [1–3, 32 (**Chapter 3**)]. Here we study what happens to the muscle composition at the physiological and structural levels during pregnancy, using females from the live-bearing fish species *Poeciliopsis gracilis* (Heckel, 1848; family Poeciliidae), as a model system. Specifically, we test three contrasting hypotheses: (i) The pregnancy will negatively affect muscle architecture by sacrificing muscle tissue (i.e. reducing fibre number, fibre thickness and total muscle mass) and, in turn, locomotor performance in live-bearing fish, (ii) the pregnancy will not affect muscle architecture and (iii) the pregnancy will induce changes to the locomotor musculature in order to (at least partly) compensate for the increase in drag [32 (**Chapter 3**)] with the aim of maintaining locomotor performance (**Chapter 4**). For this purpose, we performed a series

of pilot experiments using virgin and pregnant females from the live-bearing fish species *Poeciliopsis gracilis* (family Poeciliidae). To determine possible changes in the muscle fibre distribution and composition, we used various histological techniques to compare stained cross sections from the abdomen of pregnant and virgin females.

To evaluate if pregnancy would affect the muscle physiology and metabolism, we quantified the transcription levels of some of the genes involved in these processes using real-time quantitative PCR. The high plasticity of the muscles allows them to respond to the mechanical and neuronal stimuli of exercise and their adaptive responses are governed by (epi)genetic programs [33]. In rabbits, guinea pigs, and rats, it has been shown that hormonal and mechanical stimuli are send to the body during pregnancy and that this causes changes in the proportion and diameter of muscle fibres [20 – 23], changes that are similar to those that are caused by exercise in humans [24]. One could argue that some of the genes whose expression is affected by a physically demanding activity (e.g. exercise) might also show an altered pattern of expression during pregnancy. Therefore, we choose candidate genes based on a study on the changes in muscle development of swim-trained zebrafish, *Danio rerio* [34]. In this pilot study, we provide a first exploration of whether the expression of 'swim-related muscles' change during the pregnancy in live-bearing fish and provide a perspective for future studies.

## 2. Material and methods

### 2.1. Animals

Our laboratory stocks of *Poeciliopsis gracilis* (Heckel 1848) were kept at the CARUS Aquatic Research Facilities of Wageningen University and Research, once the females had reached sexual maturity (approximately 4–6 months after birth), 24 individuals were randomly separated into two groups. The females from one of the groups were assigned one adult male to ensure a continuous availability of sperm. Because 2 of the 4 females used in the Crossmon's Trichrome and hematoxylin-eosin staining were used in swimming performance tests which could affect the production of babies in subsequent pregnancies, we documented only the brood size of the pregnancies prior to any experiment, as the live births in each tank. The females from the other group, hereafter referred to as 'virgins', were isolated in individual tanks. Each set of fish was kept in a 10L tank that received water from a common filtering system to assure equal water quality for all tanks. Water temperature was kept at 24–25°C and photoperiod followed a 12 h light: 12 h dark-cycle. They were fed twice daily with *A. nauplii* in the morning (~8 am) and liver paste or flake paste (both feeds were alternated daily) in the afternoon (~4 pm).

### 2.2. Sample collection

We sampled at about two days before the delivery, a moment when the abdominal distention was close to the maximum. When we euthanized a pregnant female, we did

the same with a virgin one as a control, both with a lethal dose of MS-222 (Tricaine-S; Western Chemical Inc., Ferndale, WA, United States). After excess liquid was carefully removed with filter paper, the body was weighed (Mettler PE360 balance; scale accuracy 0.001 g; Mettler-Toledo B.V., The Netherlands). For details on the tissue preparation see S1 Text.

**2.3. Muscle staining to distinguish fibre types.**

For a detailed description of the muscle staining methods see S2 Text. Briefly, we applied Crossmon's Trichrome and hematoxylin-eosin staining on four pregnant and four virgin females to visualize the muscle tissue on the cross-sections taken from the region of maximum distention in the abdomen. Per female, multiple sections were cut within a slice of about 0.5 mm and we chose for analysis the three sections less affected by the mounting process. To visualize the location of the slow, intermediate and fast muscle fibres, we performed immunohistochemistry, and staining for Succinate Dehydrogenase (SDH) in a different set of fish, that consisted of three pregnant and three virgin females. For immunohistochemistry observations, we used monoclonal antibodies for the myosin heavy chain isoforms that were specific for slow and for fast muscles separately and we deduced the location of the intermediate fibres from the absence of staining. We used the SDH staining to make the distinction between the oxidative and non-oxidative muscle fibres. See S2 Text, for details on histological procedures.



**2.4. Muscle characterization**

For each female used for the Crossmon's Trichrome and hematoxylin-eosin staining (four pregnant and four virgin females), we took photographs from three perpendicular views (lateral, ventral, and frontal), following the method used by [35 (**Chapter 2**),36]. And measured standard length, height and width of the sampled region using Fiji [37].

For the Crossmon's trichrome stained sections (and two haematoxylin sections when the third Crossmon's trichrome stained section of the region was not appropriate for measurements), we chose the lateral half of the abdominal section (figure 1*a*) that was least affected by the mounting process to characterize the muscles. In cross-section, the epaxial muscles are located dorsally, separated by the horizontal septum from the ventrally located hypaxial muscles (figure 1*b*). We also observed the lateral superficialis muscles at the lateral edges of the horizontal septum. We measured the following variables using Fiji [37]: cross-sectional area, axial muscle area, epaxial muscle area, hypaxial muscle area (figure 1), and lateralis superficialis area. We calculated the ratio between axial muscles area and the cross-sectional area of the body as ratio = $\frac{\text{muscle area}}{\text{cross-sectional area}}$. The number of muscle fibres per region (epaxial, hypaxial and lateral wedge) were counted and 50 fibres from each region were randomly chosen and their individual areas calculated. Following [38], we calculated the equivalent area diameter. Assuming a circular shape, the equivalent diameter of each fibre was calculated as $2(\text{area}/\pi)^{1/2}$ [39]. To determine the occurrence of

hyperplasia and/or hypertrophy, we grouped the 50 randomly chosen muscle fibres, based on their diameters, into five classes: ≤21 µm, >21 µm & ≤41 µm, >41 µm & ≤61 µm, >61 µm & ≤81 µm, and >81 µm. [40]. We analysed the proportion of fibres corresponding to each class.

### 2.5. Quantification of transcription levels of muscle-related genes.

To evaluate the transcription levels of muscle-related genes, we sampled the caudal region (figure 1*a*) of the females used for the immunohistochemistry method and the SDH staining (three pregnant and three virgin females) plus another set of 5 pregnant and 5 virgin females, making it a total of eight pregnant and eight virgin females. We chose the genes of interest based on what is known for changes in musculature development in swim-trained zebrafish, *Danio rerio* [34]. To evaluate the production of proteins contributing to sarcomere construction and growth, we selected genes coding for proteins such as myosin (*myo*) and troponin t1 (*tnnt1*) which indicate formation of new muscle tissue. For the genes involved in muscle growth inhibition or stimulation, we selected the ones coding for the growth promotor myogenin (*myog*) and growth inhibitor myostatin (*mstn*); and for the genes involved in the energy supply of muscles and the mitochondrial metabolism, we chose the ones coding for phosphofructokinase-m (PFKM), ubiquinin (NADHd) and succinate dehydrogenase (SDH). Oxygen supply is strongly correlated to energy supply, as mitochondrial metabolism requires oxygen for the last step of the cellular respiration: the electron transport chain. This is the aerobic part of the process and produces most of the ATP. Myoglobin (*mbin*) is included as the gene of interest for examining the effects of pregnancy on this process. *Mbin* was included as an indicator of the oxygen supply as mitochondrial metabolism requires oxygen for the last step of the cellular respiration: the electron transport chain. See S3 Text, for details on the RT-qPCR analyses.

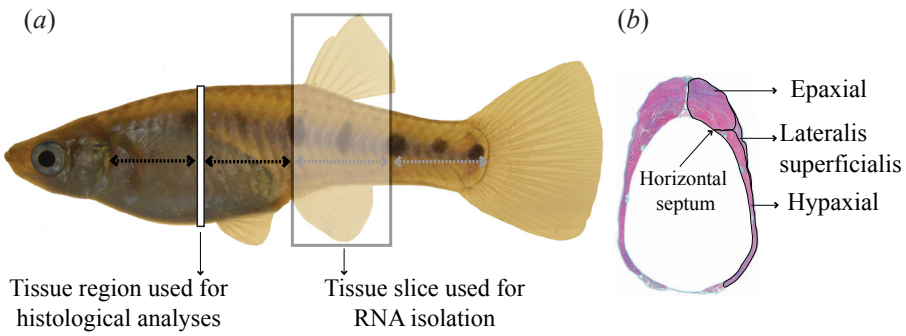



**Figure 1.** Sections used for gene expression and histological analyses. (*a*) The RNA was isolated from the post-anal region, whereas the histological sections were taken from the abdominal region from about half way between the pectoral fins and the anal pore. (*b*) Location of the epaxial, hypaxial and lateralis superficialis muscles and of the horizontal septum.

As the aim is to quantify transcription of muscle related genes, reference quantification of unaltered gene transcription is required for standardization. For this study, bèta-actin (*ActB*), ribosomal protein L7 (RPL7) and tubulin alpha (*tuba*) were selected as reference genes (see table S1 for description of the genes).

*Statistical analyses* – To compare the expression levels of the genes between virgin and pregnant specimens, Cycle threshold (Ct) values and pooled efficiencies from the output files of the qPCR runs were imported into the program Relative Expression Software Tool – REST V2.0.13 [41]. REST automatically produces output on Ct values input, comparing the relative expression levels between treatments (virgin vs pregnant) per gene to identify possible up or down regulations due to pregnancy.

## 3. Results and discussion



*Body measurements* – Most of the pregnant females grew slower, from the moment when they started reproducing until the sampling day (approximately 3–7 months), than the virgin ones (both in SL and body mass; figure 2*a*) under the same conditions. However, dividing mass by $SL^3$ (figure 2*b*), yielded similar values for pregnant and virgin females, except for one pregnant fish (light blue circle) which had a relatively high body mass over $SL^3$ ratio than the other fish, which could be explained by the bigger broods (i.e. more offspring per brood) that she produced (figure 2*c,d*). The pattern observed in figure 2*c,d*, where bigger females produced on average bigger broods is common in nature [42]. Because we did not know the size of the broods present inside the females at the moment of the experiment, we could not differentiate between the body mass and volume that corresponded to the females' reproductive allocation and to the females' somatic tissues. The reduced growth of pregnant females might be caused by the required energetic investment in their developing embryos. Furthermore, during pregnancy the growing ovary narrows the space occupied by the digestive system, which may constrain the food intake of females, resulting in an indirect cost by limiting the female's energy intake [43]. Females of *P. gracilis*, store all of the nutrients for embryo development in the yolk prior to each cycle of fertilization [35 (**Chapter 2**),44], therefore pregnant females had to invest energy in new yolked eggs. Whereas the virgin females kept the same set of eggs and did not have to invest in reproduction, having more energy available to invest in somatic growth. There was no effect of female length on maximum height and width (figure 2*e–h*), and here again, the longest pregnant female is an outlier.

Since the number and size of muscle fibres vary with body size [45–47], the larger body size of the virgins than that of the pregnant fish might potentially conceal the effect that pregnancy could have on the number and size of the muscle fibres. In the perspectives for future studies, (see the final section of this chapter) we provide suggestions on how to

avoid the potentially confounding effect of female length on muscle composition and fibre density.

*Location of slow and intermediate fibres* – To compare the muscle composition and fibre density between pregnant and virgin females, we started by mapping the different muscle regions using different histological staining methods such as Crossmon's trichrome, haematoxylin eosin, immuno-histochemistry and staining for SDH (figure 3).

For both pregnant and virgin females, the lateralis superficialis muscle (figure 3*d,e,m,n,* r.1.) presented slow fibres (stained dark brown with the S58 antibody) and intermediate fibres (beige staining, situated between the slow and the fast muscle fibres; they did not stain with the S58 nor the Zm4 antibodies). The lateralis superficialis stained dark with the SDH method confirming that the muscle fibres have a high oxidative activity (figure 3*g,h,p,q*, r.1). Besides the expected slow and intermediate fibres found in the lateralis superficialis, there was one additional region with slow and intermediate muscle fibres that was situated between the lateralis superficialis and the ventral limit of the body (figure 3*d,f,p,r*, r.2). It was present in both pregnant and virgin fish and it consisted of a single or double layer of slow fibres followed by a single or double layer of intermediate fibres. This second region might be an extension of the lateralis superficialis, since this one has shifted up, possibly because of the extended abdomen. A similar region of slow fibres is found in Antarctic notothenioid fish [39]. The presence of the fibres was suggested to correlate to a relatively high fraction of sub-carangiform swimming during cruising. So, in the case of *P. gracilis*, we suggest that this region may help to counteract the high bending stiffness caused by the reproductive tissue, facilitating body undulations especially during sustained swimming.

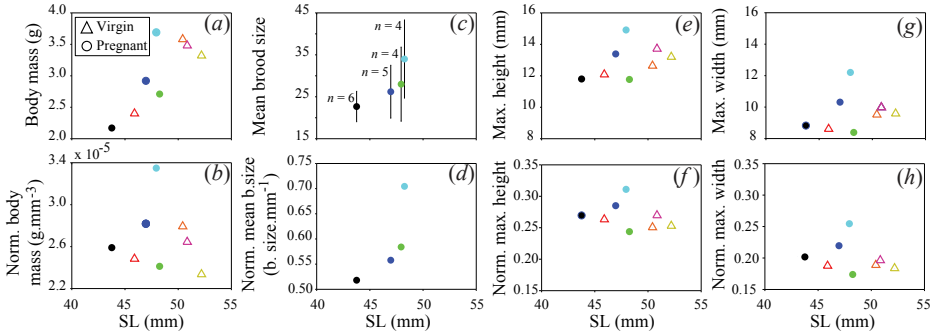



**Figure 2.** Body measurements of pregnant (filled circles; $N_{fish}$ = 4) and virgin (open triangles; $N_{fish}$ = 4) females. Each color represents one individual fish. (*a*) Body mass. (*b*) Body mass normalized by $SL^3$. (*c*) Mean brood size (i.e. number of embryos per brood). Error bars represent the standard error of the mean. *n* represents the number of broods on which each of the error bars are based. (*d*) Mean brood size normalized by SL. (*e*) Maximum height of the whole cross-section sampled. (*f*) Maximum height normalized by SL. (*g*) Maximum width of the whole cross-section sampled. (*h*) Maximum width normalized by SL.

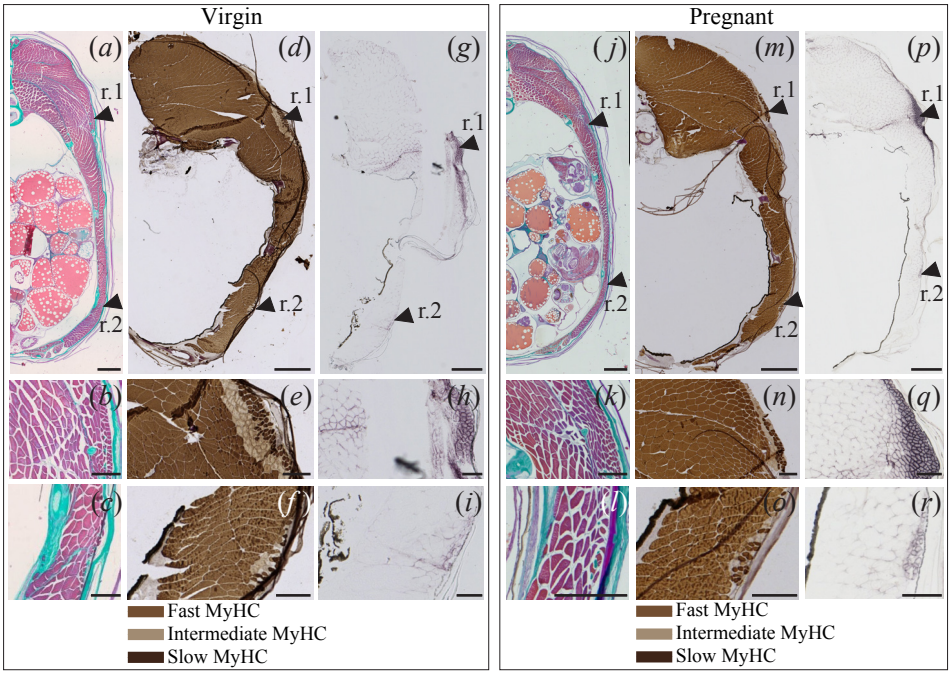



**Figure 3.** Example of hematoxylin-eosin staining, slow and fast myosin heavy chain staining, and SDH activity in abdomen, taken at corresponding locations along the body. (*a–i*) Virgin. (*j–r*) Pregnant. (*a–c, j–l*) Hematoxylin-eosin staining. (*a, j)* Overview, *(b, k*) magnification of region 1 of slow and intermediate fibres, within the lateralis superficialis, (*c, l*) magnification of region 2 of slow and intermediate fibres within the hypaxial muscle. (*d–f, m–o*) Stained for *slow MyHC* (dark brown) and fast MyHC (brown). Intermediate fibres in light brown. (*d, m*) Overview, (*e, n*) region (*1, f*) region 2. (*g–i, p–r*) SDH activity. (*g, p*) Overview, (*h, q)* region 1, (*i, r)* region 2. Scale bars: 1 mm (*a,d,g,j,m,p*), 0.25 mm (*b,c,e,f,h,i,k,l,n,o,q,r*).

*Cross-sectional body area and muscle areas* – To compare the muscle areas between pregnant and virgin females, we first evaluated the relationship between the cross-sectional area of the body and the SL of the fish and found that the cross-sectional area of the body increased with SL for all fish (figure 4*a*). After normalizing by $SL^2$, the cross-sectional area of the pregnant females seemed to be bigger possibly due to relatively larger width and height (figure 4*b*). Moreover, the total muscle area also increased with SL (figure 4*c*) and seemed to be lower for pregnant fish when normalized (figure 4*d*). This suggests that pregnant females have comparatively bigger abdomens than the virgin ones, independently of their body size, and their muscle area is comparatively smaller. This is clearer represented by the ratio between muscle and body area in figure 4*e*, where pregnant females show a relatively lower proportion of muscle per body area. However, our small sample size did not allow for more definite conclusions. This suggests that the abdomen of pregnant

females might have a higher flexural stiffness which would make it harder to bend [1,3], due to the comparatively bigger abdomen. In addition, they would have a lower proportion of muscles in that region which might affect their ability to vary body stiffness by producing active forces during stretching (implying negative mechanical work) [48]. Moreover, pregnant females would be propelling a comparatively bigger body (i.e. higher mass and drag) with a lower quantity of muscle. This might decrease the available power to propel the bigger body volume [1], unless there would be muscle compensation, such as for example muscle fibres with higher power output or a bigger volume of axial muscle in other regions along the body.

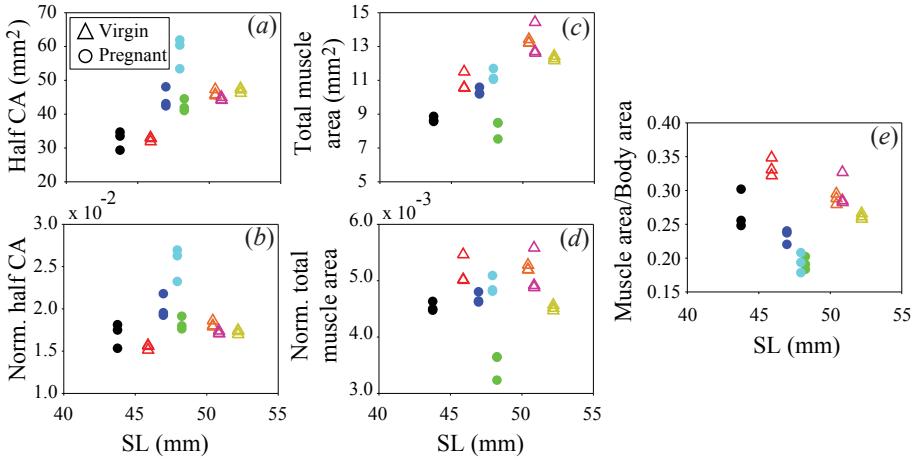



**Figure 4.** Muscle areas of pregnant (filled circles; $N_{fish}$ = 4) and virgin (open triangles; $N_{fish}$ = 4) females (there are three sections per fish). (*a*) Cross-sectional areas (CA), taken from the left or right half of the section. (*b*) Cross-sectional areas normalized by $SL^2$. (*c*) Total muscle area. (*d*) Total muscle area normalized by $SL^2$. (*e*) Ratio between total muscle area and cross-sectional area. The areas were measured on one of the two halves (right or left). Each color represents one invididual fish, and three samples were taken per fish.

The areas of the epaxial, hypaxial and lateralis superficialis muscles, seem to increase with increasing SL (figure 5*a,c,e*) and pregnant females seemed to have smaller epaxial and hypaxial muscle areas. This suggests that these two bigger muscles account for the possible differences seen in the total muscle area shown in figure 4*c,d*. However, when normalized by $SL^2$, no clear differences were observed between pregnant and virgin females (figure 5*b,d,f*), but we do not rule out potential differences that may be concealed by fish size.

*Mean fibre area* – The mean fibre area per muscle seemed to increase with increasing SL (figure 6), especially in the epaxial and hypaxial muscles (figure 6*a–d*). This might indicate that fish were growing by hypertrophy of the muscle fibres.

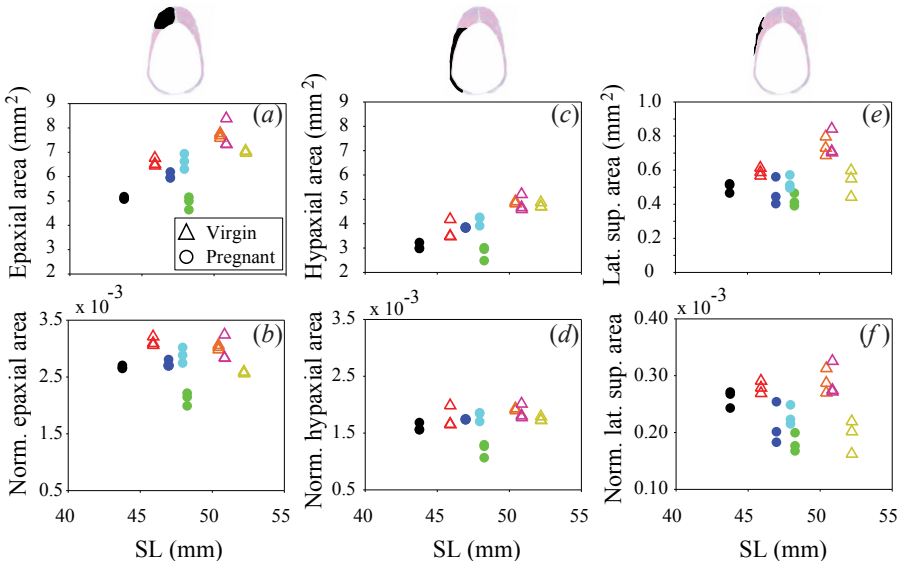



**Figure 5.** Areas per muscle of pregnant (filled circles; $N_{fish}$ = 4) and virgin (open triangles; $N_{fish}$ = 4) females. (*a*) Areas of the epaxial muscle. (*b*) Area of the epaxial muscle normalized by SL². (*c*) Area of the hypaxial muscle. (*d*) Area of the hypaxial muscle normalized by SL². (*e*) Area of the lateralis superficialis. (*f*) Area of the lateralis superficialis normalized by SL². The areas were measured on one of the two halves (right or left). There are three samples per fish. Each color represents one invididual fish, and three samples were taken per fish.

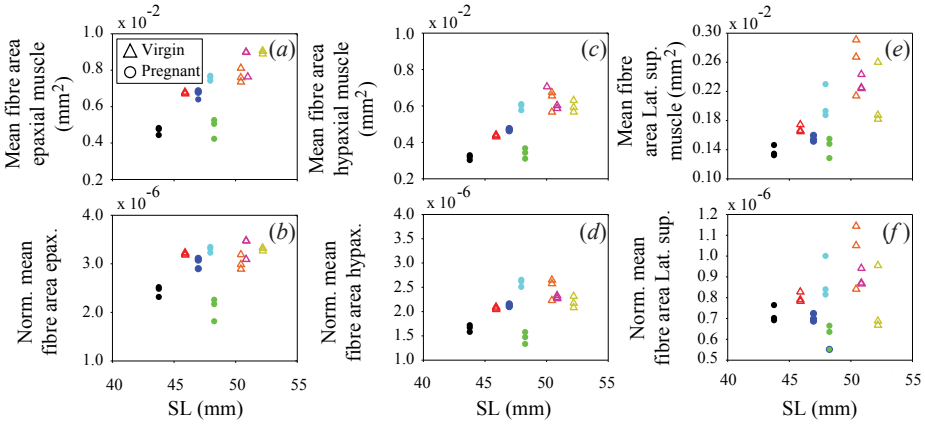



**Figure 6.** Mean fibre area per muscle, in pregnant (filled circles; $N_{fish}$ = 4) and virgin (open triangles; $N_{fish}$ = 4) females. (*a*) Mean fibre area of the epaxial *m*uscle. (*b*) Mean fibre area of the epaxial muscle normalize*d* by SL². (*c*) Mean fibre area of the hypaxia*l* muscle. (*d*) Mean fibre area of the hypaxial muscle normalized by SL². (*e*) Mean fibre area of the lateralis superficialis muscle. (*f*) Mean fibre area of the lateralis superficialis normalized by SL². Each mean was calculated from 50 random fibres per muscle, on one of the two halves (right or left). Each color represents one individual fish and three samples were taken per fish.

*Fibre number* – The total number of fibres in each of the muscles and in total, apparently decreases with increasing SL (figure 7). However, the pattern is not clear, and we could only adventure an explanation such as that shorter fish tended to grow by hyperplasia and the longer ones by hypertrophy, which is the case at least for the rainbow trout [49]. Furthermore, there seems to be no effect of pregnancy on the number of fibres, but the influence of body size might be obscuring the effect of pregnancy on fibre number. Hence, given our limited sample size, and the effect of fish length on fibre number, we are unable to draw any conclusions.

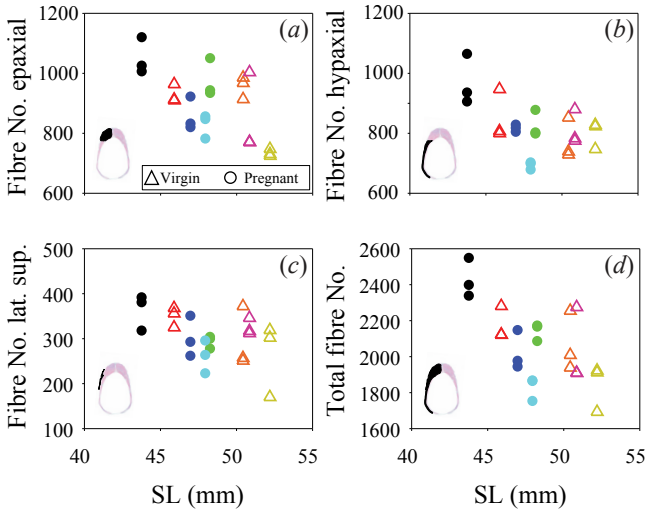



**Figure 7.** Fibre distribution in pregnant (filled circles) and virgin (open triangles) females. We analysed one half (right or left) of the cross-sectional area. Number of fibres in (*a*) epaxial, (*b*) hypaxial, (*c*) lateralis superficialis muscle, and (*d*) total number of fibres. Each color represents one individual fish and three samples were taken per fish.

*Fibre morphology* – The variety of fibre sizes observed in cross-section (figure 8) in both pregnant and virgin females reflected the muscle growth that continued past sexual maturation [50], which includes myotube formation followed by hypertrophy of the produced fibres [17,51,52]. This was especially apparent for the epaxial and hypaxial muscle where the number of thicker fibres increased with increasing SL, independently of the reproductive status of the individual (figure 8*a,b,e,f*). In contrast, the lateralis superficialis did not show a clear trend (figure 8*c,d*). The more abundant fibres had relatively small sizes. This corresponds to the predominance of slow and intermediate fibres in this muscle, which have smaller diameters than the fast ones. Overall, we propose that the bigger muscle area of the virgin females is due to bigger areas per muscle fibre and a higher proportion of thicker fibres.

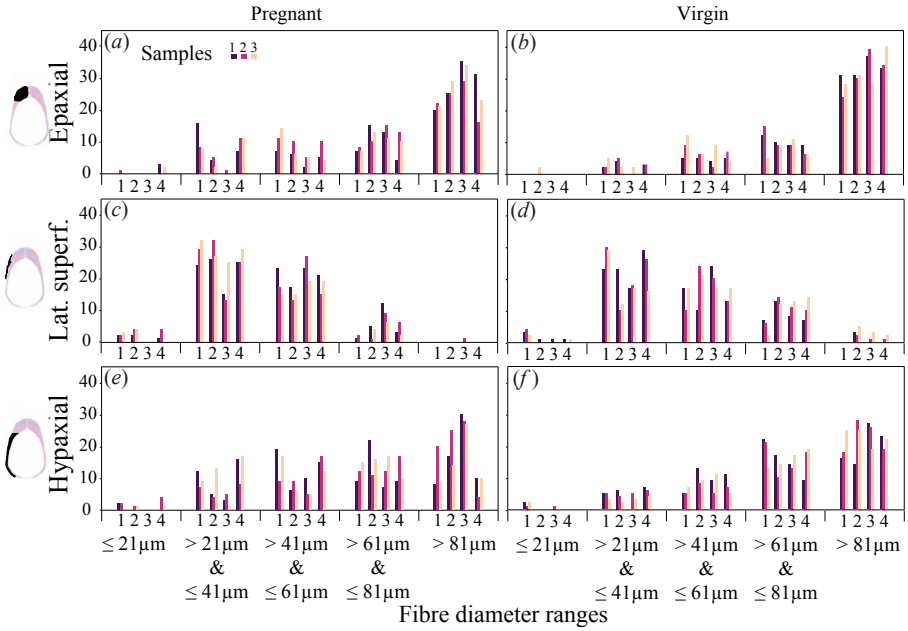



**Figure 8.** Distribution of fibres in five diameter ranges in 4 virgin and 4 pregnant females. Distribution of muscle fibre diameters in (*a, b*) epaxial muscle, (*c, d*) in lateralis superficialis muscle, and (*e, f*) in hypaxial muscle. (a, *c, e*) *c*orrespond to data from pregnant females and (*b*, *d, f*) *to* data from virgin females. The numbers on the x axes correspond to the female number, either pregnant or virgin. Fish 1 to 4 are sorted by increasing SL.

*Gene expression* – The results of the RT-qPCR analyses showed no biologically significant up- or downregulation in transcription levels of the muscle-related genes due to pregnancy (figure 9, figure S1 and table S3). If these results reflect the effect of pregnancy on the muscles, we could say that there is no effect of pregnancy on the production of sarcomeric proteins such as myosin and troponin t1, which is consistent with the absence of differences in the activity of the myogenin and myostatin, which regulate muscle growth. Neither the energy supply is altered, since Phosphofructokinase-m, ubiquinin and succinate dehydrogenase do not change activity. In addition, the oxidative capacity is also unchanged, as shown by the lack of change in myoglobin expression.

Nevertheless, these gene-expression results matched the outcomes of the histological analyses where no differences were apparent between the muscle distribution and composition of pregnant and virgin females. This suggests that: (i) females do not compensate for the effects of pregnancy on drag and performance by modifying their muscles, (ii) that virgin females, due to their high reproductive investment in yolked eggs and correspondingly alteration in body shape have undergone very similar changes in

their muscles to those of pregnant females or (iii) that the selected genes are not affected by pregnancy. However, due to the small sample sizes used, we had too limited power to conclude on any distinction in the expression level of the muscle-related genes between pregnant and virgin females.

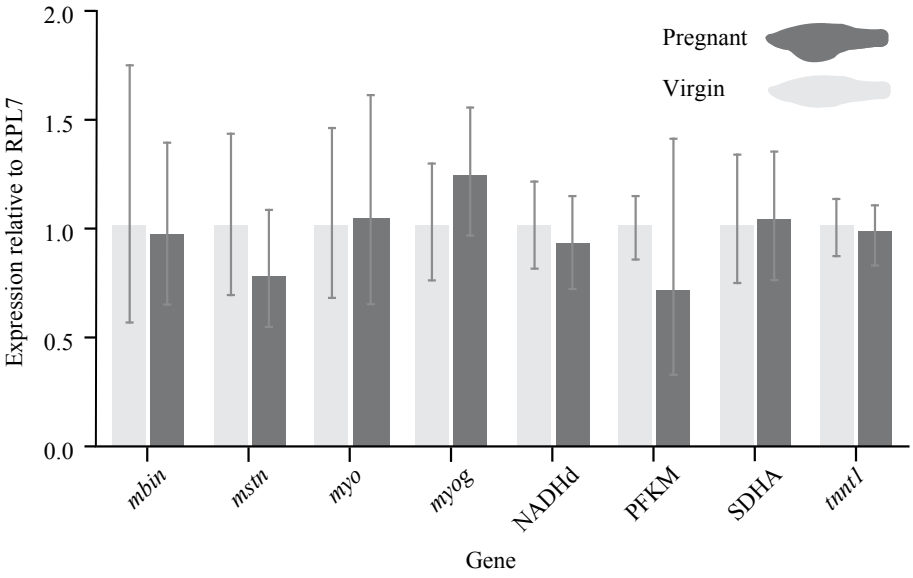



**Figure 9.** Graphical representation of the expression levels and 95%-confidence intervals per gene, normalised to the reference gene (RPL7) and control treatment per gene (virgins, set to 1.0 by default).

*Influence of reproductive traits on muscle morphology and distribution* – Musculature might adapt to the effects of pregnancy, but we could not make definite conclusions in this pilot study because our small sample size did not provide enough statistical power in combination with large variation between and within individuals. Furthermore, the difference in body size between the sampled pregnant and virgin females might have been a confounding factor because muscle characteristics vary with body size, which is hindering a clear view on the possible differences linked to the reproductive stages.

To what extent could the reproductive characteristics of *P. gracilis* play a role in lessening the effects of pregnancy on muscles? This species has a short reproductive cycle, giving birth on average every 18 days, and it is constantly pregnant. Given this short interbrood interval and continuous offspring production, it might not be energy efficient for females to modify their muscle architecture to fluctuations in their reproductive allocation. Moreover, this species carries simultaneously several litters that differ in the stage of the embryonic development [53–57], a reproductive strategy known as

superfetation. Superfetation lowers the increase in volume during pregnancy, allowing the female to keep a slim and elongated body [58,59]. And lastly, in this species most of the nutrients for the embryo development are already stored in the yolk [35 (**Chapter 2**)]. We observed that our lab-reared virgin females carried fully yolked (unfertilized) eggs (Quicazan-Rubio *et al*., personal observations), paradoxically suggesting that these virgin females may also carry a reproductive burden with them even though they never mated with a male.

Knowledge on the effect of pregnancy on muscle composition and gene expression was based on the short-horn sculpin and rats, which differ in reproductive characteristics such as the frequency at which they get pregnant, and the number of litters that they carry at a time. The short horn sculpin reproduces once a year and the rat has 5–6 litters per year. Both carry one litter at a time. By contrast, *P. gracilis* reproduces all year long and carries about two litters at a time, which requires its body to continuously change in response to pregnancy. Hence, the changes in the muscle activity and morphology throughout its pregnancy might be smaller than for the short-horn sculpin and rat. Thus, it is likely that the effect of pregnancy on muscles described in species that reproduce seasonally, have longer pregnancies, and carry only one brood at a time [20–23,26,27,60] does not apply to *P. gracilis*.



*Perspectives for future studies* – This pilot study was performed on a relatively small number of specimens per treatment. Due to time constraints and the labour-intensive nature of the histological and gene expression analyses we had to limit the number of specimens that we could analyse. However, the impression from our data is that the differences for *P. gracilis*, if existing at all, would be rather small, which means that it would take a considerably higher number of individuals to show a significant effect of pregnancy on muscle composition.

Nonetheless, the existence of pregnancy-induced muscular alterations in live-bearing fish cannot be ruled out based on our pilot study performed on a single Poeciliid species. It is possible that the muscle characteristics of *P. gracilis* during pregnancy differ at least to some extent from other live-bearing species. We therefore recommend that future studies focus on species that exhibit bigger pregnancy-induced changes in body shape during the reproductive cycle, and whose virgin females have a reproductive allocation markedly lower than the ones of *P. gracilis*. Suitable examples are Poeciliid species with high levels of placentation and low levels of superfetation. The pregnant females of such species exhibit larger changes in abdominal shape during pregnancy, and its virgin females have not suffered the abdominal volume increase due to pregnancy yet and have only had to mobilize a low amount of nutrients towards the egg [35 (**Chapter 2**)].

Together with the above recommendation, we suggest increasing the sample size in follow-up studies to up to one order of magnitude more, to reach the statistical power that will assure a reliable comparison. Furthermore, one should increase the effort in

comparing individuals of similar standard length. In this study, we controlled for age of the female, but due to different growth rates between pregnant and virgin individuals, body size varied. This could be an important contributor to the measured large variation.

The current histological methods, applied on the bigger sample size suggested above, would allow quantitative analyses of the muscle fibre types. We also suggest including the staining for ATPase since it has been shown that under the influence of functional demands, changes ocurr not only in the metabolic properties of muscle fibres, but also in their ATPase activity [61]. The quantitative analyses on slow, intermediate and fast muscle fibres would allow us to determine whether the changes seen in mammals, such as rats, guinea pigs, and rabbits during pregnancy happen also in *P. gracilis*. In rats, guinea pigs, and rabbits, which have pregnancy durations of 21–23, 59–72, and 27–42 days, respectively, the diameter of the slow muscle fibres increased whereas the one of the fast fibres decreased due to pregnancy [20–22]. This change might be due to the alteration in the mechanisms implicated in the maintenance of muscle mass. For example, the increase in oestrogen production that happens during pregnancy might favour the increase of the slow muscle fibre area because these fibres have a higher expression of oestrogen receptors compared to the fast ones [23,24]. The increase in the slow muscle area could imply that pregnant females would increase their endurance [62] and have better sustained locomotion. On the other hand, the decrease in the diameter of the fast fibres would imply lower maximum power output from these fibres (and hence lower fast-start escape performance), matching what has been seen for gravid fish [1].

Another aspect to be studied is the stretch-induced changes of the muscle fibres around the abdomen due to reproduction, as it is known for humans [26,27,60], rats [23] and have been proposed to happen also in fish [1]. We suggest evaluating the presence of these changes in pregnant fish by performing longitudinal sampling, in addition to the cross-sectional one, at different positions along the abdomen. Longitudinal samples allow visualizing the effects of stretching on the arrangement of the Z discs and the thickness and number of the sarcomeres. In the case of pregnant rats, Z discs around the abdomen are disorganized and the sarcomeres get thinner as a result of pregnancy [23]. It also increases the number of sarcomeres in series at the ends of muscle fibres [63] by means of increased synthesis of protein synthesis and addition of myotubes [64]. Although muscle output decreases during gravidity [1,27], there are no studies on the effect of muscle stretch on gravid individuals and it is suggested that the stretch due to gravidity might not be big enough to significantly reduce the ability to produce tension within the muscle, at least in humans [26].

On the other hand, other factors such as an altered line of action of the muscle might have a more negative impact on the muscle performance and its changes have been demonstrated to also happen parallel in time with the reduced abdominal muscle function capabilities in humans [26]. Therefore, we suggest measuring the angle of insertion, in

the sagittal plane, of the muscle during pregnancy. The alteration of such angle may alter the line of action of the muscle, and its capacity to produce torque [28]. In humans, the line of action of the rectus abdominal muscle during pregnancy was altered laterally and anteriorly, and therefore its torque production might be significantly reduced [26].

We studied the expression of muscle-related genes using material from the post-anal region of the fish (presumably unaffected by the abdominal distention) and obtained the histological material from the abdomen (at the position of maximum distension). To understand how the musculature changes throughout the body during pregnancy, we suggest applying the histological and molecular methods to the two regions already studied and to include other regions along the body. If females could change their post-anal musculature to compensate for a reduced locomotor performance during pregnancy, we would find a thickening of the muscle fibres which would increase their power output and allow them to propel their bigger (i.e. higher mass and larger frontal surface area) body forward. Alternatively, because reproduction and locomotion compete for resources, the size of the muscle fibres could be decreased due to a lower resource availability. Besides extending the histological and gene-expression analysis to the caudal peduncle and abdominal region respectively, another good candidate extra region would be the anterior portion of the abdomen which experiences considerable stretching during pregnancy [1].



Since the contractile properties of the muscles vary with the reproductive cycle, as has been reported for the oviparous short-horn sculpin [1], and the morphology of some of the muscle fibres vary throughout the pregnancy for rats [22], we suggest evaluating females at one more stage during pregnancy. We contemplate the possibility that we might have missed the up or down regulation of transcription levels of muscle-related genes due to timing differences, as all the samples of the pregnant specimens in our study were collected at the end of the gestation period. If the possible up regulation is only present for a short time at the onset of pregnancy to induce muscle development, followed by a slightly elevated level for maintenance, the differences in expression levels may be missed. Therefore, we suggest sampling at the onset and at the end of pregnancy, which would imply doubling the number of individuals, so that half would be tested at the beginning and the other half at the end of the cycle.

Finally, since pregnancy might stretch the muscles, and also make them work harder to propel the pregnant body, we suggest analysing genes involved in both phenomena. There are two candidate genes to account for muscle stretching, one of them codes for the slow myosin heavy chains (*slow MyHC2*), and this gene is expected to be activated when stretching happens. The second gene codes for the fast glycolytic (type IIx) myosin fibres, which have been found to be supressed when the rat's muscles are stretched [65]. Because the genes chosen to evaluate changes in the energy supply and oxidation facilitation of the muscles do not cover all the possible activities that would change due to

pregnancy, we suggest studying some of other the genes that have been found to change expression in fish subjected to swim training: genes involved in the regulation of muscle mass (IGF-1/PI3K/mTOR, *bmp, mstn*), in myogenesis and satellite cell activation (*pax3, fgf, notch, wnt, mef2, hh, ephrinb2*) and in angiogenesis (*vegf*VEGF, *hif, notch, ephrinb2, klf2*) [33].

In conclusion, besides the expected slow and intermediate fibres found in the lateralis superficialis, there was one additional region with slow and intermediate muscle fibres that was situated between the lateralis superficialis and the ventral limit of the body. The region was observed in both pregnant and virgin individuals. To our knowledge, this is the first time that this is reported for the Poeciliidae, and its specific function is yet to be evaluated. Our results suggest that pregnancy in a superfetatious lecithothrophic species, such as *Poeciliopsis gracilis*, does not appear to have significant effects on the muscular composition of females. This result is supported both by histological analyses and by assays of expression of muscle-related genes. To explore further the effects of pregnancy on muscle composition, we recommend studying species with high levels of placentation and low levels of superfetation, using only individuals of similar age and standard length. We also suggest increasing the sample size to up to one order of magnitude, and to sample at the beginning of pregnancy too. To visualize the effects of stretching, we recommend performing longitudinal histological samples and to measure the angle of insertion of the muscles. We also suggest extending the histological analyses to the caudal region of the body and the molecular analyses to the abdominal one, and possibly include the anterior region of the abdomen in both analyses too. Besides the current methods, we recommend to stain for ATPase, and to include the analysis of other genes, such as the ones coding for *slow MyHC2,* and other exercise related genes involved in the regulation of muscle mass (IGF-1/PI3K/mTOR, *bmp, mstn*), in myogenesis and satellite cell activation (*pax3, fgf, notch, wnt, mef2, hh, ephrinb2*) and in angiogenesis (*vegf*VEGF, *hif, notch, ephrinb2, klf2*).

**Ethics.** The experiments were approved by the Wageningen University Animal Experiments Committee. Permit number 2013103.

## Author contributions

E.M.Q.R, H.S., J.V., K.L., M.F., J.L.V.L., and B.J.A.P. conceptualized the experiment, and designed the experimental methodology. H.S., M.G., and J.V. performed the experiments. E.M.Q.R. and J.V. analysed the data. E.M.Q.R. and J.V. wrote the first draft of the manuscript with input from H.S., K.L., J.L.V.L., and B.J.A.P. All authors provided feedback on the manuscript.

## Acknowledgements

We thank the following people for their contributions. David Reznick provided stocks of *Poeciliopsis gracilis*. Truus Wiegers-Van der Wal, Sander Visser, Wian Nusselder, Emily

Roux, Jasper Lengkeek, and Menno ter Veld took care the fish. Miranda Goudsmith, and Kaj Lelieveldt contributed to the laboratory work. Silke Cleuren, Niek van Duijvenvoorde, Marcel Jaklofsky, Marloes E. Busscher, and Jurgen Meijer, contributed to the protocols we used in the sample collection. Useful feedback was given by Maria Forlenza and Ellen Kranenbarg-Stolte on the reference gene selection and RT-qPCR analyses, by Fida Buijse on the statistical analysis, and by Kas Koenraads on a draft of the gene-expression section.

## References

1. James RS, Johnston IA. 1998 Influence of spawning on swimming performance and muscle contractile properties in the short-horn sculpin. *J. Fish Biol.* **53**, 485–501. (doi:10.1006/jfbi.1998.0722)
2. Plaut I. 2002 Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
3. Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* **164**, 38–50. (doi:10.1086/421412)
4. Seigel RA, Huggins MM, Ford NB. 1987 Reduction in locomotor ability as a cost of reproduction in gravid snakes. *Oecologia* **73**, 481–485. (doi:10.1007/BF00379404)
5. Brodie III ED. 1989 Behavioral modification as a means of reducing the cost of reproduction. *Am. Nat.* **134**, 225. (doi:10.1086/284977)
6. Bauwens D, Thoen C. 1981 Escape tactics and vulnerability to predation associated with reproduction in the lizard *Lacerta vivipara*. *J. Anim. Ecol.* **50**, 733–743. (doi:10.2307/4133)
7. Sinervo B, Hedges R, Adolph SC. 1991 Decreased sprint speed as a cost of reproduction in the lizard *Sceloporus occidentalis*: variation among populations. *J. Exp. Biol.* **336**, 323–336.
8. Veasey JS, Houston DC, Metcalfe NB. 2001 A hidden cost of reproduction: the trade-off between clutch size and escape take-off speed in female zebra finches. *J. Anim. Ecol.* **70**, 20–24. (doi:10.1111/j.1365-2656.2001.00476.x)
9. Lee SJ, Witter MS, Cuthill IC, Goldsmith AR. 1996 Reduction in escape performance as a cost of reproduction in gravid starlings, *Sturnus vulgaris*. *Proc. R. Soc. London, B Biol. Sci.* **263**, 619–623. (doi:10.1098/rspb.1996.0093)
10. Wu W, Meijer OG, Lamoth CJC, Uegaki K, van Dieën JH, Wuisman PIJM, de Vries JIP, Beek PJ. 2004 Gait coordination in pregnancy: transverse pelvic and thoracic rotations and their relative phase. *Clin. Biomech.* **19**, 480–488. (doi:10.1016/j.clinbiomech.2004.02.003)
11. Britton RH, Moser ME. 1982 Size specific predation by herons and its effect on the sex-ratio of natural populations of the mosquito fish *Gambusia affinis* baird and girard. *Oecologia* **53**, 146–151. (doi:10.1007/BF00545657)
12. Trexler JC, Tempe RC, Travis J. 1994 Size-selective predation of sailfin mollies by 2 species of heron. *Oikos* **69**, 250. (doi:10.2307/3546145)
13. Miles DB, Sinervo B, Anthony Frankino W. 2000 Reproductive burden, locomotor performance, and the cost of reproduction in free ranging lizards. *Evolution (N. Y).* **54**, 1386–1395. (doi:10.1554/0014-3820(2000)054[1386:rblpat]2.0.co;2)

14. Brokordt KB, Himmelman JH, Guderley HE. 2000 Effect of reproduction on escape responses and muscle metabolic capacities in the scallop *Chlamys islandica* Muller 1776. *J. Exp. Mar. Bio. Ecol.* **251**, 205–225. (doi:10.1016/S0022-0981(00)00215-X)
15. Lourdais O, Brischoux F, DeNardo DF, Shine R. 2004 Protein catabolism in pregnant snakes (*Epicrates cenchria maurus* Boidae) compromises musculature and performance after reproduction. *J. Comp. Physiol. B* **174**, 383–391. (doi:10.1007/s00360-004-0424-6)
16. Selman RG, Houston DC. 1996 A technique for measuring lean pectoral muscle mass in live small birds. *Ibis (Lond. 1859).* **138**, 348–350. (doi:10.1111/j.1474-919X.1996.tb04353.x)
17. Johnston IA, Bower NI, Macqueen DJ. 2011 Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol.* **214**, 1617–1628. (doi:10.1242/jeb.038620)
18. Love RM. 1980 *The chemical biology of fishes*. London: Academic Press.
19. Chapman RF. 2013 *The insects: structure and function*. New York: Cambridge University Press.
20. Lalatta-Costerbosa G, Barazzoni AM, Lucchi ML, Bortolami R. 1987 Histochemical types and sizes of fibers in the rectus abdominis muscle of guinea pig: Adaptive response to pregnancy. *Anat. Rec.* **217**, 23–29. (doi:10.1002/ar.1092170105)
21. Lalatta-Costerbosa G, Barazzoni AM, Lucchi ML, Bortolami R. 1988 Fiber sizes and histochemical characteristics of the rectus abdominis muscle of the rabbit under conditions of pregnancy and mechanically induced stress. *Anat. Rec.* **222**, 136–144. (doi:10.1002/ar.1092220205)
22. Martin WD. 1979 A study of the effect of pregnancy on muscle fibers of the rectus abdominis muscle of the rat. *Anat. Rec.* **195**, 455–462. (doi:10.1002/ar.1091950306)
23. Vesentini G *et al.* 2018 Morphological changes in rat rectus abdominis muscle induced by diabetes and pregnancy. *Brazilian J. Med. Biol. Res.* **51**, 1–10. (doi:10.1590/1414-431X20177035)
24. Wiik A, Gustafsson T, Esbjornsson M, Johansson O, Ekman M, Sundberg CJ, Jansson E. 2005 Expression of oestrogen receptor alpha and beta is higher in skeletal muscle of highly endurance-trained than of moderately active men. *Acta Physiol. Scand.* **184**, 105–112. (doi:10.1111/j.1365-201X.2005.01433.x)
25. Goldspink DF. 1980 Physiological factors influencing protein turnover and muscle growth in mammals. In *Development and specialization of skeletal muscle* (ed DF Goldspink), pp. 65–89. Cambridge: Cambridge University Press.
26. Gilleard WL, Brown JMM. 1996 Structure and function of the abdominal muscles in primigravid subjects during pregnancy and the immediate postbirth period. *Phys. Ther.* **76**, 750–762. (doi:10.1093/ptj/76.7.750)
27. Coldron Y, Stokes MJ, Newham DJ, Cook K. 2008 Postpartum characteristics of rectus abdominis on ultrasound imaging. *Man. Ther.* **13**, 112–121. (doi:10.1016/j.math.2006.10.001)
28. van Leeuwen JL, van der Meulen T, Schipper H, Kranenbarg S. 2008 A functional analysis of myotomal muscle-fibre reorientation in developing zebrafish *Danio rerio*. *J. Exp. Biol.* **211**, 1289–1304. (doi:10.1242/jeb.012336)
29. Beamish FWH. 1978 Swimming Capacity. In *Fish Physiology* (eds WS Hoar, D.F.Randall), pp. 101–187. New York: Academic Press, Inc. (doi:10.1016/S1546-5098(08)60164-8)

30. Booth RK. 1998 Swimming performance of Anadromous Atlantic salmon, *Salmo salar* L., during their spawning migration in the Exploits River, Newfoundland, Canada. PhD dissertation. University of Waterloo, Waterloo, ON, Canada.
31. Perez HM, Brokordt KB, Martínez G, Guderley HE. 2009 Locomotion versus spawning : Escape responses during and after spawning in the scallop *Argopecten purpuratus* and after spawning in the scallop *Argopecten purpuratus*. *Mar. Biol.* **156**, 585–1593. (doi:10.1007/s00227-009-1194-1)
32. Quicazan-Rubio EM, van Leeuwen JL, van Manen K, Fleuren M, Pollux BJA, Stamhuis EJ. 2019 Coasting in live-bearing fish: the drag penalty of being pregnant. *J. R. Soc. Interface* **16**. (doi: 10.1098/rsif.2018.0714)
33. Palstra AP, Rovira M, Rizo-Roca D, Torrella JR, Spaink HP, Planas JV. 2014 Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. *BMC Genomics* **15**, 1136. (doi:10.1186/1471-2164-15-1136)
34. van der Meulen T, Schipper H, van den Boogaart JGM, Huising MO, Kranenbarg S, van Leeuwen JL. 2006 Endurance exercise differentially stimulates heart and axial muscle development in zebrafish (*Danio rerio*). *Am. J. Physiol. Integr. Comp. Physiol.* **291**, R1040–R1048. (doi:10.1152/ajpregu.00116.2006)
35. Fleuren M, Quicazan-Rubio EM, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS One* **13**, e0195976. (doi:10.1371/journal.pone.0195976)
36. Voesenek CJ, Pieters RPM, van Leeuwen JL. 2016 Automated reconstruction of three-dimensional fish motion, forces, and torques. *PLoS One* **11**, 8–10. (doi:10.1371/journal.pone.0146682)
37. Schindelin J *et al.* 2012 Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682. (doi:10.1038/nmeth.2019)
38. Alami-Durante H, Fauconneau B, Rouel M, Escaffre AM, Bergot P. 1997 Growth and multiplication of white skeletal muscle fibres in carp larvae in relation to somatic growth rate. *J. Fish Biol.* **50**, 1285–1302. (doi:10.1111/j.1095-8649.1997.tb01653.x)
39. Dunn JF, Archer SD, Johnston IA. 1989 Muscle fibre types and metabolism in post-larval and adult stages of notothenioid fish. *Polar Biol.* **9**, 213–223. (doi:10.1007/BF00263769)
40. Dal Pai-Silva M, Carvalho RF, Pellizzon CH, Dal Pai V. 2003 Muscle growth in Nile tilapia (*Oreochromis niloticus*): histochemical, ultrastructural and morphometric study. *Tissue Cell* **35**, 179–187. (doi:10.1016/S0040-8166(03)00019-3)
41. Pfaffl MW, Horgan GW, Dempfle L. 2009 Relative expression software tool (REST).
42. Roff DA. 1992 *The evolution of life histories: theory and analysis*. New York: Chapman & Hall.
43. Weeks SC. 1996 The hidden cost of reproduction: reduced food intake caused by spatial constraints in the body cavity. *Oikos* **75**, 345. (doi:10.2307/3546263)
44. Reznick DN, Mateos M, Springer MS. 2002 Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298**, 1018–1020. (doi:10.1126/science.1076018)
45. Weatherley AH, Gill HS, Rogers SC. 1980 The relationship between mosaic muscle fibres and size in rainbow trout (*Salmo gairdneri*). *J. Fish Biol.* **17**, 603–610. (doi:10.1111/j.1095-8649.1980.tb02792.x)

46. Weatherley AH, Gill HS, Lobo AF. 1988 Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *J. Fish Biol.* **33**, 851–859. (doi:10.1111/j.1095-8649.1988.tb05532.x)
47. Johnston IA, Fernandez DA, Calvo J, Vieira VLA, North AW, Abercromby M, Garland TJ. 2003 Reduction in muscle fibre number during the adaptive radiation of notothenioid fishes: a phylogenetic perspective. *J. Exp. Biol.* **206**, 2595–2609. (doi:10.1242/jeb.00474)
48. van Leeuwen JL, Lankheet MJM, Akster HA, Osse JWM. 1990 Function of red axial muscles of carp (*Cyprinus carpio*): recruitment and normalized power output during swimming in different modes. *J. Zool.* **220**, 123–145. (doi:10.1111/j.1469-7998.1990.tb04298.x)
49. Mommsen TP, Moon TW. 2001 Hormonal regulation of muscle growth. In *Muscle development and growth* (ed IA Johnston), Academic Press.
50. Johnston IA, Abercromby M, Vieira VLA, Sigursteindóttir R, Kristjánsson BK, Sibthorpe D, Skúlason S. 2004 Rapid evolution of muscle fibre number in post-glacial populations of Arctic charr *Salvelinus alpinus*. *J. Exp. Biol.* **207**, 4343–60. (doi:10.1242/jeb.01292)
51. Stickland NC. 1983 Growth and development of muscle fibers in the rainbow trout (*Salmo gairdneri*). *J. Anat.* **137**, 323–333.
52. Brooks S, Johnston IA. 1993 Influence of development and rearing temperature on the distribution, ultrastructure and myosin sub-unit composition of myotomal muscle fibres types in the plaice *Pleuronectes platessa*. *Mar. Biol.* **117**, 501–503.
53. Turner CL. 1937 Reproductive cycles and superfetation in Poeciliid fishes. *Biol. Bull.* **72**, 145–164. (doi:10.2307/1537249)
54. Scrimshaw NS. 1944 Superfetation in Poeciliid fishes. *Copeia* **1944**, 180–183. (doi:10.2307/1437814)
55. Pires MN, Banet AI, Pollux BJA, Reznick DN. 2011 Variation and evolution of reproductive strategies. In *Ecology and Evolution of Poeciliid Fishes* (eds JP Evans, A Pilastro, I Schlupp), pp. 28–37. Chicago: The University of Chicago Press.
56. Thibault RE, Schultz RJ. 1978 Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution (N. Y).* **32**, 320. (doi:10.2307/2407600)
57. Zúñiga-Vega JJ, Macías Garcia C, Johnson JB. 2010 Hypotheses to explain the evolution of superfetation in viviparous fishes. In *Viviparus fishes. II* (eds MC Uribe, HJ Grier), pp. 241–253. New Life Publications.
58. Frías-Alvarez P, Zúñiga-Vega JJ. 2016 Superfetation in live-bearing fishes is not always the result of a morphological constraint. *Oecologia* **181**, 645–658. (doi:10.1007/s00442-015-3477-1)
59. Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009 Evolution of placentas in the fish family Poeciliidae: An empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **40**, 271–289. (doi:10.1146/annurev.ecolsys.110308.120209)
60. Kostyo JL. 1956 A study of the glycogen levels of the rat uterus and certain skeletal muscles during pregnancy. **60**, 33–37.
61. Guth L, Yellin H. 1971 The dynamic nature of the so-called "fiber types" of mammalian skeletal muscle. *Exp. Neurol.* **31**, 277–300. (doi:10.1016/0014-4886(71)90196-8)
62. Sänger AM, Stoiber W. 2001 Muscle fiber diversity and plasticity. In *Fish Physiology*, pp. 187–250.(doi:10.1016/S1546-5098(01)18008-8)

63. Williams PE, Goldspink G. 1978 Changes in sarcomere length and physiological properties in immobilized muscle. *J. Anat.* **127**, 459–468.
64. Dix DJ, Eisenberg BR. 1990 Myosin mRNA accumulation and myofibrillogenesis at the myotendinous junction of stretched muscle fibers. *J. Cell Biol.* **111**, 1885–1894. (doi:10.1083/jcb.111.5.1885)
65. Goldspink G, Scutt A, Martindale J, Jaenicke T, Turay L, Gerlach G-F. 1991 Stretch and force generation induce rapid hypertrophy and myosin isoform gene switching in adult skeletal muscle. *Biochem. Soc. Trans.* **19**, 368–373. (doi:10.1042/bst0190368)

## Supplementary material S1. Tissue preparation

For the Crossmon's Trichrome and hematoxylin-eosin staining, we euthanized 4 pregnant and 4 virgin females were with a lethal dose of tricaine mesylate (0.25 g/0.5 L) and trimmed just anteriorly to the pectoral fins, and removed the caudal fin. Subsequently, we cut the trunk cross-sectionally at the level of the anal pore and cut the two resulting blocks in half with cross-sectional cuts. Each section was weighed and the material was placed in bouin fixative for about 1 month to soften the bony structures. Subsequently the fixative was removed by transferring the samples in a solution of ethanol 70% that was renewed multiple times for maximum 2 months. Then the samples were washed in water, and dehydrated in a series of graded alcohols (70, 80, 96, and 100%) and xylene applications, after which the two blocks anterior to the anal pore were embedded in paraffin wax (Paraclean by VWR, The Netherlands).

For the immunohistochemistry method, the staining for the succinic dehydrogenase activity, and the gene expression method, we worked with another set of 3 pregnant and 3 virgin females. For the gene expression method we used additional females (5 pregnant and 5 virgin), for a total of 8 pregnant and 8 virgin females. We euthanized all females with a lethal dose of tricaine mesylate (0.25 g/0.5 L). The abdominal cavities of these individuals were opened ventrally, the internal organs were removed and weighed. Subsequently the abdominal skin flaps were placed in their original position to preserve as much as possible the normal shape of the fish body, and the fish were quickly frozen in melting isopentane. After freezing, the fish were warmed up to -20°C, trunks were trimmed just anteriorly to the pectoral fins, and the caudal fin was removed. Subsequently, the trunks were cross-sectionally cut at the level of the anus after which the two resulting blocks were cut in half with cross-sectional cuts and stored at -80°C.

## Supplementary material S2. Histological preparations

*Crossmon's Trichrome and hematoxylin-eosin staining* – Next, within a slice of about 1000 µm, located on the abdominal region at half way between the pectoral fins and the anal pore, we cut 5 µm sections, using a Microm HM350 microtome (Adamas Instr. BV, The Netherlands). For morphometric analyses, half of the sections were stained with Mayer's hematoxilin-eosin, and the other half with Crossmon's trichrome staining. We chose the best three sections per individual for the analyses.

*Immunohistochemistry (IHC)* – The two blocks stored at -80°C, were glued to the cryostat holder with KP-Cryo Compound (Klinipath, Duiven, The Netherlands). Subsequently,

the same KP-Cryo compound was applied on the inside bend of the tissue to provide more firmness around the ventral cut. Then, the block containing the abdomen was cut in half resulting in a left and a right portion. Both portions were used to cut 10 µm thick sections with a CM3050 S cryostat (Leica, Germany), from the end located at about half way between the pectoral fins and the anal pore. Subsequently, the sections were placed onto Polysine coated slides (Polysine®, Gerhard Menzel GmbH, Germany). Before staining, the sections were air dried and fixated in acetone (4°C) for 7 minutes and left to dry at room temperature for 15 minutes. Next, we applied methanol with hydrogen peroxidase for blocking the endogenous peroxidase. Then they were rinsed in PBST (PBS Tween, at 0.05% concentration) twice for five minutes. The sections were pre-incubated for 30 minutes with a blocker solution PBST-B (PBST with BSA-C (2%) (Aurion, The Netherlands) [1] to diminish the background coloration in the reactions. The blocking solution was removed using a pipette before applying the primary antibody.

To identify the fibre types, we used monoclonal antibodies specific for the myosin heavy chain (MHC) isoforms of slow and fast muscle fibres. For the slow muscle fibres, we used S58 (DSHB Developmental Studies Hybridoma Bank) which has proven to work on several other teleost species, including Poeciliids [1–7]. For the fast muscle fibres we used Zm4 (ZIRC, Zebrafish International Resource Center) which has worked in zebrafish [8,9]. We stated the presence of the intermediate fibres as those that didn't respond to neither of the two antibodies used. To enhance the detection in the immunohistochemical reaction, we used VECTASTAIN® Elite® ABC KIT (HRP, Mouse IgG) (Vector Laboratories, USA). The biotinylated conjugate from the kit is coupled to the primary antibodies and the avidin-biotin-HRP complex formed is coupled in turn to the conjugate. After some washing steps the 3,3-diaminobenzidine (DAB) turned into a brown precipitated product by the attached HRP-enzyme, facilitating the detection of the specific muscle fibres by the primary antibodies used. It created a clear overview of the positions of the muscle fibre on a cross-section.

We applied 100 µl of a 1:120 dilution of S58 and 100 µl of a 1:10 dilution of Zm4 onto the sections and they were left overnight at 4°C. After that the slides were rinsed again with the buffer PBST three times for 5 minutes. Subsequently, we added the conjugate and applied VECTASTAIN for 30 min at room temperature to couple the complex to the secondary antibody and then rinsed it with PBST buffer two times for 5 min each and subsequently with Tris-HCl buffer (pH 7.6) twice for 5 min each. The colouring reaction was performed with DAB, H2O and This-HCl for 15 min and it was stopped by rinsing with demineralised water twice for 5 min each. After we counterstained using Mayer's Haematoxylin for 1 min (VWR, The Netherlands), we rinsed the sections with tap water for 10 min. The sections were dehydrated using a series of Ethanol dilutions followed by 3 steps of xylene. The sections were mounted using DEPEX (VWR, The Netherlands). All tests were performed in duplicate and with a negative control. On the negative control we

followed identically steps as with the other slides, but no primary antibody was applied, instead it was incubated in PBST with five times diluted blocker solution from the kit in the PBST.

*Succinic Dehydrogenase (SDH)* – We used staining for SDH to make the distinction between the oxidative and non-oxidative muscle fibres. Sections were stained using the method described by [5]. Briefly, we incubated the sections in the dark at 28˚C for 30 minutes in a medium containing 37.5 mM sodium phosphate buffer, pH 7.6, 75 mM sodium succinate, 5 mM sodium azide and 0.4 mM tetranitro blue tetrazolium. The reaction was stopped with 10 mN HCl.
*Imaging of histological preparations* – The IHC sections were examined with a Nikon Mikrophot FXA upright microscope. And images were taken with an attached Olympus DP50 color camera. The Crossmon's trichrome and hematoxylin-eosin staining images were taken with a Leica Dm6b upright microscope coupled with a Leica DFC450c color camera. Using 10x and 20x objectives we made images of different regions of one section and aligned the images using the tiles scan function of Leica, obtaining a single image with the full section.

## Supplementary material S3. Quantitative transcriptions levels of muscle-related genes

As the genomes of *P. gracilis* had not yet been sequenced, and no genomic data was available for any of its genes encoding for muscle-related proteins, information about the DNA sequences was deduced from related species. These species were necessary to have the appropriate parts of the *P. gracilis* genome sequenced. Preferably the mRNA or cDNA of those genes were known, as these indicate that the genes indeed code for proteins.

### *RNA isolation*

After being euthanized, the 8 pregnant and 8 virgin females were stored at -80° Celsius. A single transversal tissue slice, ranging 40-80 mg, was cut from the tail region right behind the anal pore (figure 1). Per specimen, RNA was isolated from these samples using the RNeasy Universal Mini Kit (Qiagen, Venlo, the Netherlands), with homogenisation with a Tissuelyser II (Qiagen), followed by measuring the RNA concentrations with a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Ermelo, the Netherlands) and evaluation for integrity by agarose gel electrophoresis.

**cDNA synthesis**

Samples of 1000 ng RNA were reverse-transcribed into cDNA using the QuantiTech Reverse Transcription protocol for two-step RT-PCR (Qiagen, Venlo, the Netherlands). A non-reverse-transcriptase control was included for each sample.

**Sequencing muscle-related genes**

PCR primers were designed on the conserved parts of the aligned sequences of related species, with preference to the locations in the 5' and 3' UTR's in order to pick up the whole CDS of the genes in the species of interest. These related species were *Poecilia reticulata, P. formosa, Oryzias latipes, Xyphophorus maculatus*. Primers were designed using the program Primer3plus (Untergasser et al. 2007) and obtained at Eurogentec (Liege, Belgium).

PCR was performed on cDNA, the PCR products with expected lengths were purified with the QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands), ligated into pGEM-TEasy vectors and bacteria of the E. coli strain JM109 High Efficiency Competent Cells (Promega, Leiden, The Netherlands, cat.no. L2001) were transformed with these vectors according to the pGEM-T Easy Vector Systems protocol (Promega, Leiden, The Netherlands). Per gene, four to eight colonies of transformed bacteria were selected for colony PCR, colony PCR products of correct lengths were randomly selected, purified via a Sephacryl S-400 HR filtration process (GE Healthcare, Hoevelaken, The Netherlands) and sent to GATC-Biotech AG (Constance, Germany) for sequencing.

The obtained sequences were edited and aligned with the program Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, USA). The final sequences (table S2) were entered as a nucleotide BLAST-search [12] to verify the acquisition of the correct genes. These sequences do not always cover the whole gene, but are of sufficient length for qPCR primer design (table S2). The qPCR primers were designed by primer-blasting highly conserved interspecies sequence parts (obtained from Sequencher 4.10.1) and selecting the best primer out of the suggestions with the programme Oligoanalyzer 3.1.

**Table S1.** Description of selected genes of interest and reference genes.

| Gene | Description |
|---|---|
| Sarcomere proteins | |
| Myosin *(myo)* | The term myosin actually refers to a whole family of motor proteins, but is often used to address their role in muscle activity. These specific types of myosin molecules primarily belong to myosin class II [10]. Slight differences in these molecules are found between white muscle (fast twitch) and red muscle (slow twitch) fibres. However, these different muscle fibre type specific genes cannot be found in Poecillidae related species and thus more general primers were designed that pick up more general myosin sequences. |
| Troponin t1 *(tnnt1)* | In vertebrates, troponin molecules are only present in skeletal muscles and, thus, also in the myomeres of fish. Troponin refers to a complex of three regulatory proteins that facilitate proper interaction between myosin and actin molecules. It is involved in the formation of the tropomyosin-actin complex which consists of troponin C, T and I subunits (respectively, TnC, TnT and TnI). The troponin subunits slightly differ between white (fast twitch) and red (slow twitch) muscle fibres [11]. White muscle fibres contain fast troponin C2 (*tnnc2*), fast skeletal troponin T3 (*tnnt3*), and fast-twitch skeletal muscle isoform troponin I2 (*tnni2*). Red muscle fibres contain slow troponin C1 (*tnnc1*), slow skeletal troponin T1 (*tnnt1*), and slow-twitch skeletal muscle isoform troponin I1 (*tnni1*) [12]. Unpublished research by Busscher showed the presence of *tnnt1* in the species of interest [13]. However, evidence of the presence of the different isoforms is lacking for the Poeciliidae family. Therefore, the aim was to design primers specific to slow skeletal muscles, but we couldn't be certain of their specificity. |
| Muscle growth | |
| Myogenin *(myog)* | The transcription factor myogenin is involved in myogenesis of skeletal muscles. This growth promotor is not specific for white or red muscle fibres, but can still give information about the change in total muscle development during gestation [12]. |
| Myostatin *(mstn)* | This protein is a member of the TGF-β family and is also involved in myogenesis. In contrast to the muscle growth promotor myogenin, this protein inhibits skeletal muscle differentiation and growth [14]. |
| Energy supply | |
| Phosphofructo-kinase-m (PFKM) | Phosphofructokinase-m is a protein involved in glycolysis, i.e. the first step of cellular respiration. The products formed in glycolysis are used in the citric acid cycle and electron transport chain, where more ATP is produced [15]. |
| Ubiquinin (NADHd) | NADH dehydrogenase is the first membrane enzyme complex of the electron transport chain. It is involved in the transport of hydrogen molecules over the inner membrane of the mitochondria, helping to form the electrochemical proton gradient needed for ATP synthase to produce ATP [15]. |
| Succinate dehydrogenase (SDH) | Succinate dehydrogenase is involved in step two and three of cellular respiration. It is the only protein involved in both the citric acid cycle and the electron transport chain (as the second membrane enzyme) [15]. |
| Oxidation facilitation | |
| Myoglobin *(mbin)* | This is the oxygen-transporting protein found within both white and red muscle fibre tissues. Higher transcription levels in pregnant fish can be an indication of its muscle tissue becoming more aerobic [16]. |
| Reference genes | |
| Bèta-actin *(ActB)* | Besides its role in sarcomere formation, actin is also present in the cytoskeleton of cells. This specific actin type is called bèta-actin and generally has a very constant expression rate. |
| Ribosomal protein L7 (RPL7) | Ribosomes catalyse protein synthesis and consist of a small (40S) and a large (60S) subunit in eukaryotes. These organelles are involved in translation of RNA into proteins. The subunits themselves consist of many ribosomal RNAs and proteins. One of those ribosomal proteins is the 60S ribosomal protein L7. |
| Tubulin alpha *(tuba)* | Tubulins are globulin proteins comprising five different families, with α-tubulin and β-tubulin being the most prevalent ones. Dimers of these types of tubulins build up microtubules, which are in their turn important components of the common cytoskeleton. |

**Table S2.** Final alignment of clones. Indicated are the qPCR primers.

| Gene | Aligned obtained sequences (5' - > 3') |
|---|---|
| *ActB* | >ActB_finalsequence<br>CCACACCCAAAGTTCAGCCATGGAAGATGAAATCGCCGCACTGGTTGTTGACAACGGATCCGGTA<br>TGTGCAAAGCCGGATTCGCCGGAGACGACGCCCCTCGTGCTGTCTTCCCATCCATCGTTGGTCGCC<br>CAAGGCATCAGGGCGTGATGGTTGGTATGGGCCAGAAGGACAGCTATGTAGGTGATGAAGCCCAG<br>AGCAAGAGAGGTATCCTGACCCTGAAGTACCCCATCGAGCACGGTATTGTGACCAACTGGGATGA<br>CATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGAGAGTTGCCCCTGAGGAGCACCCCG<br>TCCTGCTCACAGAGGCCCCTCTGAACCCCAAAGCCAACAGGGAGAAGATGACCCAGATCATGTTT<br>GAGACCTTCAACACCCCCGCCATGTACGTTGCCATCCAGGCCGTGCTGTCCCTGTACGCTTCTGGT<br>CGTACCACTGGTATCGTCATGGACTCCGGTGATGGTGTGACCCACACAGTGCCCATCTATGAGGGC<br>TACGCCCTGCCCCATGCCATCTTGCGTCTGGACTTGGCCGGCCGCGACCTCACAGACTACCTCATGA<br>AGATCCTGACAGA:GCGTGGCTACTCCTTCACCACC:ACAGCCGAGAGGGAAATCGTGCGTGACATC:<br>AAGGA:GAA:GCTGTGCTA:CG:TCGCCCTGGACT:TCGAGCAGGAGATGGGTACCGCTGCCTCC:TCTT<br>CATCCCTGGAGAA:GAGCTACGAAGCTCCCTGACGGACAGGTCATCACCATCGGCAATGAGAGGTT<br>CCGTTGCCCAGAGGCCCTCTTCCAGCCTTCCTTCCTTGGTATGGAGTCCTGCGGAATCCAYGAGACC<br>ACCTACAACAGCATCATGAAGTGCGACGTCGACATCCGTAAGGACCTGTACGCAAACACCGTGCTG<br>TCTGGAGGTACCACCATGTACCCTGGCATTGCTGACAGGATGCAGAAGGAGATCACAGCCCTGGCC<br>CCATCCACCATGAAGATCAAGATCATCGCCCCACCAGAGCGTAAATACTCTGTCTGGATCGGAGGC<br>TCCATCCTGGCCTCCCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGATGAGTCTG<br>GCCCCTCCATCGTCCACCGCAAATGCTTCTAAACAGACTGTTCCTCCTCCCCGTTCCCCAAACACCCA<br>ACAACTTCAGCTCTGTGCAGAACAACCACATTTCTCATACACACTCAGGCGCAGAGCCTAGACGACA<br>ACTCATTGGCATGGCTTCAGTTATTTTTGGCGCTTGACTCAGGATTT:AAAAAAAA::CTGGAACGATG<br>AAGGAGACG:GTAA |
| *mbin* | >Mbin_finalsequence<br>TTATTGTTCAACCAGAAAAGCCCAGCTCTTTGTAGGTGGTGTCGATGTCAGCGATGACRACGGCCAT<br>CACGTTCCTCAGRGCCTGCTGRCCGGCAGCGTCCAAGCCGGCCTTCTCCCCCATGAYTTTAGCGATCA<br>CCTCAGTAATCAGCCTGAAGTTATTGATTGGGATCTTATGCTTGGTGGCGTGMGTGTTGGCCAGGGG<br>TTTGAGGATGGCGGCGTGGTTGCCTTTGGCCTTTAGGAGCTCACCGAGCTTCTTCAGCACAGTGGCG<br>CCGTGGGCAGAAACAGGTGCATTGCCGGCCAGGTCTCCCTTGGCAATSCCAGCAAACTTYGGGAACA<br>GCTTCTGGGTTTCTGGGTGCTCATGGAATAAGCGGGTCAGAACCAGATTCCCATGGGTGTTGTAATC<br>TGCTTCCACCGGACCCCAGTGCTTCAGAACCATATCAAAATCCGCCAT |
| *mstn* | >Mstn_finalsequence<br>TACGACGTGCTGGGAGACGACAGCCGGGATCCGGTACCGGAGGAGGACGACGAGCACGCGACCACC<br>GAGACTCTGATGATCATGGCCACTGAACCCGACCCGGCGGTCCAGGMGGACGGRGAACCGACCTGC<br>TGCTTCTTCTCCTTCACGCAGAAGCTGCAGGCCACGCGGATCGTGCGCGCGCAGCTGTGGGTGCACCT<br>GCGCCCCGCGGCCGAGGCGACCACCGTGTTCCTGCAGATCTCCCGCCTCATGCCGGCCACGGACGGC<br>GGCTGGAGGCACGTCCGGATCCGCTCCCTGAAGATCGACGTGAACGCCGGRSTGAGCTCGTGGCAGA<br>GCATCGACGTGAAGCAGGTGGTGGCGGTKTGGCTGCGGCAGCCGGAGACCAACTGGGGCATCGAGA<br>TCAAGGCCTTCGACTCGCGGGGGAAAGACCTGGCGGTGACCGCGGTGCAGGCCGGGGAGGACGGGC<br>TGCAACCGTTCATGGAGGTGAAGGTGTCTGAGGGCCCCCGGCGGGYCCGGAGAGACTCGGGTCTGGA<br>CTGCGATGAGAACTCCCCSGAGWCSCKCTGCTGCCGCTACCCGCTCACCGTGGACTTCGAGGACTTTG<br>GCTGGGACTGGATTATCGCCCCGAAGCGCTACAAGGCCAACTACTGCTCCGGGGAGTGCGAGTACAT<br>GCACCTGCAGAAGTACCCACACACCCACCTGGTGAACAAGGCCAACCCCCGGGGGTCCGYGGGCCY<br>CTGCTGCACCCCCACCAAGATGTCCCCCATCAACATGCTCTACTTCAACCGCCAGGAACAGATCATCT<br>ACGGTAAGATCCCAA |
| *myo* | >MYO_finalsequence<br>TTCCTGGCTCTCCTCATACTTCTGCTTCCATTCTGCCAGGACCTTGTCAAAGTTCCTCTGCTTCTTGTCA<br>AGGTTGGCAGCCAGAGAATTAGCTCTCTCCACATCTGTCATGAGGTCYTCCACCTCACCYTGGAGCCTC<br>TGCTTGGTCTTCTCCAAAGAGGCACACTTGGAGTTCACAGCCTCAATGGATTCCTCAGCMTCCTGCAAG<br>CGCTGGGCAAGCTTTTTCTTGGCCTCCTCCAGCTCCTCAGTGCGYTGGATGGCATCAGTCTCATATTTGG<br>ACCTCCACTGAGCCACCTCACTGTTGGCCTTAGACATGCCTCTCTGSAGCTCAGCCTTGGCCTCCTGCTC<br>CTCCTCAAACTGCTCTCTGAGCAGATCACAGTCATGGCGGGCTGACTGAACWGCATGGGCCAGRGCAT<br>TCTTGGCCTTCACTTCCTCCTCAATGTGTCTCTTGAGCTCCTCAATCTGCTGAGTGAAAGCCTGCTTGCCT<br>CTTGTCAGCTGAGAAACAAGAGCTTCTTTCTCCTCAAGYTGGCGAGAAAACTCACCATTCTCTGTCTGGA<br>GTCTTGCTTTCTGTGCATTAATGTCATTCAGCTGGCGGACATTCTCATCATTTTTAGCTTTGATTTCACTAA<br>GTTGGTCCTCAAGAGTTCTGCACATTTTCTCCAAGTTGCCCTTGGCTTTGGCAACAGCCTCCATGTTGCTG<br>GACAGGTCATCAATCTCCATCTTGTACTCACTCTTCTCCTTCTCCAGCTTCTGCTTGACGCGCTGCAGGTTG<br>TCGATCTGCTCTCCCAGCTCAGCAACGCTGTCGGCCTGCTTCTTGCGGAGAGGTGCTGCGGTRGCTTCATG<br>CTGCAGCGTGGACTCTTCAAGGTCACGACGCAGCTTCTGGAACTCAGCCTCACGCTTCTTGTTCATCTCAA<br>TCTGAGCAGCTGTGGCTCCACCAGCCTCCTCAAGCCTCTCGCTGATCTCCTCAAGCTCCCTGGAGAGGTCA<br>GCTCTCTGCTTCTCTACCTTAGCCCTAGCAGCCCTCTCAGCCTCAATCTCCTCTTCCAGCTCCTCAAT |
| *myog* | >Myog_finalsequence<br>CTCCAGCCTGTCRCCTCATTCTGAGACCCACTGCCCTGGTCAGTGCCTGCCCTGGGCCTGTAAGCTGTGC<br>AAAAGAAAGACTGTGACCATGGATCGCCGGAGAGCAGCAACGCTGAGGGAGAAGAGACGCCTGAAGAA<br>GGTGAACGAGGCCTTCGATGCTCTGAAGAGGAGCACCTTGATGAACCCCAACCAGAGGCTGCCCAAAGT<br>GGAGATCTTGCGGAGCGCCATACAGTACATCGAACGGCTACAAGCGCTGGTGTCCTCTCTCAACCAGCAG<br>GACACTGAGACAGGACAG |
| *NADHd* | >NADHd_finalsequence<br>CAGGGATGTGAGGGAACATCAGSCATGTTTCCATCATCCCTCACAGGAACTGGATAACTGTATGGYGTGRC<br>TTCATTTATCATTCCTGTGTATTTTGTATAGGGGCTGATGAATGGAAGAATAAYACCTAMAAGGCCAATCC<br>CACAGGAKGCCACKATAACTGGCTCCTTATTCCAAGCATTTTTCAGGAAAGCTCCAATTCCTGCCA |

| | |
|---|---|
| *PFKM* | >PMFK_finalsequence<br>GACCAGAACCAGAACCATGGCGCAACCYGTTAAAACCGACCCCACTAAGATGGGTGAGGGTCGGGCGATT<br>GCAGTGCTGACTTCTGGAGGCGATGCTCAGGGAATGAATGCGGCTGTGAGAGCCACCGTTCGAGTGGGGTT<br>GTACACTGGAGCCAAGGTKTACTTTGTCCATGAGGGTTACCAGGGCCTGGTTGACGGGGGAGACAATATCC<br>GCCCAGCTACCTGGGAAAGTGTGTCAATGATGCTGCAGCTGGGTGGGACTGTGATCGGTAGCGCCCGCTGT<br>CAGGACTTCCGCACCAAGGAGGGGCGCACCAAGGCGGCCTGTAACTTRGTCAAGCTGGGCATCACCAACTT<br>GTGCGTTATTGGAGGTGATGGCAGTCTCACAGGTGCCAACCAGTTCAGGACGGAGTGGAAAGACCTGCTGG<br>CTGACCTGGTTAAAGCAGGAAAAATCACAGCAAATGAAGCAAAGAACTCCTCCCACCTCAACATTGTSGGC<br>ATGGTGGGCTCCATCGACAATGACTTCTGTGGAACTGATATGACCATTGGCACTGACAGCGCCCTGCATCGC<br>ATTATTGAAATAGTAGATGCCATCACAACCACGGCACAGAGTCACCAGAGGACCTTCATCTTGGAGGTAATG<br>GGTAGGCACTGTGGCTACCTTGCCTTGGTGACTGCTCTGGCCTGTGGTGCCGACTGGGTCTTCATCCCAGAGA<br>TGCCTCCAGATGAAGGATGGGAGGAACATCTGTGTAGAAGACTTACARATCAAAGATSTCGTGGTTCCCGTT<br>TGAATATCATCATTGTTGCAGAGGGAGCAATAGATCGCAAAGGAAAACCAATCACCTGTGACCTAGTCAAA<br>CAGCTGGTGTCAAAGCAGCTGGGCTTCGACACCCGAACCACCATCCTGGGCCATGTGCAAAGAGGAGGAAC<br>CCCCTCTGCTTTTGACAGGATCCTGGCAAGCAGGATGGGTGTGGAGGCTGTCATGGCTCTGCTGGAAGCCAC<br>ACCAGATACTCCTGCMTGTGTGGTCAGCCTGTCTGGAAACATGGCTGTCAGGCTGCCTCTAATGGAATGTGTG<br>CAAGTGACTAAAGACGTCACCACAGCCATGGCTGAAGGACGGTATGACGATGCGGTGATGCTCAGGGGAAA<br>GAGCTTTGAGAACAACTGGAACACGTATAAAATGCTTGCCCATGTGAACCCTCCAGAAACAAAGAGCAATAT<br>CAACATTGCCATACTGAACGTCGGCGCTCCATGTGCAGGMATGAATGCTGCAGTACGTGCAGCAGTCAGGRT<br>TGGGCTSCTTCAGGGCCACCAGATGCTGGCGGTGCATGAAGGCTTCGATGGCTTGGCACATGGGMTGATTGA<br>GCCAATTGGTTGGTCTGGAGTGGCAGGATGGACTGGAAAGGGCGGTTCCATGCTTGGSACCAAGAGGACAC<br>TGCCTAARCAGYTTCTGGAGGAGATCAGYCTGAACATCACAAAGTTCAACATCCATGGTATTGTCRTTATCGGAGG<br>CTTTGAGGCATTCCTGGGAGGTCTAGAGATGRTGGAAGCTAGASAGAAGTACGAGGAAYTGTGTATTCCCCTC<br>GTCGTTGTTCCTGCTACCGTCTCCAATAATGTCCCTGGATCTGACTTCAGCATAGGCGCAGACACAGCCCTCAA<br>CACCATAACCATGACATGTGACCGGATCAAACAGTCTGCTGCCGGCACCAAGAGGAGAGTGTTCATTGTTGAG<br>ACCATGGGGGGATACTGTGGYTACCTGGCAACCATGGCTGGGTTGGCATCTGGAGCAGATGCGGCCTACATTT<br>ATGAGGAACCGTTCARCATYCATGACCTCGAGCTGAACGTTGATCATCTGTTGGAAAARATGAAGACAACAGT<br>GAAGAGGGGGCTAATTCTGAGAAATGAGAAGTGCAACGYAAACTACACGACTGATTTCATCTTCAACCTGTAT<br>TCAGAGGAGRGSAAGGGYGTGTTTGACTGYAGAAAAAATGTTCTTGGTCATATGCAGCAGGGYGGRACACCCA<br>GTCCTTTTGATAGGAACTTTGGCACAAAGATGGGAATCAAGTCTGTTCTTTGGTTGACTGACAARCTRAAGGAA<br>TGCTACAGACATGGTCGCATCTTTGCCAACTCKAAGGACTCAGCCTGTGTGCTGGGAATGAGGAAAAGAGCGT<br>TGGTGTTCCAGCCTTTGGCAGACCTAARAACTGAGACTGACATTGAACACCGTATTCCAAAGRCCCAGTGGTGG<br>CTGAAGCTGAGGCCCATCTTGAAGATCYTGGCCAAGTACAAGATCAACTTGGACACCTCAGAGAGGACTGCAA<br>TGGAACATGTCATCAAGAAGAGAGGCTTAGTTCCTCAGTAGC |
| *rpL7* | >RPL7_finalsequence<br>TTGCAATGGCGGACGCAGAAAAAAAAGGTTCCGTCGGTCCCTGAGAGCCTTTTAAAAAGGCGAAAGGCCTTC<br>GCCGCCATGAAGGCCATGCGTATCAAGAAGCTWCTGGYGGAGAAGAAGGCCCGGAARGTGACCAGGAAACT<br>GATCTACAAGAGGGCTGAAAAGTACCACAAGGAGTACAGGCAGATGTACAGGCGTGAGATCCGCATGTCTCG<br>TATGGCTCGCAAAGTGGGGAACTACTATGTGCCACCTGAGCCCAAACTGGCCTTTGTCATCAGGATCAGAGGT<br>ATCAACGGTGTCCCACCAAAGGTCCGCAAGGTTCTGCAGCTGCTCCGTCTGCGCCAGATCTTCAACGGTGTGTT<br>TGTCAAGCTGAACAAGGCTTCTATCAACATGCTTAGGATTGCAGAGCCTTATATTGCTTGGGGATACCCCAACC<br>TGAAGTCTGTGCGTGAGCTCATCTACAAACGTGGCCATGGCAAAGTGAAGAAGATGCGTATTGCCCTCACAGA<br>CAACGCTCTGATAGAGAAGCCCCTTGGCAAATAYGGCATCATCTGCATTGAGGACCTTATTCACGAGATCTACA<br>CAGTTGGCAAGAACTTCAAGATGGCCAACAACTTCCTGTGGCCTTTCAAGCTGTCATCGCCACGTGGKGGTATG<br>AACAAGAAGACCACACACTTTGTGGARGGAGGTGACGCTGGAAACAGGGAGGACCAGATCAACAGGCTGATC<br>CGAAGGATGAACTAAGTAA |

| | |
|---|---|
| *SDH* | >SDH_finalsequence<br>GTCCCGTCTCCTCTCCA:GAGGGTGCTCTCTAACACGAAAGCGGTCCCTGCTGCTGCAGTTCAGGGTTCCAGG<br>AACTTTCACTTCTCCATCTACGGCAAGAAGAAAAACGTCAAAGTCGCAGATGACATCTCCACTCAGTATCCT<br>GTCGTGGATCACGAGTTTGATGCCGTGGTGGTGGGAGCCGGAGGAGCCGGCCTTCGGGCCGCTTTTGGCCTG<br>TCAGAGGCCGGCTTCAACACCGCCTGCGTCACCAAGCTCTTCCCCACCAGGTCTCACACCGTCGCTGCACAG<br>GGTGGGATCAACGCGGCTCTTGGTAACATGGAGGAGGATGACTGGCGCTGGCATTTCTACGACACGGTGAA<br>GGGATCTGATTGGCTGGGAGACCAGGACGCCATCCATTACATGACAGAGCAGGCTCCTGCAGCCGTAGTGG<br>AGCTGGAAAACTTTGGCATGCCGTTCAGCCGCACCGAAGACGGGAAGATCTACCAGAGAGCCTTCGGGGGT<br>CAGAGTCTCAAGTACGGGAAGGGGGGCCAGGCCCACCGCTGCTGCTGTGTGGCGGACCGGACGGGACACTC<br>CCTGCTGCATACGCTTTATGGAAGGTCACTCCGCTATGACACCAGCTACTTTGTGGAGTACTTTGCCCTGGAT<br>CTGCTGATGGAAAACGGAGAGTGTAAAGGAGTTATTGCCCTGTGCATGGAGGACGGCTCCATCCACCGCTTC<br>AGAGCCCAGAACACCGTCATCGCCACCGGTGGTTACGGAAGGACGTATTTCAGCTGCACGTCCGCCCACACA<br>AGCACAGGAGACGGAAACGCCATGGTGACCAGAGCCGGCCTGCCGTGCCAGGATTTGGAGTTTGTGCAGTTC<br>CATCCCACTGGGATCTACGGGGCCGGCTGCCTGATCACGGAGGGTTGCCGTGGCGA:GGGAGGAATCCTGAT<br>CAACAGCGAGGGCGAGCGCTTCATGGAGCGTTACGCCCCCAACGCCAAAGACCTGGCTTCCAGA:GACGTGG<br>TGTCCCGCTCCATGACCATAGAGATCAGAGAGGGCAGAGGCGTGGGTCCGGACAAGGACCACGTGTACCTG<br>CAGCTCCACCACCTGCCTCCGCAGCAGCTGGCCACCAGGCTGCCCGGGATCTCCGAGACGGCCATGATCTTC<br>GCTGGAGTCGACGTGACTAAAGAGCCCATCCCCGTCCTGCCCACGGTTCACTACAACATGGGAGGAATTCCC<br>ACCAACTACAAGGGACAGGTGATCGATCACACCAACGGCGAGGACAAGGTGGTTCCTGGGCTGTACGCCTG<br>CGGTGAGGCGGCTTGTGCCAGCGTGCACGGCGCTAACAGGCTGGGCGCTAACTCCCTGCTGGACCTGGTGGT<br>GTTCGGGAGAGCCTGCGCGCTAACGATCGCAGAGGAGCACAAACCGGGAGAGAAGCTGTCCCCGCTGAAGC<br>CCAGCGCAGGAGAGGAGTCTGTGTCCAACCTGGACAAGCTGAGATTTGCCAATGGAAGTCTGAGAACATCA<br>GAGATCAGGCTGAACATGCAGAAGACCATGCAGAACCACGCCGCCGTCTTCCGTACCGGCTCTGTTCTGAAG<br>GAGGGATGCGATAAGATGGCCGACGTCTACCAGAGCATGGAGAACATCAAAACGTTTGACAGAGGCATCGT<br>GTGGAACACAGAYTTGGTGGAGTCACTGGAGCTGCAAAACCTGATGCTGAACGCCGTCCAAACCATCAACG<br>CTGCTGAGCAAAGAAAGGAGAGCAGGGGCGCCCACGCCAGAGAGGACTTCAAAGATCGTATCGACGAGTA<br>CGACTACTCCAAGCCCCTGGAGGGTCAGCAGAAGAAGCCCTTCGAGCAGCACTGGAGGAAGCACACCATGT<br>CATACGTCGACCCCAAGACCGGAAAGGTGACGCTGAGGTACCGCCCCGTCATCGACAGCTCTCTGAACGAA<br>CAGGACTGCGCCCACGTTCCTCCTGCCATCCGCTCGTACT |
| *tnnt1* | >TNNT1_finalsequence<br>MGATGAGGCCAAGAAGAAGAAGARAGTGCTGTCTGGCATGGGAGCCAACTTTGGAGGTTTCCTGGCCAAAG<br>CCGAGTCGAGGAAGAGCAAGCGCCTGACGGGCAGGGAGATCAAGAGGAAGACGCTGGCCGACAGACGGCA<br>GCCTCTAGGCATCGACAACATGAGGGAGGACGGCCTGAGAAACCGGGCCCAGGAGATGTGGAACTGGATCC<br>ATCAGCTGGAGTCCGAGAAGTTCGACTTCATGGAGCAAATGAAGCACCAGAGATATGAGATTACTGTGTTAC<br>TGAACAGAATCCAACATGC |
| *tuba* | >Tuba_PG_final<br>GACGTGGGTCACATTTCACCATCTGATTGGCCGGCTCGAAGCAGGCGTTGGTTATTTCCGCCACAGTCAGCTG<br>CTCGTGATAGGCTTTCTCTGCGGAGATGACCGGGGCGTAGGTGGCCAGAGGGAAGTGGATTCTCGGATAGGG<br>CACCAGGTTGGTCTGGAACTCCGTCAGATCTACGTTCAGTGCTCCGTCGAAGCGCAGGGAGGCGGTGATGGA<br>GGAGACGATCTGGCTGATGAGGCGATTCAAGTTGGTGTACGACGGGCGTTCGATGTCGAGGTTCCGGCGGCA<br>GATATCATAGATGGC<br>>Tuba_PT_final<br>GACGTGGGTCACATTTCACCATCTGATTAGCTGGTTCAAAGCATGCGTTGGTGATGTCAGCCACAGATAGTTGT<br>TCMTGGTAGGCTTTCTCAGCAGAGATCACTGGAGCATAAGTGGCCAGAGGGAAGTGRATACGAGGATATGGCA<br>CCAAGTTGGTCTGGAACTCGGTCAGGTCAACATTCAGGGCTCCATCAAAACGGAGAGATGCTGTGATTGAAGA<br>CACAATCTGGCCAATAAGCCTGTTCAGGTTGGTGTAGGTTGGTCTCTCAATGTCAAGATTTCTGCGGCAGATAT<br>CATAGATGGC<br>>Tuba_HF_final<br>GACGTGGGTCACATTTCACCAWCTGATTSGCCGGCTCAAAGCAGGMRTTTGTGATCTCAGCYACYGWGAGCTG<br>CTCATGGTAAGCCTTCTCTGCAGAGATCACAGGGGCGTAGGTGGCCAGAGGGAAGTGGRTGCGAGGGTATGGC<br>ACCAAGTTGGTCTGGAACTCRGTCAGATCAACATTAAGGGCACCATCAAAACGGAGAGACGCAGTGATGGAGG<br>ACACGATCTGRCYGATCAGCCTGTTCAGGTTGGTGTAGGTGGGGCGTTCGATGTCGAGGTTCCTGCGGCAGATA<br>TCATAGATGGC<br>>Tuba_PJ_final<br>GACGTGGGTCACATTTCACCAWCTGATTGGCCGGCTCGAAGCAGGAATTTGTGATCTCAGCGACCGTCAGCTGC<br>TCATGGTAAGCCTTCTCTCTGCAGAGATCACTGGGGCGTATGTGGCCAGGGGGAAGTGGRTACGAGGATATGGCAC<br>CAAGTTGGTCTGGAACTCAGTCAGATCAACATTCAGGGCACCATCAAAACGGAGAGACGCAGTGATGGAGGACA<br>CGATCTGGCTGATCAGCCTGTTCAGGTTGGTGTAGGTGGGGCGTTCGATGTCGAGGTTTCTGCGGCAGATATCAT<br>AGATGGC |

### Real Time quantitative PCR

RT-qPCR runs were performed on a Rotor-Gene Q (Qiagen) with the Rotor-Gene SYBR® Green PCR Kit (Qiagen) as detection chemistry. Each reaction contained 6.25 µl Rotor-Gene SYBRGreen MM, 3,75 µl primers (end concentration 1 µM) and 2,5 µl cDNA (25 x diluted after synthesis). Cycling conditions were according to the manufacturer's protocol (RotorGene SYBRGreen Handbook, Qiagen). The three reference genes were included in each run to allow an adequate comparison in the analysis phase. Non-template and non-RT controls were included and no amplifications above background levels were observed.

### Data analyses

The results of the RT-qPCR runs were analysed with the Rotor-Gene Q series software – version 2.3.1 and REST 2009 (Qiagen), with which the melting temperature curves were checked for consistencies between the samples. The fluorescence curves were examined to ensure the specificities of the amplifications. All primers showed to be specific and had high efficiencies. The Ct-values, i.e. the number of PCR cycles needed for the signal to exceed a predetermined threshold value, and amplification efficiencies of all samples were obtained with Comparative Quantitation Analysis from the Rotor-Gene Q software and were exported into Microsoft Excel (2016) files. The primer efficiencies per primer set were pooled throughout the entire experiment.

### Reference gene selection

Three tools were used to select the best reference gene: *BestKeeper* [17], *geNorm V3.4* [18] and *NormFinder* [19]. All three programs identified *RPL7* as the best reference gene, better than *ActB* and *tuba.*

**Table S3. REST-RG relative expression output of analysis via 2000 iterations.** No sample groups are significantly different expressed compared to their control groups.

| Gene | Type | Reaction Efficiency | Expression | SE | 95% C.I. | P(H1) |
|---|---|---|---|---|---|---|
| ***mbin*** | TRG | 0,7619 | 0,981 | 0,463 - 2,164 | 0,264 - 4,543 | 0,945 |
| ***mstn*** | TRG | 0,7956 | 0,785 | 0,441 - 1,380 | 0,301 - 2,009 | 0,241 |
| ***myo*** | TRG | 0,7844 | 1,057 | 0,579 - 1,841 | 0,226 - 2,982 | 0,823 |
| ***myog*** | TRG | 0,7881 | 1,259 | 0,910 - 1,790 | 0,612 - 2,711 | 0,115 |
| ***NADHd*** | TRG | 0,8019 | 0,929 | 0,682 - 1,303 | 0,548 - 1,720 | 0,512 |
| ***PFKM*** | TRG | 0,7425 | 0,682 | 0,631 - 1,204 | 0,077 - 1,566 | 0,220 |
| ***SDHA*** | TRG | 0,8019 | 1,009 | 0,625 - 1,534 | 0,463 - 2,019 | 0,962 |
| ***tmmt1*** | TRG | 0,7906 | 0,942 | 0,769 - 1,126 | 0,648 - 1,315 | 0,395 |
| ***rpL7*** | REF | 0,8025 | 1,000 | | | |

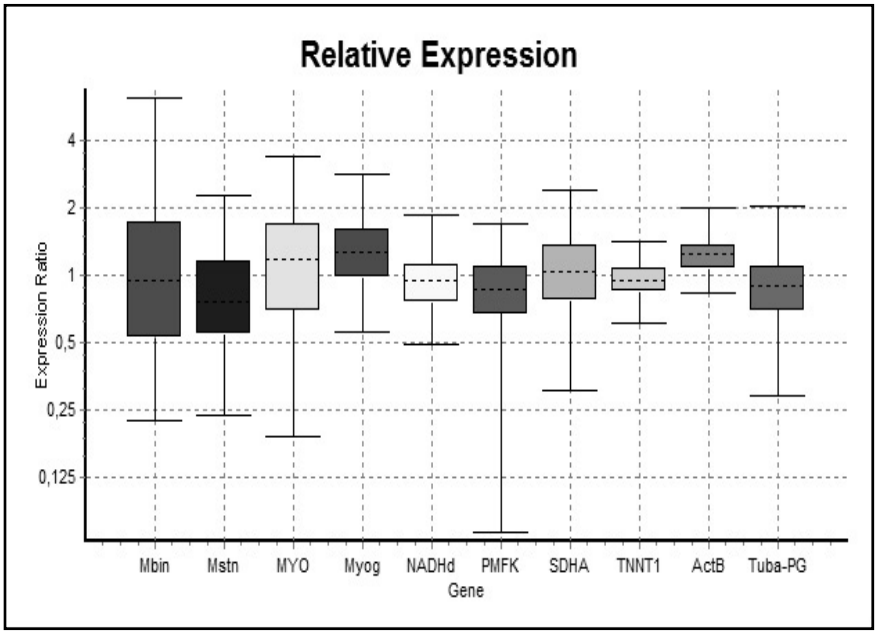





**Figure S1.** REST-RG relative expression output graph of analysis via 2000 iterations. Control groups are set at 1 as default. No sample groups are significantly different expressed compared to their control groups.

## References. Supplementary Material


1. Devoto SH, Melançon E, Eisen J, Westerfield M. 1996 Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* **122**, 3371–3380.
2. Hammill E, Wilson RS, Johnston IA. 2004 Sustained swimming performance and muscle structure are altered by thermal acclimation in male mosquitofish. *J. Therm. Biol.* **29**, 251–257. (doi:10.1016/j.jtherbio.2004.04.002)
3. Stoiber W, Haslett JR, Steinbacher P, Freimüller M, Sänger AM. 2002 Tonic fibres in axial muscle of cyprinid fish larvae: their definition, possible origins and functional importance. *Anat. Embryol. (Berl).* **205**, 113–124. (doi:10.1007/s00429-002-0232-2)
4. Johnston IA, Fernandez DA, Calvo J, Vieira VLA, North AW, Abercromby M, Garland TJ. 2003 Reduction in muscle fibre number during the adaptive radiation of notothenioid fishes: a phylogenetic perspective. *J. Exp. Biol.* **206**, 2595–2609. (doi:10.1242/jeb.00474)
5. Jaspers RT, Testerink J, Della Gaspera B, Chanoine C, Bagowski CP, van der Laarse WJ. 2014 Increased oxidative metabolism and myoglobin expression in zebrafish muscle during chronic hypoxia. *Biol. Open* **3**, 718–27. (doi:10.1242/bio.20149167)
6. Johnston IA, Abercromby M, Vieira VLA, Sigursteindóttir R, Kristjánsson BK, Sibthorpe D, Skúlason S. 2004 Rapid evolution of muscle fibre number in post-glacial

populations of Arctic charr *Salvelinus alpinus*. *J. Exp. Biol.* **207**, 4343–60. (doi:10.1242/jeb.01292)
7. Marschallinger J, Obermayer A, Steinbacher P, Stoiber W. 2013 The zebrafish myotome contains tonic muscle fibers: Morphological characterization and time course of formation. *J. Morphol.* **274**, 320–330. (doi:10.1002/jmor.20095)
8. Bird NC, Windner SE, Devoto SH. 2012 Immunocytochemistry to study myogenesis in zebrafish. In *Myogenesis: Methods in Molecular Biology* (ed JX DiMario), pp. 153–169.(doi:10.1007/978-1-61779-343-1_9)
9. Barresi MJF, D'Angelo JA, Hernández LP, Devoto SH. 2001 Distinct mechanisms regulate slow-muscle development. *Curr. Biol.* **11**, 1432–1438. (doi:10.1016/S0960-9822(01)00428-6)
10. Sellers JR. 2000 Myosins: a diverse superfamily. *Biochim. Biophys. Acta - Mol. Cell Res.* **1496**, 3–22. (doi:10.1016/S0167-4889(00)00005-7)
11. Gomes AV, Potter JD, Szczesna-Cordary D. 2002 The role of troponins in muscle contraction. *IUBMB Life (International Union Biochem. Mol. Biol. Life)* **54**, 323–333. (doi:10.1080/15216540216037)
12. NCBI. 2015 MYOG myogenin (myogenic factor 4) [*Homo sapiens* (human)]. See http://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=4656.
13. Busscher ME. 2015 Primer design for muscle related genes in the live-bearing fish family Poeciliidae. BSc Thesis. Wageningen University.
14. Mouisel E *et al.* 2014 Myostatin is a key mediator between energy metabolism and endurance capacity of skeletal muscle. *Am. J. Physiol. Integr. Comp. Physiol.* **307**, R444–R454. (doi:10.1152/ajpregu.00377.2013)
15. Silverthorn DU. 2007 *Human physiology: an integrated approach*. San Francisco: Pearson Education Inc.
16. van der Meulen T, Schipper H, van den Boogaart JGM, Huising MO, Kranenbarg S, van Leeuwen JL. 2006 Endurance exercise differentially stimulates heart and axial muscle development in zebrafish (*Danio rerio*). *Am. J. Physiol. Integr. Comp. Physiol.* **291**, R1040–R1048. (doi:10.1152/ajpregu.00116.2006)
17. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004 Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**, 509–515. (doi:10.1023/B:BILE.0000019559.84305.47)
18. Vandesompele J, De Preter K, Pattyn F, Poppe B, van Roy N, De Paepe A, Speleman F. 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **18**, research0034.1-research0034.11.
19. Andersen CL, Jensen JL, Ørntoft TF. 2004 Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250. (doi:10.1158/0008-5472.CAN-04-0496)

# Chapter 6

## General Discussion

## 1. Introduction

A live-bearing mode of reproduction implies morphological and physiological constraints. For animals that live in water the constraints are influenced by the characteristics of the aqueous environment. To explore the effects of pregnancy on the morphology and performance of swimming animals, we based our research on the live-bearing fish family Poeciliidae. In **Chapter 2**, we explored the morphological aspect of the *Locomotor cost hypothesis* by comparing the changes in body shape of two closely related species, one placentotrophic (*Poeciliopsis turneri*) which has a well-developed placenta, and one lecithotrophic (*Poeciliopsis gracilis*), which lacks a placenta and allocates all the nutrients needed by the embryo in the eggs prior to fertilization [1,2]. The *Locomotor cost hypothesis* predicts that females with a placenta would have a less-impaired swimming performance than those without it, which would increase their survival rate [3]. It proposes that placental females should have more slender bodies than females without a placenta [3–6 (**Chapter 2**)]. In **Chapter 3, 4** and **5**, we focused on the lecithotrophic species *P. gracilis* and tested the effect of the changes in body shape caused by the reproductive allocation increase (RAI, the increase of the proportion of body mass dedicated to reproduction) on the production of drag (**Chapter 3**). Then, we reported the effect of pregnancy on escape response (**Chapter 4**) and on the muscle morphology and genes associated with the muscle function (**Chapter 5**). In the present chapter, I will discuss the connection between the findings of this thesis and the results from other studies and I will present perspectives for future work.

## 2. How does the placenta affect body shape during pregnancy?

Our results in **Chapter 2** are the first empirical evidence supporting the *Locomotor costs hypothesis* about the changes in body shape due to the presence of a placenta. Females from the placentotrophic species *P. turneri* were more slender than females from the closely related lecithotrophic species, *P. gracilis*, especially at the early stages of pregnancy, and the difference diminished over the course of gestation. The more slender body of *P. turneri* during early pregnancy may reflect its lower reproductive allocation at this stage. The advantages of this more slender body shape lie in smaller cross-sectional and surface areas which might produce a lower drag ([7,8], **Chapter 3**), a lower abdominal volume which enhances local axial bending [7,9–11], and a lower mass which is more easily accelerated [8]. As shown in this thesis, these morphological advantages of a more slender body occur only at the early stages of pregnancy and diminish over the course of gestation.

Additionally, the virgin individuals of the placental species, with their more slender body shape compared to the virgin individuals from the non-placental species, may also benefit from lower locomotor costs thanks to their proportionally smaller cross-sectional

and surface areas [12,13], and to the lower limitations for body axial bending [8,11].

Although our results support the *Locomotor cost hypothesis* for the evolution of the placenta, we only examined one of the three independent origins of this organ in the genus *Poeciliopsis* [1,2]. It is necessary to verify the potential advantage of the placenta within other evolutionary origins before we can conclude that the placenta may have evolved because it reduces RA, increases slenderness and improves swimming performance and fitness [3].

Certainly, there are limitations when working with a single origin of the placenta and with only two species. First, any studied characteristic of the two species might differ by chance.. Secondly, the characteristics of two related species cannot be assumed to be statistically independent. To overcome such difficulty, one could apply the Phylogenetically Independent Contrasts method [14]. It is based on the logic of Felsenstein 1985 [15], under which the character values of phylogenetically related species are transformed to be independent using the knowledge of the species' relationship [14]. We suggest applying the Phylogenetically Independent Contrasts method for exploring the adaptive origin of the placenta, by analysing different origins of the organ within the Poeciliidae family and possibly covering other clades that might have evolved a placenta under similar environmental conditions, such as sharks [16].



Our two-species analysis of the effect of the placenta on the body shape of females, was most likely affected by other reproductive strategies, in particular superfetation. Superfetation is the ability of females to carry simultaneously multiple litters at different levels of embryonic development [17,18]. This strategy diminishes the absolute change in body shape compared to what a species without superfetation would experience during a whole pregnancy [19,20]. *P. turneri* presents a slightly higher level of superfetation than *P. gracilis*, and this might lessen the magnitude of the body-shape changes during a pregnancy period. Because several of the species of the Poeciliidae present a combination of reproductive strategies, we suggest using the multispecies approach explained above to account for the effects of these other traits in future studies. These studies would also benefit from the use of our non-invasive photogrammetry method to record and evaluate body-shape changes.

This morphological study quantified changes in body shape. Nonetheless, to explore the actual function of these changes, we need to test whether more slender body shapes are advantageous for the female.

### 3. How does a pregnancy affect the drag on a female's body?

One of the reasons why the more slender bodies are supposed to represent lower costs for locomotion is because they have smaller cross-sectional and surface areas and therefore are expected to produce lower drag than thicker bodies carrying higher RA [3,7,8]. We

tested this assumption in **Chapter 3** and confirmed that the increase in RA resulted in increased drag. More specifically, we found that drag grew exponentially with the increase of RA, while it grew in a power fashion with speed. The visualization of the flow around the fish showed that flow separation, one of the main sources of body drag, happened behind the abdominal region and that the separation increased with bigger abdomens.

Both the drag results and the flow visualization findings were derived for straight bodies measured at constant ambient speeds. However, in real life Poeciliid fish combine bursts of BCF swimming, where they undulate their bodies, with coasting phases when they experience deceleration. Studying the drag produced during free swimming is experimentally challenging and would require, for example, the placement of pressure and shear stress sensors over the full body surface. Besides the technical difficulties that this represents, this would alter the drag production itself as seen on tagged animals [21,22] and might affect the axial bending of the fish. Another option is the numerical calculation of drag, which requires simulations of a complex event and it is prone to errors due to the complex behaviour of the flow around the fish. However, ultimately we want to derive the locomotor cost of a certain reproductive strategy. A direct way to evaluate this cost, is to measure the oxygen consumption which enables an estimate of the active metabolic rate as an approximation of the energetic costs of locomotion during sustained swimming. Studies on pregnant Poeciliid fish indicate that late in pregnancy females have a high active metabolic rate [23,24], but see [25]. To reveal the costs due to pregnancy, we suggest the comparison of the locomotor costs of pregnant and virgin females. Finally, to explore the differences in energy requirements between reproductive strategies, we once again suggest comparative studies between different origins of the same trait.

All these measures, be they morphological, biomechanical or physiological, must be analysed in light of their effect on swimming performance and ultimately fitness, which is finally on which natural selection acts.

## 4. How does a pregnancy affect the escape response of fish?

The escape response is a repeatable and ecologically relevant performance indicator and we evaluated it on pregnant and virgin females of *P. gracilis* (**Chapter 4**), expecting that pregnancy and the stage of pregnancy would have a significant effect on this behaviour. We predicted that the increase in reproductive allocation of the pregnant females over a pregnancy would cause a decrease of swimming performance. And we assumed that virgin females carry low to none reproductive allotments and therefore their morphology and physiology would not yet be affected by a reproductive burden. Contrary to our predictions, we found that the stage of pregnancy and the reproductive stage (virgin vs pregnant) do not seem to have a significant effect on the escape response of *P. gracilis*, at least for the relatively small brood sizes (mean RAI: 13.5%) observed in our experiments.

Most of the studies on swimming performance of pregnant females in the Poeciliidae family have reported negative effects caused by pregnancy [7,8,26,27]. To my knowledge, there are only two studies showing no effect of the reproductive stage on the locomotor costs during sustained swimming [25,26]. This analyses were done in *Poecilia reticulata* and *Gambusia affinis*, which in other experiments showed negative effects of pregnancy on locomotion [7,8].

We found an unexpectedly high RA in virgin females (figure 1), which might be one of the reasons for the absence of detectable differences in the escape response between pregnant and virgin individuals. In **Chapter 2,** we had already found that the difference in body shape between pregnant and virgin females from this species was relatively low, compared to what is seen for the placental species *P. turneri*. The high RA of virgin *P. gracilis* may represent a considerable burden, and for these virgin females the costs may principally come from having to propel a relatively thick body, but not from other costs of reproduction such as the continuous production of yolked eggs.

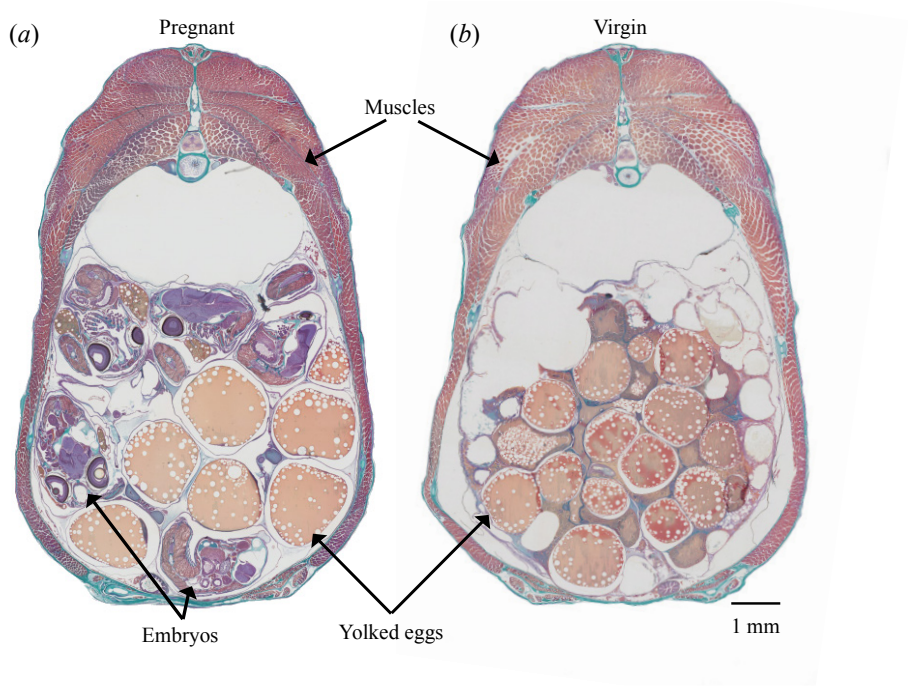



**Figure 1.** Cross-sectional cuts on the abdominal region of a (*a*) pregnant individual at about 2 days before delivery, and (*b*) a virgin individual. Both sections are stained with Crossmon trichome staining.

Another reason for the lack of differences in the escape response, especially between the end of pregnancy and the day of delivery, could be the small body shape variation of *P. gracilis* throughout pregnancy which might be partially caused by superfetation. Because superfetation is expected to lower the peak investment on reproduction [3], the changes in female body shape decrease, diminishing the differences in locomotor costs. All the above reasons point towards relatively small differences in morphology and locomotor performance between pregnant and virgin females of *P. gracilis*.

The absence of significant differences in the escape response might further have been caused (at least partially) by the large variation in the escape behaviour presumably due to a suboptimal stimulus, which may not always have elicited maximum responses. Experimentally, it is difficult to consistently elicit a maximum escape response because individuals can adapt to the stimulus, responding with sub-optimal behaviours. Furthermore, we found that the escape response varied greatly between events of the same individual and between individuals [28]. This variation is important, because it might mask the possible differences in escape performance between pregnant and virgin females. A combination of different stimuli might be the best approach given the variation between and within individuals and their possible adaptation to the stimulus. Furthermore, to diminish the variation of the responses, I suggest restricting the initial direction of the fish with respect to the stimulus by applying a slow water flow, which would maintain the fish in a consistent direction [29]. This set up would require a constant flow with minimum wall effect.

Our findings may have some bearing on the *Locomotor cost hypothesis* and the evolution of the placenta. They suggest that for a lecithotrophic superfetatious species with smaller changes in RA than a placental species (**Chapter 2**), the increase in locomotor cost might be much smaller than for a species with a placenta [3]. Our study design did not allow to test the potentially advantageous effects of the placenta, because we covered a single lecithotrophic species. Consistent with the *Locomotor cost hypothesis*, however, we show that the low RAI in lecithotrophic species corresponds to small (and in our study non-significant) changes on the locomotor performance throughout pregnancy (**Chapter 4**). Furthermore, the finding that virgin females carry a big RA and generate similar escape performances to those of the pregnant females (figure 1) suggests that the locomotor costs caused by carrying unfertilized yolked eggs affects also the locomotor performance of the virgins.

Considering the above, one might argue that the placenta lowers the locomotor costs of females even before fertilization of their eggs, because they are exempt from the burden of carrying already all the nutrients needed for embryo development, and can therefore keep a more slender body shape than virgin females who lack a placenta. This is an argument that has not been proposed before and lends additional support to the *Locomotor cost hypothesis*, because the placenta would also lower the locomotor costs

of virgin females from placental species. Certainly, this premise needs to be proven by carrying out multispecies tests with different origins of the reproductive traits of interest, as mentioned before.

Escape responses and any other swimming behaviours are powered by the fish muscles. And the morphological alterations that we have explored in **Chapters 2** and **3**, together with pregnancy related physiological changes, might alter the muscle morphology. In addition, the expression of genes known to be involved in muscle function, may change with pregnancy and would potentially affect the locomotor performance of the females.

## 5. How does pregnancy affect muscles morphology and the expression of genes that are related to muscle function?

In **Chapter 5**, we presented the first description of the morphology of the axial musculature of pregnant female fish and of the expression of genes related to the muscles. We expected that pregnancy would induce changes in these traits, because in several other clades there is mobilization of nutrients and energy during gravidity from the muscles to the reproductive tissues (fish [30,31], scallops [32], birds [33,34], some insects [35], and snakes [36]). Moreover, the abdominal growth during gestation in some viviparous species stretches the muscle fibres, diminishing the performance of muscles during and even some time after the gravidity period (humans [37–39], rats [40] and possibly fish [11]). Contrary to the expectations, we found no significant differences between pregnant and virgin females in the morphology and the expression of genes relevant to muscle function. However, the unexpectedly high reproductive allocation of virgin females (figure 1) decreases the morphological difference between them and the pregnant ones, which can result in similar production of drag (**Chapter 3**), and in general, similar locomotive needs (**Chapter 4**). This might result in similar muscle architecture to cover those needs (**Chapter 5**). However, large litters were rare in the studied population and our results suggest that thicker females, carrying larger litters kept a comparatively similar quantity of muscle as those with smaller litters. Hence, bigger broods would presumably lower significantly the maximum speed of the female, negatively affecting its swimming performance (**Chapter 4**), because the female would need to move a bigger mass against the higher drag (**Chapter 3**), using a lower proportion of muscles.

Furthermore, we report on an extra region of red and pink muscle fibers present in the belly region of pregnant and virgin individuals. This was another unexpected finding. Like the lateralis superficialis muscle, it might be involved in the undulatory motions of the body, and it might be an extension of the lateralis superficialis muscle. A similar region of slow fibres is found in Antarctic notothenioid fish [41], where it presumably contributes to the undulation in the sub-carangiform swimming during cruising. In *P. gracilis*, the region might facilitate the undulation of the relatively thick bodies of the virgin and pregnant females. However, males also possess this second region

of slow and intermediate muscle fibers (though this region is comparatively smaller in males), arguing against the idea that this region evolved to facilitate sustained swimming of females during pregnancy. This finding indicates the importance of including males in studies that focus on the potential adaptive benefits of muscles to the live-bearing mode of reproduction. Furthermore, these studies on the females should be extended to species with a placenta, because this will grant the possibility of exploring the muscle condition of virgin females which have not yet experienced the burden of producing and carrying yolked eggs, resulting in a significantly more slender body shape (**Chapter 2**).

## 6. Conclusion

We presented different methods for the quantification of the locomotor costs of pregnancy. Our results shed light onto the beneficial effects of having a placenta, which is that species with a placenta have, on average, more slender body shapes throughout pregnancy than those without it. We also quantified for the first time the drag that female fish experience with different reproductive investments showing that the increase in reproductive allocation causes an exponential increase in drag. Furthermore, our study shows that virgin females from lecithotrophic species may experience similar morphological and performance changes to their conspecific pregnant females, because they already carry fully yolked eggs before mating. Our biomechanical approach is essential for the understanding of the evolution of complex traits such as placentation. Nevertheless, to answer such evolutionary questions in the future it is necessary to work with an experimental design that includes different origins of the studied trait and that takes into account the phylogenetic information of each of the species/populations explored.

## References

1. Reznick DN, Mateos M, Springer MS. 2002 Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298**, 1018–1020. (doi:10.1126/science.1076018)
2. Pollux BJA, Meredith RW, Springer MS, Garland TJ, Reznick DN. 2014 The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature* **513**, 233–236. (doi:10.1038/nature13451)
3. Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009 Evolution of placentas in the fish family Poeciliidae: An empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **40**, 271–289. (doi:10.1146/annurev.ecolsys.110308.120209)
4. Thibault RE, Schultz RJ. 1978 Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **32**, 320. (doi:10.2307/2407600)
5. Pires MN, Arendt J, Reznick DN. 2010 The evolution of placentas and superfetation in the fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae: subgenera *Micropoecilia* and *Acanthophacelus*). *Biol. J. Linn. Soc.* **99**, 784–796. (doi:10.1111/j.1095-8312.2010.01391.x)

6. Fleuren M, Quicazan-Rubio EM, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS One* **13**, e0195976. (doi:10.1371/journal.pone.0195976)
7. Plaut I. 2002 Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
8. Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* **164**, 38–50. (doi:10.1086/421412)
9. Noren SR, Redfern JV, Edwards EF. 2011 Pregnancy is a drag: hydrodynamics, kinematics and performance in pre- and post-parturition bottlenose dolphins (*Tursiops truncatus*). *J. Exp. Biol.* **214**, 4151–4159. (doi:10.1242/jeb.059121)
10. Langerhans RB, Reznick DN. 2010 Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics. In *Fish Locomotion*, pp. 200–248. (doi:10.1201/b10190-8)
11. James RS, Johnston IA. 1998 Influence of spawning on swimming performance and muscle contractile properties in the short-horn sculpin. *J. Fish Biol.* **53**, 485–501. (doi:10.1006/jfbi.1998.0722)
12. Pettersson LB, Hedenström A. 2000 Energetics, cost reduction and functional consequences of fish morphology. *Proc. R. Soc. B Biol. Sci.* **267**, 759–764. (doi:10.1098/rspb.2000.1068)
13. Langerhans RB. 2009 Morphology, performance, fitness: functional insight into a post-Pleistocene radiation of mosquitofish. *Biol. Lett.* **5**, 488–491. (doi:10.1098/rsbl.2009.0179)
14. Garland TJ, Adolph SC. 1994 Why not to do two-species comparative studies: limitations on inferring adaptation. *Physiol. Zool.* **67**, 797–828. (doi:10.1086/physzool.67.4.30163866)
15. Felsenstein J. 1985 Phylogenies and the comparative method. *Am. Nat.* **126**, 1–25.
16. Buddle AL, van Dyke JU, Thompson MB, Simpfendorfer CA, Whittington CM. 2019 Evolution of placentotrophy: using viviparous sharks as a model to understand vertebrate placental evolution. *Mar. Freshw. Res.* **70**, 908. (doi:10.1071/MF18076)
17. Turner CL. 1937 Reproductive cycles and superfetation in Poeciliid fishes. *Biol. Bull.* **72**, 145–164. (doi:10.2307/1537249)
18. Scrimshaw NS. 1944 Superfetation in Poeciliid fishes. *Copeia* **1944**, 180–183. (doi:10.2307/1437814)
19. Zúñiga-Vega JJ, Reznick DN, Johnson JB. 2007 Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. *Oikos* **116**, 995–1005. (doi:10.1111/j.2007.0030-1299.15763.x)
20. Frías-Alvarez P, Macías Garcia C, Vázquez-Vega LF, Zúñiga-Vega JJ. 2014 Spatial and temporal variation in superfoetation and related life history traits of two viviparous fishes: *Poeciliopsis gracilis* and *P. infans*. *Naturwissenschaften* **101**, 1085–1098. (doi:10.1007/s00114-014-1247-2)
21. van der Hoop JM. 2017 Effects of added drag on cetaceans: fishing gear entanglement and external tag attachment. PhD dissertation. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution. USA.
22. Suzuki I, Sato K, Fahlman A, Naito Y, Miyazaki N, Trites AW. 2014 Drag, but not buoyancy, affects swim speed in captive Steller sea lions. *Biol. Open* **3**, 379–386.

(doi:10.1242/bio.20146130)
23. Timmerman CM, Chapman LJ. 2003 The effect of gestational state on oxygen consumption and response to hypoxia in the sailfin molly, *Poecilia latipinna*. *Environ. Biol. Fishes* **68**, 293–299. (doi:10.1023/A:1027300701599)
24. Seebacher F, Ward AJW, Wilson RS. 2013 Increased aggression during pregnancy comes at a higher metabolic cost. *J. Exp. Biol.* **216**, 771–776. (doi:10.1242/jeb.079756)
25. Svendsen JC, Banet AI, Christensen RHB, Steffensen JF, Aarestrup K. 2013 Effects of intraspecific variation in reproductive traits, pectoral fin use and burst swimming on metabolic rates and swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *J. Exp. Biol.* **216**, 3564–74. (doi:10.1242/jeb.083089)
26. Belk MC, Tuckfield RC. 2010 Changing costs of reproduction: age-based differences in reproductive allocation and escape performance in a livebearing fish. *Oikos* **119**, 163–169. (doi:10.1111/j.1600-0706.2009.17742.x)
27. Fleuren M. 2017 Reproductive adaptations to reduce locomotor costs in viviparous fish (Poeciliidae). PhD thesis. Wageningen University and Research, Wageningen, The Netherlands. (doi:10.18174/424276)
28. Eaton RC, DiDomenico R, Nissanov J. 1988 Flexible body dynamics of the goldfish C-start: implications for reticulospinal command mechanisms. *J. Neurosci.* **8**, 2758–2768. (doi:10.1523/JNEUROSCI.08-08-02758.1988)
29. Tytell ED, Lauder GV. 2008 Hydrodynamics of the escape response in bluegill sunfish, *Lepomis macrochirus*. *J. Exp. Biol.* **211**, 3359–69. (doi:10.1242/jeb.020917)
30. Johnston IA, Bower NI, Macqueen DJ. 2011 Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol.* **214**, 1617–1628. (doi:10.1242/jeb.038620)
31. Love RM. 1980 *The chemical biology of Fishes*. London: Academic Press.
32. Brokordt KB, Himmelman JH, Guderley HE. 2000 Effect of reproduction on escape responses and muscle metabolic capacities in the scallop *Chlamys islandica* Muller 1776. *J. Exp. Mar. Bio. Ecol.* **251**, 205–225. (doi:10.1016/S0022-0981(00)00215-X)
33. Selman RG, Houston DC. 1996 A technique for measuring lean pectoral muscle mass in live small birds. *Ibis (Lond. 1859).* **138**, 348–350. (doi:10.1111/j.1474-919X.1996.tb04353.x)
34. Veasey JS, Houston DC, Metcalfe NB. 2001 A hidden cost of reproduction: the trade-off between clutch size and escape take-off speed in female zebra finches. *J. Anim. Ecol.* **70**, 20–24. (doi:10.1111/j.1365-2656.2001.00476.x)
35. Chapman RF. 2013 *The insects: structure and function*. New York: Cambridge University Press.
36. Lourdais O, Brischoux F, DeNardo DF, Shine R. 2004 Protein catabolism in pregnant snakes (*Epicrates cenchria maurus* Boidae) compromises musculature and performance after reproduction. *J. Comp. Physiol. B* **174**, 383–391. (doi:10.1007/s00360-004-0424-6)
37. Coldron Y, Stokes MJ, Newham DJ, Cook K. 2008 Postpartum characteristics of rectus abdominis on ultrasound imaging. *Man. Ther.* **13**, 112–121. (doi:10.1016/j.math.2006.10.001)
38. Kostyo JL. 1956 A study of the glycogen levels of the rat uterus and certain skeletal muscles during pregnancy. **60**, 33–37.
39. Gilleard WL, Brown JMM. 1996 Structure and function of the abdominal muscles in primigravid subjects during pregnancy and the immediate postbirth period. *Phys.*

*Ther.* **76**, 750–762. (doi:10.1093/ptj/76.7.750)
40. Vesentini G *et al.* 2018 Morphological changes in rat rectus abdominis muscle induced by diabetes and pregnancy. *Brazilian J. Med. Biol. Res.* **51**, 1–10. (doi:10.1590/1414-431X20177035)
41. Dunn JF, Archer SD, Johnston IA. 1989 Muscle fibre types and metabolism in post-larval and adult stages of notothenioid fish. *Polar Biol.* **9**, 213–223. (doi:10.1007/BF00263769)

# Summary

A live-bearing mode of reproduction implies morphological and physiological constraints, which in fish might be influenced by the physical characteristics of water: a higher density which favours hydrodynamic body shapes and requires an extensive muscle system to propel the bodies through the dense liquid. This thesis aims to improve the understanding of the relationship between the morphological changes caused by pregnancy and the swimming performance of fish. I based my research on the live-bearing fish family Poeciliidae, which presents a wide variation of maternal provisioning that range from feeding their internally developing embryos exclusively from the nutrients that have been stored in the egg-yolk prior to fertilization (lecithotrophy), to almost exclusively nourish them through a placenta (placentotrophy).

A live-bearing mode of reproduction may increase the volume and mass of the female throughout the reproductive cycle increasing the costs of locomotion. Placentation may have evolved to reduce these costs as is proposed by the Locomotor cost hypothesis. In **Chapter 2**, we explored the morphological aspects of this hypothesis, which predicts that females with a placenta have slender bodies than those without it, especially at the early stages of pregnancy, and that the difference diminishes over the course of gestation. This may indicate that females with a placenta would have a less-impaired swimming performance than those without it, which would increase their survival rate. We tested this hypothesis by comparing the three-dimensional body shape changes between two closely related species, one placentotrophic (*Poeciliopsis turneri*) and one lecithotrophic (*Poeciliopsis gracilis*). For this, we developed and used a non-invasive photogrammetry method to document the three-dimensional shape and volume changes through pregnancy. Our findings supported the *Locomotor costs hypothesis* by showing that females with a placenta possessed more slender bodies in the early stages of pregnancy compared to the non-placental one, and that this advantage diminished over the course of gestation. Moreover, body thickness increased faster in the placental species than in the non-placental one. This provided the first empirical evidence for a possible adaptive morphological advantage of the placenta in live-bearing fish.

In both lecithotrophy and placentotrophy, the growing embryos increased the volume of the female and consequently its body surface and cross-sectional area. This could increase body drag, presumably causing a decrease of the maximum swimming speed and increasing the metabolic costs of locomotion. Ultimately this may decrease the fitness of the female by making her an easier predation target. However, the effects of pregnancy and the level of reproductive investment on the production of drag have not been evaluated so far in live-bearing fish. In **Chapter 3**, we examined the effect of the reproductive allocation increase (RAI, the increase in the proportion of body mass

dedicated to reproduction) on the body drag of pregnant fish. We generated 3D printed models of females of *P. gracilis* in a straight body posture with different levels of RAI. And then we measured the drag and visualized the flow around them in a flow tunnel at different speeds. We confirmed that higher RAI resulted in increased drag, and more specifically we found that drag grew exponentially with higher RAI, while it grew in a power fashion with the increase of speed. The visualization of the flow around the fish showed that flow separation, one of the main sources of body drag, happened behind the abdominal region and that the separation increased with bigger abdomens.

All of these measurements, be they morphological, biomechanical or physiological, must be analysed taking into account their effect on swimming performance and ultimately fitness, which is finally on which natural selection acts. In **Chapter 4**, we analysed the effect of pregnancy on the escape response, the main defence of many taxa against predator attacks. We elicited and measured the escape response of pregnant and virgin females of *P. gracilis* before and just after giving birth, and filmed it with three orthogonal high-speed cameras. We expected that pregnancy and the stage of gestation would have a significant effect, as it is the case for other fish species, and we assumed that virgin females carry low to none reproductive allotments and therefore their morphology and physiology would not be affected by the burden of reproduction. Contrary to our predictions, we found that the stage of pregnancy and the reproductive stage (virgin vs pregnant) did not seem to have a significant effect on the escape response of *P. gracilis*, at least for the relatively small brood sizes (mean RAI: 13.5%) happening throughout the experiments. The absence of significant differences could be due to the small body shape variation of *P. gracilis* throughout pregnancy which might be partially caused by superfetation (the ability of females to carry simultaneously multiple litters at different levels of embryonic development). Moreover, we found an unexpectedly high RA in virgin females, which might be one of the reasons for the absence of detectable differences between pregnant and virgin individuals. This suggests that the higher locomotor costs caused by carrying the unfertilized yolked eggs might affect also the locomotor performance of the virgin females. These findings may have some bearing on the Locomotor cost hypothesis and the evolution of the placenta because they suggest that: (i) for a lecithotrophic, superfetatious species which has smaller changes in RA than a species with a placenta (**Chapter 2**), the increase in locomotor cost might be much smaller than for a species with a placenta, and (ii) that the placenta might lower the locomotor costs of females even before fertilization of their eggs, because they are exempt from the burden of carrying already all the nutrients needed for the embryo development, and can therefore keep a more slender body shape than virgin females who lack a placenta. The absence of significant differences in the escape response might further have been caused (at least partially) by the large variation in the escape responses presumably due to a suboptimal stimulus.

Escape responses and any other swimming behaviours are powered by the fish

muscles. During pregnancy, the mobilization of energy from muscles to reproductive tissues and the extension of the abdomen, might negatively affect the contractile properties of the muscles and their power output as it has been shown for gravid females of other clades. Yet, no information is available for live-bearing fish on the possible morphological and physiological adaptations to their reproductive mode. In **Chapter 5**, we described for the first time the muscle morphology and the activity of genes that express essential components for muscle function for gravid fish. For pregnant and virgin females of *P. gracilis*, we stained with histological methods transverse sections of the abdominal region and performed real-time quantitative PCR on the muscles in the caudal peduncle to quantify the transcription levels of genes that are particularly expressed in muscles. Our expectation was that pregnancy would induce changes in the muscle morphology and gene expression. Contrary to the expectations, we found no significant differences between pregnant and virgin females. However, the unexpectedly high reproductive allocation of virgin females decreases the morphological difference between them and the pregnant ones, which can result in similar production of drag (**Chapter 3**), and in general, similar locomotive needs (**Chapter 4**). And this might result in similar muscle architecture to cover those needs (**Chapter 5**). Another unexpected result was the finding of a newly reported extra zone of red and pink muscle fibers present in the belly region of pregnant and virgin individuals. However, males also possess this second region (though it is comparatively smaller), arguing against the idea that this region evolved to facilitate sustained swimming of females during pregnancy.

Our study shows that pregnancy causes changes in the body shape of females and that those changes might be related to the reproductive strategy of the species. We demonstrated how increasing reproductive allocation affects the drag production during coasting, and we expect that the scaling of drag, depending on RAI and speed that we found, may be indicative for the undulatory swimming as well. Moreover, our findings suggest that the lower morphological changes experienced by lecithotrophic, superfetatious species might be reflected in smaller or no significant effects on the muscle morphology and escape response. Our biomechanical approach is essential for the understanding of the evolution of complex traits such as placentation. However, to verify the morphological and performance advantages that different reproductive traits confer and that result in higher fitness, it is necessary to perform multispecies comparisons addressing different origins of the reproductive trait.

# Samenvatting

Een levendbarende reproductiemodus impliceert morfologische en fysiologische beperkingen, die bij vissen mede worden beïnvloed door de fysische eigenschappen van water: de hoge dichtheid bevordert een gestroomlijnde lichaamsvorm en vereist een effectief spiersysteem voor de voortstuwing van het vissenlichaam door de dichte vloeistof. Dit proefschrift heeft tot doel de relatie te onderzoeken tussen de morfologische veranderingen veroorzaakt door zwangerschap en de zwemprestaties van vissen. Ik baseerde mijn onderzoek op de levendbarende familie Poeciliidae, die een grote variatie aan maternale voorzieningen vertoont, variërend van het voeden van hun intern ontwikkelende embryo's met voedingsstoffen die uitsluitend vóór de bevruchting in de eidooier worden opgeslagen (lecithotrofie), tot het bijna uitsluitend voeden via een placenta (placentotrofie).

Een levendbarend voortplantingsmechanisme kan het volume en de massa van de moeder tijdens de voortplantingscyclus verhogen, waardoor de kosten van voortbeweging toenemen. Placentatie is mogelijk geëvolueerd om deze kosten te verlagen, zoals wordt voorgesteld in de hypothese van voortbewegingskosten. In **Hoofdstuk 2** verkenden we de morfologische aspecten van deze hypothese, die voorspelt dat vrouwelijke vissen met een placenta vooral in de vroege stadia van de zwangerschap slankere lichamen hebben dan degenen zonder een placenta, en dat het verschil in de loop van de zwangerschap afneemt. Dit kan erop duiden dat placentotrofe vissen een minder aangetaste zwemprestatie hebben dan lecithotrofe vissen, hetgeen hun overlevingskansen zou kunnen verhogen. We testten deze hypothese door de driedimensionale vormveranderingen van het lichaam te vergelijken tussen twee nauw verwante soorten, een placentotrofe (*Poeciliopsis turneri*) en een lecithotrofe (*Poeciliopsis gracilis*). Hiervoor hebben we een niet-invasieve fotogrammetriemethode ontwikkeld en gebruikt om de driedimensionale vorm- en volumeveranderingen tijdens de zwangerschap te documenteren. Onze bevindingen ondersteunen de hypothese van de voortbewegingskosten: vrouwelijke vissen met een placenta hadden een slanker lichaam in de vroege stadia van de zwangerschap in vergelijking met de lecithotrofe vissen, en dit voordeel nam in de loop van de zwangerschap af. Bovendien nam de dikte van het lichaam sneller toe in de placentotrofe vissen dan in de lecithotrofe vissen. Dit leverde het eerste empirische bewijs voor een mogelijk adaptief morfologisch voordeel van de placenta in levendbarende vissen.

Bij zowel lecithotrofie als placentotrofie verhoogden de groeiende embryo's het volume van de moeder en bijgevolg het lichaamsoppervlak en het oppervlak van de dwarsdoorsnede. Dit zou de hydrodynamische weerstand van het lichaam kunnen verhogen, hetgeen vermoedelijk een afname van de maximale zwemsnelheid en verhoging van de metabole kosten van voortbeweging veroorzaakt. Uiteindelijk kan

dit de 'fitness' van de moeder verminderen doordat zij een gemakkelijker te vangen prooi is. De effecten van zwangerschap en de daaraan gerelateerde reproductieve investeringen op de hydrodynamische weerstand waren echter nog niet geëvalueerd in levendbarende vissen. In **Hoofdstuk 3** onderzochten we het effect van de toename van de reproductieve investeringen (Eng: reproductive allocation increase (RAI), de toename van het lichaamsmassa dat aan reproductie gewijd is) op de hydrodynamische weerstand van zwangere vissen. We produceerden 3D-geprinte modellen van vrouwelijke vissen met verschillende RAI-niveaus van de soort *P. gracilis* in een rechte lichaamshouding. Daarna maten we de weerstand en visualiseerden we de stroming om de modellen in een stroomtunnel met verschillende snelheden. We vonden inderdaad dat een hogere RAI resulteerde in een verhoogde weerstand; de weerstand nam exponentieel toe met een hogere RAI en via een machtsfunctie met een hogere snelheid. De visualisatie van de stroming rond de vis liet zien dat loslating van de stroming, een van de belangrijkste bronnen van hydrodynamische weerstand van het lichaam, plaatsvond achter de buikstreek en dat de loslating toenam met een groter abdomen.

In de analyse van al deze metingen, of ze nu morfologisch, biomechanisch of fysiologisch zijn, dient rekening te worden gehouden met het effect van de parameters op de zwemprestaties en het het genereren van vruchtbare nakomelingen, hetgeen uiteindelijk is waarop de natuurlijke selectie werkt. In **Hoofdstuk 4** hebben we het effect van zwangerschap op de ontsnappingsrespons geanalyseerd, de belangrijkste verdediging van veel taxa tegen aanvallen van roofdieren. We hebben de ontsnappingsrespons van zwangere vissen van de soort *P. gracilis* vóór en vlak na de bevalling uitgelokt en gefilmd met drie orthogonaal opgestelde hogesnelheidscamera's. Op dezelfde dagen hebben we de ontsnappingsreacties van maagdelijke vissen gefilmd. We verwachtten dat zwangerschap en het stadium van de zwangerschap een significant effect zouden hebben, zoals het geval is bij andere vissoorten, en we veronderstelden dat de maagden weinig of geen reproductieve investeringen hebben en daarom zouden hun morfologie en fysiologie niet beïnvloed worden door een reproductieve last. In tegenstelling tot onze voorspellingen, vonden we dat het stadium van zwangerschap en de reproductieve fase (maagd versus zwanger) geen significant effect had op de ontsnappingsreactie van *P. gracilis*, althans voor de relatief kleine broedgroottes (gemiddelde RAI: 13,5%) die optraden tijdens de experimenten. De afwezigheid van significante verschillen kan samenhangen met de geringe veranderingen in lichaamsvorm van *P. gracilis* tijdens de zwangerschap, welke gedeeltelijk zouden kunnen worden veroorzaakt door superfetatie (het vermogen van vrouwen om tegelijkertijd meerdere broedsels te dragen op verschillende niveaus van embryonale ontwikkeling). Bovendien vonden we een onverwacht hoge reproductieve investering (Eng: reproductive allocation, RA) bij maagdelijke vrouwen, wat een van de redenen kan zijn voor de afwezigheid van detecteerbare verschillen tussen zwangere en maagdelijke vissen. Dit suggereert dat de voortbewegingskosten die worden veroorzaakt

door het dragen van de onbevruchte dooiereieren ook van invloed kunnen zijn op de voortbewegingsprestaties van de maagden. Deze bevindingen kunnen van invloed zijn op de hypothese van de voortbewegingskosten en de evolutie van de placenta, omdat ze suggereren dat: (i) voor een lecithotrofe soort met superfetatie, en daarmee kleinere veranderingen in RA dan voor een soort met een placenta (**Hoofdstuk 2**), de toename in voortbewegingskosten kleiner kan zijn dan voor een soort met een placenta, en (ii) dat de placenta zelfs vóór de bevruchting de voortbewegingskosten van vrouwelijke vissen zou kunnen verlagen, omdat ze slechts in zeer geringe mate voedingsstoffen hoeven op te slaan voor de embryonale ontwikkeling, waarmee ze een slankere lichaamsvorm kunnen aanhouden dan maagden zonder placenta. De afwezigheid van significante verschillen in de ontsnappingsrespons zou verder (tenminste gedeeltelijk) kunnen zijn veroorzaakt door de grote variatie in de ontsnappingsreacties, vermoedelijk als gevolg van een suboptimale stimulus.

Spieren leveren het mechanische vermogen dat nodig is voor de ontsnappingsreacties en ander zwemgedrag. Tijdens de zwangerschap kan de mobilisatie van energie van spieren voor voortplantingsweefsel en de extensie van de buik een negatief effect hebben op de samentrekkende eigenschappen van de spieren en hun vermogensleverantie, zoals is aangetoond voor zwangere vrouwen van andere taxa. Toch is er geen informatie beschikbaar voor levendbarende vissen over de mogelijke morfologische en fysiologische aanpassingen aan hun wijze van voortplanten. In **Hoofdstuk 5** beschreven we voor de eerste keer de spiermorfologie en de expressie van genen die belangrijke componenten aanleveren voor spierfunctie van zwangere vissen. Voor zwangere en maagdelijke vrouwen van *P. gracilis* kleurden we met specifieke histologische methoden transversale secties van het abdominale gebied en met real-time kwantitatieve PCR bepaalden we de transcriptieniveaus van genen die in het bijzonder tot expressie worden gebracht in spieren in de staartwortel. Wij verwachtten dat zwangerschap veranderingen zou veroorzaken in de spiermorfologie en genexpressie. We vonden daarentegen geen significante verschillen tussen zwangere en maagdelijke vrouwen. De onverwacht hoge reproductieve investeringen door de maagden vermindert echter het morfologische verschil met de zwangere vissen, hetgeen kan resulteren in een vergelijkbare productie van hydrodynamische weerstand (**Hoofdstuk 3**), en in het algemeen, vergelijkbare eisen aan de locomotie (**Hoofdstuk 4**). Dit kan tevens resulteren in een vergelijkbare spierarchitectuur om aan die eisen te voldoen (**Hoofdstuk 5**). Een ander onverwacht resultaat was de ontdekking van een nieuwe extra zone van rode en roze spiervezels die aanwezig is in het buikgebied van maagden en zwangere vissen. Mannelijke vissen beschikken echter ook over dit tweede gebied (hoewel het relatief kleiner is) hetgeen het idee weerspreekt dat dit gebied is geëvolueerd om het zwemmen van vrouwtjes tijdens de zwangerschap te vergemakkelijken.

Ons onderzoek toont aan dat zwangerschap veranderingen in de lichaamsvorm van vrouwelijke vissen veroorzaakt en dat die veranderingen mogelijk verband houden

met de reproductieve strategie van de soort. We hebben aangetoond in welke mate de investeringen in voortplantingsweefsels de hydrodynamische weerstand tijdens het 'uitglijden' van de vis beïnvloedt, en we verwachten dat de gevonden RAI- en de snelheidheidsafhankelijke schaling van de hydrodynamische weerstand ook indicatief kan zijn voor undulerend zwemmen. Bovendien suggereren onze bevindingen dat de geringere morfologische veranderingen die optreden bij lecithotrofe soorten met superfetatie resulteren in kleinere of geen significante effecten op de spiermorfologie en de ontsnappingsreactie. Onze biomechanische benadering is essentieel voor het begrijpen van de evolutie van complexe eigenschappen zoals placentatie. Ter verificatie van de morfologische en prestatievoordelen die het gevolg zijn van verschillende reproductieve eigenschappen en welke kunnen resulteren in een hogere 'fitness' is het echter noodzakelijk om vergelijkingen met meerdere soorten uit te voeren, met verschillende oorsprongen van de reproductieve eigenschappen.

# Resumen

Un modo de reproducción vivíparo conlleva restricciones morfológicas y fisiológicas, que en los peces podrían verse influenciadas por las características físicas del agua: una densidad más alta que favorece las formas hidrodinámicas del cuerpo y que requiere un sistema muscular extenso para impulsar los cuerpos a través del denso líquido. Esta tesis tiene como objetivo mejorar la comprensión de la relación entre los cambios morfológicos causados por el embarazo y el rendimiento de natación de los peces. Basé mi investigación en la familia de peces vivíparos Poeciliidae, que presenta una amplia variedad de aprovisionamiento materno que va desde alimentar a sus embriones en desarrollo exclusivamente a partir de los nutrientes que se han almacenado en la yema de huevo antes de la fertilización (lecitotrofía), hasta nutrirlos casi exclusivamente a través de una placenta (placentotrofía).

Un modo de reproducción vivíparo puede aumentar el volumen y la masa de la hembra a lo largo del ciclo reproductivo, lo que aumenta los costos de locomoción. La placentación puede haber evolucionado para reducir estos costos, tal como lo propone la hipótesis del costo locomotor. En el **Capítulo 2**, exploramos el aspecto morfológico de esta hipótesis, que predice que las hembras con una placenta tendrían cuerpos más delgados que las que no la tienen, especialmente en las primeras etapas del embarazo, y que la diferencia disminuiría en el transcurso de la gestación. Esto puede indicar que las hembras con placenta tendrían un mejor desempeño en la natación que las que no la tienen, lo que aumentaría su tasa de supervivencia. Probamos esta hipótesis comparando los cambios de forma tridimensional del cuerpo entre dos especies estrechamente relacionadas, una placentotrófica (*Poeciliopsis turneri*) y una lecitotrófica (*Poeciliopsis gracilis*). Para esto, desarrollamos y utilizamos un método de fotogrametría no invasivo para documentar los cambios tridimensionales de forma y volumen durante el embarazo. Nuestros hallazgos apoyaron la hipótesis de los costos locomotores al mostrar que las hembras con placenta poseían cuerpos más delgados en las primeras etapas del embarazo en comparación con las no placentarias, y que esta ventaja disminuía durante el transcurso de la gestación. Además, el grosor del cuerpo aumentaba más rápidamente en las especies placentarias que en las no placentarias. Esto proporcionó la primera evidencia empírica de una posible ventaja morfológica adaptativa de la placenta en peces vivíparos.

Tanto en la lecitotrofía como en la placentotrofía, los embriones en crecimiento aumentaron el volumen de la hembra y, por consiguiente, la superficie de su cuerpo y el área de la sección transversal. Esto podría aumentar la resistencia hidrodinámica del cuerpo, probablemente causando una disminución de la velocidad máxima de natación y aumentando los costos metabólicos de la locomoción. En última instancia, esto puede disminuir la adecuación biológica de la hembra, o 'fitness', al hacer que sea un objetivo

de depredación más fácil. Sin embargo, los efectos del embarazo y el nivel de inversión reproductiva en la producción de arrastre hidrodinámico no se han evaluado hasta el momento en peces vivíparos. En el **Capítulo 3**, examinamos el efecto del aumento de la asignación reproductiva (RAI, por sus siglas en inglés, que es el aumento en la proporción de masa corporal dedicada a la reproducción) sobre el arrastre hidrodinámico de peces preñados. Generamos modelos impresos en 3D de hembras de *P. gracilis* en una postura corporal recta con diferentes niveles de RAI. Y luego medimos el arrastre y visualizamos el flujo alrededor de ellos en un túnel de flujo a diferentes velocidades. Confirmamos que una mayor RAI daba como resultado un mayor arrastre, y más específicamente encontramos que el arrastre creció exponencialmente con una mayor RAI, mientras que creció cuadráticamente con el aumento de la velocidad. La visualización del flujo alrededor de los peces mostró que la separación del flujo, una de las principales fuentes de arrastre del cuerpo, ocurrió detrás de la región abdominal y que la separación aumentó con abdómenes más grandes.

Todas estas mediciones, ya sean morfológicas, biomecánicas o fisiológicas, deben analizarse teniendo en cuenta su efecto sobre el rendimiento de la natación y, en última instancia, sobre la adecuación biológica, que es finalmente sobre la que actúa la selección natural. En el **Capítulo 4**, analizamos el efecto del embarazo en la respuesta de escape, la principal defensa de muchos taxones contra los ataques de depredadores. Obtuvimos y medimos la respuesta de escape de las hembras preñadas y vírgenes de *P. gracilis* antes y justo después de dar a luz, y la filmamos con tres cámaras ortogonales de alta velocidad. Esperábamos que el embarazo y la etapa de gestación tuvieran un efecto significativo, como es el caso de otras especies de peces, y asumimos que las hembras vírgenes no tienen ninguna asignación reproductiva y, por lo tanto, su morfología y fisiología no se verían afectadas por la carga de reproducción. Contrariamente a nuestras predicciones, encontramos que la etapa del embarazo y la etapa reproductiva (virgen vs. embarazada) no parecen tener un efecto significativo en la respuesta de escape de *P. gracilis*, al menos para los tamaños de camada relativamente pequeños (media de la RAI: 13.5%) que sucedieron a lo largo de los experimentos. La ausencia de diferencias significativas podría deberse a la pequeña variación en la forma del cuerpo de *P. gracilis* durante el embarazo que podría ser causada en parte por la superfetación (la capacidad de las hembras de llevar simultáneamente múltiples camadas en diferentes niveles de desarrollo embrionario). Además, encontramos una RA inesperadamente alta en las hembras vírgenes, lo que podría ser una de las razones de la ausencia de diferencias detectables entre las hembras embarazadas y vírgenes. Esto sugiere que los costos locomotores más altos causados por transportar los huevos con yema no fertilizados podrían afectar también el rendimiento locomotor de las hembras vírgenes. Estos hallazgos pueden tener alguna relación con la hipótesis del costo locomotor y la evolución de la placenta porque sugieren que: (i) para una especie con superfetación y lecitotrófica que tiene cambios más pequeños en la AR

que una especie con placenta (**Capítulo 2**), el aumento del costo de locomoción puede ser mucho menor que para una especie con placenta, y (ii) que la placenta podría reducir los costos de locomoción de las hembras incluso antes de la fertilización de sus huevos, ya que están exentas de la carga de llevar ya todos los nutrientes necesarios para el desarrollo embrionario, y por lo tanto pueden mantener una forma del cuerpo más delgada que las hembras vírgenes que carecen de placenta. La ausencia de diferencias significativas en la respuesta de escape podría haber sido causada además (al menos parcialmente) por la gran variación en las respuestas de escape, probablemente debido a un estímulo subóptimo.

Las respuestas de escape y cualquier otro comportamiento de natación son propulsados por los músculos de los peces. Durante el embarazo, la movilización de energía de los músculos a los tejidos reproductivos y la extensión del abdomen, podrían afectar negativamente las propiedades contráctiles de los músculos y su potencia, como se ha demostrado para las hembras grávidas de otros clados. Sin embargo, no hay información disponible para los peces vivíparos sobre las posibles adaptaciones morfológicas y fisiológicas a su modo reproductivo. En el **Capítulo 5**, describimos por primera vez la morfología muscular y la actividad de los genes que expresan la función muscular de las peces grávidas. Para hembras embarazadas y vírgenes de *P. gracilis*, se tiñeron secciones transversales de la región abdominal con métodos histológicos y se realizó una PCR cuantitativa en tiempo real en los músculos del pedúnculo caudal para cuantificar los niveles de transcripción de genes que se expresan particularmente en los músculos. Nuestra expectativa era que el embarazo induciría cambios en la morfología muscular y la expresión genética. Contrariamente a lo esperado, no encontramos diferencias significativas entre hembras embarazadas y vírgenes. Sin embargo, la asignación reproductiva inesperadamente alta de las hembras vírgenes disminuyó la diferencia morfológica entre ellas y las embarazadas, lo que puede resultar en una producción similar de arrastre (**Capítulo 3**), y en general, en necesidades de locomotoras similares (**Capítulo 4**). Y esto podría resultar en una arquitectura muscular similar para cubrir esas necesidades (**Capítulo 5**). Otro resultado inesperado fue el hallazgo de una zona adicional de fibras musculares rojas y rosadas presentes en la región del vientre de hembras embarazadas y vírgenes, que se reporta por primera vez. Sin embargo, los machos también poseen esta segunda región (aunque es comparativamente más pequeña), argumentando en contra de la idea de que esta región evolucionó para facilitar la natación sostenida de las hembras durante el embarazo.

Nuestro estudio muestra que el embarazo causa cambios en la forma del cuerpo de las hembras y que esos cambios podrían estar relacionados con la estrategia reproductiva de la especie. Demostramos cómo el aumento de la asignación reproductiva afecta la producción de arrastre durante el deslizamiento por inercia, y esperamos que la proporción del arrastre, dependiendo de la RAI y la velocidad que encontramos, también sea indicativa de lo que pasa en la natación no periódica. Además, nuestros hallazgos

sugieren que los cambios morfológicos más leves experimentados por las especies con superfetación y lecitotrofía podrían reflejarse en efectos más pequeños o no significativos sobre la morfología muscular y la respuesta de escape. Nuestro enfoque biomecánico es esencial para la comprensión de la evolución de rasgos complejos como la placentación. Sin embargo, para verificar las ventajas morfológicas y de rendimiento que confieren los diferentes rasgos reproductivos y que resultan en una mejor adecuación biológica, es necesario realizar comparaciones entre múltiples especies que aborden diferentes orígenes del rasgo reproductivo.

# Acknowledgements

I am very grateful to those who made this PhD possible, and most importantly who made this experience a very enriching one. First of all, I thank my promotor Johan van Leeuwen and co-promotor Bart Pollux. They, despite the difficulties experienced during my PhD, persevered by my side, guiding me through each new endeavour, also during the time when we had an ocean in between. They contributed immensely to my professional and personal growth. Dear Johan, thank you from the bottom of my heart for welcoming me at EZO. I am forever grateful to you for your constant guidance, for always making time for me, for helping me take my projects to the next level and for encouraging me to embrace the challenges. You lead by example, showing me that adapting to changes opens up new opportunities. Dear Bart, you introduced me to the world of ecology and evolution of fish. Thank you for dedicating your time and sharing your knowledge also at periods when fieldtrips and several occupations came your way.

While attending a seminar at the Naturalis Museum in Leiden, I met Eize Stamhuis and later we worked together in the drag project at his lab in the Ocean Ecosystems Group, at the University of Groningen. Eize, you trusted me and welcomed me in your lab. Your keen mentorship and great lab meetings helped me take firmer steps in my scientific path, and I am forever grateful to you for that. Thank you for caring, thank you for your friendship.

In the EZO Group, I lived my first experiences as a foreign student, right before starting my MSc. in California. I cherished that time and came back to pursue my PhD. I worked together with Mike Fleuren. Working as a team with you, Mike, has been one of the best learning experiences for me. Overcoming our differences, bringing our strengths to the table, and supporting each other, taught me the great value of varied teams. Thanks to your insights and personal way of working, you helped me see my blind spots. Also, your perseverance and work towards the completion of your degree, showed me that I could do it too, despite the difficulties. Dear Henk, your constant support and openness to talk about life and photography, always cheered me up. Thank you for all your work on so many aspects of my PhD, including your guidance on how to proceed with the muscle tests and how to supervise students. All the laughs and all the candies sweetened even more the shared times. Thank you so much for being my paranymph. Remco, I much appreciated all your help with the preparation of the setups. Your work always surpassed the expectations and kept the experiments going. Cees, a big thank you for your continuous support and lots of patience when I needed help with the codes. Your keen feedback always improved the projects. Karen, deep thanks for all your work on the muscle project. I enjoyed our conversations about life, family, and about the activities to keep our lives balanced. Annemarie, thank you for your calmness and sweetness when helping me through the various administrative steps of this academic path. Coming from a faraway

country, I deeply appreciated your care on making sure I had everything I needed to do my work. The candies and conversations at your office brighten my day too :). Ansita, I was so lucky to have you as my office mate and become friends with you. Thank you for being my paranymph and for all your support through the years! Our trips to Utrecht and to the African and Brazilian dance classes were such a great highlight of that time when we decided to broaden our Pakistani and Colombian moves. Uroš, Ursinho. Our chats were amusing and fun from the very beginning, some lack logic, but let's don't lose that ;). Your words and help throughout my PhD, accompanied with your pumpkin soup and Tiramisu meant a lot to me. Sebas, I much appreciated the countless conversations we had and the times shared. Your devoted work with the seahorses and sepias and your organizational skills were such a good example. Thank you for the traditions that you initiated at EZO. Maurijn Yomiiiii! Your creative soul brought amusement to the office and many fish. Your knowledge of fish work, and image-design tools was much appreciated, and I will always remember you when the "Nossa" song will come up in the radio and I will dance it for the good times. Sander K, I deeply enjoyed our conversations and feel so grateful for your advices. Your keen feedback on my projects helped me to see more broadly my subject, thank you. Please say hi to Tura and Mylene from Elsa, the non-"frozen" one :). Sander G, you welcomed me from the first time I visit EZO, thank you for your shared loved for birds, I remember with much joy the time we visited Leiden and photographed the numerous hummingbird wings. Martin, thank you for your insightful advices. Kees S, thank you for your sweet company during coffee breaks. Florian, from your arrival to the group, your fresh ideas and energy spread. I felt very lucky to have brainstorms with you about my projects, and you were very generous with your time. Julian, I so enjoyed discovering your great humour and much appreciated our conversations about science and life. Dear Pim, already from my first visit to Ocean Ecosystems, you made me feel welcome and your enthusiasm about life was contagious. I was very happy that you decided to do your PhD at EZO. Wouter, thank you so much for sharing your knowledge and support on subjects that went beyond me. Your devotion to your work, shown sometimes by several mosquito bites, was inspiring. Myrthe, your enthusiasm on fish ecology and evolution was motivating and I thank you for the ideas and tea shared.

I owe a big thank to my students Silke, and Niek, the apprentices Miranda, Jurgen, and Kaj, and to Jeffrey, for all your great work on the muscle project. Miranda, thank you for the conversations. I was very happy to see you again visiting us at EZO. Annemarie, your pilot work on *Poeciliopsis gracilis* helped to lay the basis of some of the sections of this thesis, thank you.

Menno, Truus, Wian, Sander, Emily, and Jasper at CARUS, words are not enough to thank you for taking care of each of our fish, and of me. Your kindness, and lively conversations made my stay at CARUS an enjoyable one. Truus, you took me under your wing from the beginning, and patiently taught me how to work with fish. Thank you for

making me feel always welcomed. Kobika, your kindness and our conversations warmed my heart, thank you. And to Rob Steenmans, deep thanks for making sure that the fish, with which I am forever grateful, were properly treated.

My Zodiac friends. Evita, thank you for your friendship, for the countless dinners, coffee chats, parties, the laughs and the moments of sadness shared. Marcelita, Raka, Soumya, you made me feel home, and I loved the amusing moments at your offices and houses. Thank you Edo, Brunito, Marcel, Linda, Marjoelin, Anja, Nico, Ellen, Loes, for all those moments shared, and for the ceramics days! Jan Wouter, thank you so much for all these years of friendship. From the time when we collaborated in the hummingbird project at EZO, we became very good friends and you introduced me to the Dutch culture and helped me navigate through it. David Lentink, thank you for giving me the opportunity to meet the EZO group through the hummingbird project and for, together with Swanne Gordon, helping me refine the proposal and prepare for the interview for the grant that supported my PhD. Maria and Geert, I enjoyed our conversations and the activities shared.

And to my other friends in Wageningen, Ivo Roessink, I enjoyed our conversations about ecology and life and also playing underwater hockey with you. The Heiduikers underwater hockey team reminded me how to play as a team, to keep trying until reaching the goal, and to be brave, while being also kind. Luis García, our conversations about fish and ships inspired me, and I learnt a lot from you. Patricia Frias-Alvarez, your visit to EZO opened my mind to what my fish would be doing in nature, and I thank you for seeding that curiosity in me. Aage, Pieternel, and my dear housemates at the corridor of Plantsoen, thank you for the fun times shared, and all your care. Natalie, I learnt a lot from your fun plans, discipline, and care for your friends. Dear Soumayeh and Lucille, thank you for opening my world to your cultures, thank you for the growth. Vale A., Domenico, Vale M., Thibaut, Walter, Danilo, Alberto, Raphael, Carla, Niccolo, Noella, Lavi, Francesco, Erika, Arie, and Jime, thank you for all the great times.

A special word of appreciation for my Colombian family in Wageningen: Diana, Theo, Monica, Daniel, Rebecca, Ferdie, Jose, Paola, Beatriz, Paula, Cesar, Carlos, Isa, Emma, Marcela, Claudia, Edward in Almere, and Andres and Lore in Eindhoven. Zonia, Leo, Zarita, Zamuel, Suzana, Andrea, Matthijs, Amalia, Carolina, Mario, vecinos, how lucky to have lived close to you and feel the warmth and love when visiting your homes. Monica, thank you for your words and support and for the laughs that reminded me that life is very enjoyable. Diana, parcerita, thank you for your honesty, support, company and inspiration.

In the Ocean Ecosystems Group, at the University of Groningen, I got to meet very special people. Klaas, your infinite curiosity and devotion to new projects always amazed me, thank you for guiding me. I also want to thank my office mates Patrick and Gemma who shared their knowledge and stories, and Cindy, Dennis, Peter, Willem, Jan, Friso, Sam, Azim and Xiaoyin. I feel very fortunate for the great times shared with you all.

While visiting Groningen, I also met amazing people outside work. Miranda, thank you for housing me and for becoming a very good friend. Jorge, Ailine, and Laura, thank you for opening your house and hearts! Jorge, thank you for your friendship and for introducing me to meditation. Marlenny, señorina, you have been a very bright light in my path during the PhD, thank you for sharing your experience with me. And thanks to the Colombian community in Groningen who Andrés Q. so kindly introduced me to: Liliana, Cris, Diego, Maryam, Alfredo, Vivis, Gabriela, Andres, David, Alejandro, Jonathan, and Esméé from The Netherlands. And thanks to Rodolfo for inviting me to fun plans in Groningen.

I am very fortunate to have friends who also from the distance played an important part on this journey's success and enjoyment. Liliana, Marie and Ana, my oxytocin friends, who help me put life into perspective. Swanne, Andres, Juliana, Pamela, Mauricio, Rebecca, Alison, Christopher, Anke, David, Alejandro, Kristiina, Eva, Sofia, Ruth, Jazmin, Carolina, Danijela, Carolina, Laura, Juliana, Laura, Andrea, Alejandro and Prof. Ricardo Ramírez. Quiero agradecer también a mis amigos del equipo de hockey subacuático Titanes, en especial a Edward, Sandra, Alexandra, Cony, Jeisson, Amar, Giova, Henry y Orlando por su buenísima energía y compañía. Miles de gracias a las personas maravillosas que me recibieron en Suesca con tanto cariño: Doña María, Doña Alicia, Doña Judith, Lady, Natalie, Iván, Deyanira y Esperanza. Y a Maité, la peludita de cuatro patas, por su compañía y afecto. Gracias también a mi Alma Mater, la Universidad Nacional de Colombia, y en especial a las funcionarias de la biblioteca de Posgrados de Ciencias Humanas por recibirme día a día durante los últimos meses de mi tesis y hacer mi estancia agradable.

I want to express my gratitude towards Mauricio Quimbaya and Adriana Gomez at Universidad Javeriana, and to Mateo Pradilla at Universidad Nacional for the opportunities they gave me to work on projects in the interface between Biology and Enginnering. I want to thank Remco Hamoen for his great job with the computer tomographies, and the team of Oceanz for producing the beautiful 3D printed fish models.

I would like to thank Colciencias, who funded my PhD and made this dream possible and the Department of Animal Sciences at Wageningen University for funding my research visit to Groningen.

I have tried to remember all the people who have contributed to the completion of this milestone, but it is certain that I have incurred in some omission.

Finalmente, agradezco inmensamente a mi familia: a mi mamá, por siempre apoyarme y entender el camino que he ido eligiendo, a mi papá por siempre animarme a seguir adelante, a mis hermanos Ingrid y Daniel, por ser mis confidentes, por apoyarme y por caminar juntos. Gracias familia por tanto amor y comprensión, y por tenerme paciencia en los momentos en que más lo necesité. Soy muy afortunada por ser parte de esta familia. ¡Los amo!

# Personalia

## Curriculum vitae

Elsa Magnolia Quicazán Rubio was born on the 24th of March 1980 in Bogotá, Colombia. From an early age, she was in contact with nature. She used to visit her grandparents farm in the Andean mountains where she observed her veterinarian cousin, Marta Trespalacios, catching bugs and caring for animals such as ducks and rabbits. Elsa studied biology at the National University of Colombia. During her undergrad studies, she developed a fascination for animal locomotion. She joined the Biophysics group, led by Dr Ramon Fayad, where she studied animal flight. To complement her knowledge, she attended a fluid-mechanics class, where she was the only biologist, and felt always welcomed.

Her interests pointed also towards the arts, and attended drawing and designing classes at the university. She volunteered as drawing instructor for the fishermen of a northern island in Colombia. Swimming is an important part of her life and during her BSc, she became co-founder of Titanes, the Bogota underwater hockey team. There, her love for swimming, team work, and competition was fulfilled.

Continuing her interest on animal locomotion, she pursued a BSc thesis on hummingbird flight, with support and interest from the Mechanical Engineering Department. She was guided by her Biology thesis director, Dr Gary Styles, an external director, Dr Douglas Altshuler, and Dr Ricardo Ramirez, professor of mechanical engineering, who welcomed Elsa in his lab. After her BSc, she fulfilled short-term administrative and educational positions.

Her BSc project on hummingbird flight resulted in a collaboration with the Experimental Zoology Group (EZO), Wageningen University, the Netherlands. Around that time, she started her MSc project at the University of California, Riverside, supervised by Dr Douglas Altshuler. She studied the muscle activity and wing beat kinematics of flying hummingbirds. At the end of her MSc project, she obtained a fellowship from the Colombian funding institution, Colciencias, to pursue her doctoral studies.

Returning to her love for swimming, she studied the effects of pregnancy on the swimming performance of fish during her PhD project, supervised by Prof. Johan van Leeuwen and Dr Bart Pollux, EZO. She learned how to rear and work with fish. Building setups, measuring and analysing kinematic and morphological variables, supervising students and working in teams was also at the core of her experience. And thanks to the interdisciplinary nature of EZO, there was always someone to consult for advice and to work with.

Towards the end of her thesis, Elsa worked with Prof. Eize Stamhuis, in the Ocean Ecosystem Group, at the University of Groningen. Eize and Klaas van Manen guided her through drag- and flow measurements of models of pregnant fish. They, and the Ocean Ecosystem group, provided her with enriching conversations on biomechanics and ocean ecology subjects.

Elsa finished her thesis in Bogotá and Suesca (Colombia). Bogotá offered her the advantages of a big metropolis, and Suesca offered her the fresh air and views of the countryside, experiencing rural country life. During various visits to Colombia and the last months of her thesis, she attended scientific and social events that reinforced her desire to be a bridge between the natural sciences and engineering in Colombia. The rising interest in interdisciplinary research, provided the opportunity to write two syllabi, on biomechanics and bioinspired design, for the Mechanical Engineering major of the Universidad Javeriana, Cali, Colombia. Also, this rising interest gave her the opportunity to be a referee during the TPI (Taller de Proyectos Interdisciplinarios) + Expoideas event organized by the Engineering Department at the National University of Colombia.

## Publications

**Quicazan-Rubio EM**, van Leeuwen JL, van Manen K, Fleuren M, Pollux BJA, Stamhuis EJ. 2019 Coasting in live bearing fish: the drag penalty of being pregnant. *J. R. Soc. Interface* **16**, 20180714. (doi:10.1098/rsif.2018.0714)

Fleuren M, **Quicazan-Rubio EM**, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? Evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS One* **13**, e0195976. (doi:10.1371/journal.pone.0195976)

Fleuren M, van Leeuwen JL, **Quicazan-Rubio EM**, Pieters RPM, Pollux BJA, Voesenek CJ. 2018 Three-dimensional analysis of the fast-start escape response of the least killifish, *Heterandria formosa*. *J. Exp. Biol.* , jeb.168609. (doi:10.1242/jeb.168609)

Kruyt JW, **Quicazan-Rubio EM**, van Heijst GF, Altshuler DL, Lentink D. 2014 Hummingbird wing efficacy depends on aspect ratio and compares with helicopter rotors. *J. R. Soc. Interface* **11**, 570–81. (doi:10.1098/rsif.2014.0585)

Altshuler DL, **Quicazan-Rubio EM**, Segre PS, Middleton KM. 2012 Wingbeat kinematics and motor control of yaw turns in Anna's hummingbirds (*Calypte anna*). *J. Exp. Biol.* **215**, 4070–4084. (doi:10.1242/jeb.075044)

## Abstracts

**Quicazan-Rubio, EM,** van Manen, K, Pollux, BJA, Stamhuis, EJ, and van Leeuwen, JL. Drag forces and PIV measurements on model fish. *In: Tercer Congreso Internacional sobre Tecnologias Avanzadas de Mecatrónica, Diseño y Manufactura (AMDM 2016). Memorias; Cali, Colombia.* April 13–15, 2016.

**Quicazan-Rubio, EM,** Fleuren, M, Voesenek, CJ, van Leeuwen, JL, Pollux, BJA. Análisis en 3D de la respuesta de escape para estudiar la evolución de la placentación en peces poecílidos. *In: IV Congreso Colombiano de Zoología. Resúmenes; Cartagena D.T, Colombia.* December 1–5, 2014.

**Quicazán-Rubio, EM,** Kruyt, JW, van Heijst, GF, Altshuler, DL, Lentink, D. La eficacia del ala de colibrí depende de la razón de aspecto y se compara con las hélices de helicópteros. *In: IV Congreso Colombiano de Zoología. Resúmenes; Cartagena D.T, Colombia.* December 1–5, 2014.

**Quicazan-Rubio, EM,** Fleuren, M, van Leeuwen, JL, Pollux, BJA. The use of 3D recording of fast-start escape response to study the evolution of placentation in Poeciliid fish. *In: SICB 2014 Annual Meeting Abstracts; Austin, TX, USA.* January 3–7, 2014.

Fleuren, M, **Quicazan-Rubio, EM,** Voesenek, CJ, van Leeuwen, JL, Pollux, BJA. Reconstructing changes in 3D body shape during the pregnancy of three species of viviparous fish (Poeciliidae): effects of placentation and superfetation. In: *SICB 2014 Annual Meeting Abstracts; Austin, TX*, *USA.* January 3–7, 2014.

Kruyt, JW, **Quicazan-Rubio, EM,** van Heijst, GJF, Altshuler, DL, Lentink, D. Small aspect ratio differences impact hover efficacy among 12 hummingbird species. *In: SICB 2013 Annual Meeting Abstracts; San Francisco, CA*. January 3–7, 2013.

Kruyt, JW, **Quicazan-Rubio, EM,** Gussekloo, SWS, van Leeuwen, JL, van Heijst, GJF, Altshuler, DL, Lentink, D. Comparing aerodynamic performance of 13 hummingbird species from Colombia and California. In: *Proceeding of the Annual Main Meeting of the Society for Experimental Biology, p. 134. Glasgow, Scotland.* 1–4 July, 2011.

Lentink, D, Kruyt, JW, **Quicazan-Rubio, EM,** Gussekloo, SWS, Altshuler, DL, van Leeuwen, JL. Comparative aerodynamic performance of hummingbird wings from Colombia. In: *Proceedings of the Society for Integrative and Comparative Biology – 2010. Annual Meeting, Seattle, WA, USA*. January 3–7, 2010.

**Quicazán R., EM,** and Stiles, FG. Comparaciones Aerodinámicas de Alas de Colibríes: *Colibrí coruscans, Metallura tyrianthina* y *Acestrura mulsanti*. *Acta Biológica Colombiana* 11,168. 2006.

Arias, J, Barbosa, S and **Quicazán, E.** Evaluación de dos Aislamientos de *Trichoderma* en el Control de Algunos Hongos Patógenos. *Acta Biológica Colombiana* 5,12. 2000.

**WIAS TSP (Training and supervision plan)**

| | ECTS[1] |
|---|---|
| **Basic package** | |
| WIAS Introduction Course | 1.5 |
| Course on philosophy of science and/or ethics | 1.5 |
| **Scientific Exposure** | |
| Four international conferences including:<br>- Annual Meeting of the Society for Experimental Biology,<br>- Annual Meeting of the Society for Integrative and Comparative Biology,<br>- IV Colombian Congress of Zoology,<br>- III Congreso Internacional sobre Tecnologías Avanzadas de Mecatrónica, Diseño y Manufactura (AMDM2016). | 5.2 |
| WIAS Science day | 0.3 |
| PE&RC Day 2013 "Biomimicry" | 0.3 |
| MATLAB Academic Tour 2014 at Wageningen University | 0.2 |
| Four oral/poster presentations | 4.0 |
| **In-Depth Studies** | |
| Locomorph Summer School on Morphology and Morphosis in Animals and Robots 2012 | 2.1 |
| Statistics for the Life Sciences | 2.0 |
| Advanced statistics courses: Design of Experiments | 1.0 |
| **Statutory courses** | |
| Use of Laboratory Animals | 3.0 |
| **Professional Skills Support Courses** | |
| High-Impact Writing in Science | 1.2 |
| Project and Time Management | 1.5 |
| Surviving Peer Review Course | 0.3 |
| **Research Skills Training** | |
| Preparing own PhD research proposal | 6.0 |
| External training period – University of Groningen – Ocean Ecosystems Department | 2.0 |
| **Didactic Skills Training** | |
| Two lectures for Developmental Biology of Animals. EZO30306 | 2.7 |
| One practical for Behavioural Ecology | 0.7 |
| Supervision of 2 BSc and 1 MSc (Capita selecta) students | 4.0 |
| **Total ECTS** | **42.5** |

Herewith the WIAS Graduate School, declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of WIAS.

[1]One ECTS (European Credit Transfer and Accumulation System) equals a workload of 28 hours.

The research described in this thesis was financially supported by the Administrative Department of Science, Technology and Innovation (COLCIENCIAS), and by the Netherlands Organization for Scientific Research (NWO)

Financial support from the Experimental Zoology group of Wageningen University for printing this thesis is gratefully acknowledged.