

Understanding Sexual Conflict and Environmental Effects on Reproductive Strategies in Mosquitofish (*Gambusia holbrooki*)

Fatema Akhter

Supervisors: Professor Michael D Jennions &
Dr. Megan L Head



Division of Ecology and Evolution
Research School of Biology
The Australian National University

A thesis submitted for the degree of Doctor of Philosophy
June 2026

© Copyright by Fatema Akhter, 2026
All Rights Reserved

Declaration

I, Fatema Akhter, declare that this thesis is based on my own research. All work presented in this thesis have been conducted and written by me. In case of collaborative research, the extent of my contribution, and the contributions of the collaborators has been clearly indicated. I confirm that no part of this thesis has been submitted to any other institution for any kind of award or degree.

Total Word Count: 53420

Fatema Akhter

June 2026

Publication during candidature

1. **Akhter F**, Head ML, Jennions MD & Chung MHJ (2026). Disentangling the life-history costs of direct male-male competition and sperm production. *Evolutionary Ecology*, 40, 17. <https://doi.org/10.1007/s10682-026-10383-6>.

Acknowledgements

All praises and gratitude to Allah (SWT) for showing His blessing and mercy, which helped me to successfully complete this thesis. I am grateful to Him for giving me the opportunities, strength, patience and perseverance which helped me to overcome the challenges that I faced during this journey. I extend my sincere gratitude to the holy Prophet Muhammad (Peace be upon him), whose way of life has been a continuous guidance and inspiration for me.

Securing a PhD degree from abroad is challenging, especially when pursuing it in another language. With the unwavering support and guidance of my supervisors, Michael Jennions and Megan Head, I was able to successfully complete this journey and navigate all the challenges. Without their support I cannot imagine where I would be today.

My primary supervisor, **Michael Jennions** is one of the amazing people I have ever met. He is multi-talented and kind-hearted and was always there for me. I started my PhD without my family nearby, and during that time he supported me mentally and showed greater understanding. He was always available, even outside of his working hours. I learned a lot of things from him, he taught me how to think outside the box and helped me to improve my writing. Every discussion we had taught me something new. I also learned from him how to give an engaging presentation and connect with people. I feel truly blessed and lucky to have him as my supervisor. My journey finished smoothly only because of him.

I am deeply grateful to my co-supervisor, **Megan Head**, who was always there to relieve my stress. She thinks straightforwardly and always gave me the best and simple solutions for every problem that I faced while running the experiments. She is an amazing scientist, and her passion towards her work inspired me to develop my interest in insect research as well. She is hard -working and always strives to explore new ideas. Without her

guidance and supervision, I would not been able to complete my experiments and get some simple but interesting results.

I am deeply grateful to **Meng-Han Joseph Chung** for his continuous guidance and encouragement. I learned lots of basic analysis tools from him. His insightful comments during the experimental design and data analysis helped me to see my research from different perspectives. I greatly appreciate his patience and support with R analysis.

I was fortunate to have **Ondi Crino** and **Daniel Noble** as members of my supervisory panel. Their constructive suggestion regarding my experimental design were invaluable, and I am grateful to them for their continuous encouragement throughout my journey.

People from the Jennions and Head Lab: **Lauren, David, Upama, Ivan, Chenke, Diego** and **Mahmud** - thank you all for being there for me throughout my PhD journey. I truly appreciate every conversation we had, whether about research, struggles during experiment, or about future. Your support made this journey much more enjoyable.

Finally, I am expressing my deepest appreciation to my husband (**Showev**) and my son (**Fatin**) for their unwavering support while I worked in the lab during the weekends and public holidays. I am grateful to them for never complaining about my absence during the times they needed me most. Without their support this journey would have become stressful. I am also grateful to my mother for taking care of my little girl (**Fatiha**), while I was writing my thesis and preparing for my exit seminar. My heartfelt thanks also go to my brother (**Mainuddin**) and my mother-in-law for their mental and emotional support throughout this journey.

ABSTRACT

Sexual conflict is very common and is considered as a natural phenomenon, which arises when males and females show divergency in their reproductive interests. Environmental factors such as changes in temperature, salinity and water clarity can significantly influence the level of sexual conflict and can also influence the reproductive strategies of each sex. In species, such as mosquitofish (*Gambusia holbrooki*), where mating is frequently coercive and male harassment is intense, social interactions and environmental conditions both play critical roles in shaping reproductive outcomes. To investigate how sexual conflict and environmental factors (such as salinity, temperature, turbidity) interact to influence reproductive strategies, physiological stress, and life-history traits in males and females, I ran a set of laboratory experiments using mosquitofish.

In this thesis, I present four experimental studies examining; the cost related to harassment and multiple mating (Chapter 2); the cost of fighting and sperm production (Chapter 3); the effect of turbidity on female stress level (Chapter 4); and the effect of salinity and temperature on male reproductive strategies (Chapter 5).

Chapter 1 introduces the concepts of reproduction, sexual conflict and how different environmental factors can influence reproduction in fish.

Chapter 2 differentiates the cost of sexual harassment and multiple mating (polyandry) in female mosquitofish (*Gambusia holbrooki*). In polyandrous situation both things happen simultaneously, which is why it is difficult to know which one is more expensive. I differentiated the cost by controlling the harassment level and mating rates by using three treatments. I used ablation technique to control the rate of mating. I found that female who faced more harassment, grow more slowly and produce a smaller number of eggs.

Chapter 3 differentiates the cost of sperm production from the cost of male-male fighting.

As both things usually happen at once, I used the ablation technique (physically cutting the gonopodia) to stop sperm transfer to the female. To find out the most expensive one, I designed three treatments with varying number of rivals and with different sperm ejaculation ability. I found that sperm production is more expensive than fighting and competing with rivals.

Chapter 4 investigates the effect of turbid water on the level of male harassment and female stress level and how the level of harassment influences the female stress level. By using four types of treatment (clear water or turbid water; and one male or three males), I found that reduced water turbidity (clear water) elevates female physiological stress (cortisol level), while male harassment levels do not affect female physiological stress. My findings highlight the importance of abiotic conditions in modulating the intensity of sexual conflict.

Chapter 5 explores how temperature and salinity influence male life history and reproductive traits, including sperm quantity and velocity. I designed four treatments (two temperatures and two salinity levels) and found that in saline water males produce less sperm than in freshwater and mature earlier at a smaller size. Overall, salinity has more severe effect than temperature, but when salinity and high temperature combine then the effects are more complicated.

TABLE OF CONTENTS

Declaration.....	ii
Publication.....	iii
Acknowledgement.....	iv-v
Abstract.....	vi-vii
Chapter 1	
General Introduction.....	1-14
Chapter 2	
Teasing apart the cost of sexual harassment and multiple mating in females.....	15-51
Introduction.....	15-20
Materials and Method.....	21-26
Statistical analysis.....	26-27
Results.....	27-34
Discussion.....	34-41
Conclusion.....	41-42
References.....	43-51
Chapter 3	
Disentangling the life-history costs of direct male-male competition and sperm production.....	52-91
Abstract.....	52-53

Introduction.....	54-58
Materials and Method.....	58-64
Predictions.....	64-65
Statistical analysis.....	65-66
Results.....	67-74
Discussion.....	74-80
Conclusion.....	80-81
References.....	82-91

Chapter 4

Reduced water turbidity rather than male harassment elevates female physiological stress in a fish with intense sexual conflict.....	92-120
Introduction.....	92-96
Materials and Method.....	96-101
Statistical analysis.....	101-102
Results.....	102-105
Discussion.....	105-108
Conclusion.....	109
References.....	110-120

Chapter 5

How do temperature and salinity affect male reproductive traits?- An experimental test in mosquitofish.....	121-157
---	---------

Introduction.....	121-125
Materials and Method.....	125-130
Statistical analysis.....	130-132
Results.....	132-139
Discussion.....	140-145
Conclusion.....	145
References.....	146-157
 Chapter 6	
General Conclusion.....	158-161
 Appendix A	
Reduced water turbidity rather than male harassment elevates female physiological stress in a fish with intense sexual conflict (Chapter 4).....	162-187
 Appendix B	
Chapter 2: Supplementary material.....	188-197
 Appendix C	
Chapter 3: Supplementary material.....	198-212
 Appendix D	
Chapter 4: Supplementary material.....	213-217
 Appendix E	
Chapter 5: Supplementary material.....	218-221

LIST OF FIGURES

Chapter 2

Figure 1: Experimental design with three treatments.....	23
Figure 2: Effects of sexual harassment and polyandry on the number of rapid approaches by female <i>G. holbrooki</i> towards males.....	28
Figure 3: Effects of sexual harassment and polyandry on the number of times that female <i>G. holbrooki</i> rejected males.....	29
Figure 4: Effects of sexual harassment and polyandry on the number of times that female <i>G. holbrooki</i> received gonopodial thrusts from males.....	30
Figure 5: Plots showing the effect of sexual harassment and polyandry on a female's reproductive performance.....	31
Figure 6: Plots showing the effect of sexual harassment and polyandry on a female's relative reproductive investment.....	32
Figure 7: Violin plot showing the effect of sexual harassment and polyandry on the total number of eggs.....	33
Figure 8: Plots showing the effect of sexual harassment and polyandry on female life history traits.....	34

Chapter 3

Figure 1: Experimental design manipulating the presence of rivals and the male's ability to release sperm.....	60
Figure 2: The effect of direct male-male competition on mating and fighting behaviour.....	69

Figure 3: The effect of direct male-male competition on (i) body size and (ii) immune response.....	70
Figure 4: The effect of direct male-male competition on sperm traits.....	70
Figure 5: The effect of past sperm investment on mating and fighting behaviour.....	72
Figure 6: The effect of past sperm investment on (i) growth and (ii) immune.....	73
Figure 7: The effect of greater sperm investment on sperm traits.....	74
 Chapter 4	
Figure 1: Experimental design with four treatments.....	97
Figure 2: Timeline of the experimental protocol.....	101
Figure 3: Effect of water turbidity and male density on the amount of sexual harassment received by females.....	103
Figure 4: Effect of water turbidity and male density on the cortisol level of females....	104
 Chapter 5	
Figure 1: Full experimental design with two temperatures (26°C and 31°C) and two salinity treatments (0‰ and 20‰) to create four treatment groups.....	130
Figure 2: The effect of salinity and temperature on male size at maturity.....	133
Figure 3: The effect of salinity and temperature on male time to maturity.....	134
Figure 4: The effect of salinity and temperature on gonopodium length.....	135

Figure 5: The relationship between body size and gonopodium length in saline and fresh water.....	135
Figure 6: The effect of salinity and temperature on male adult growth.....	136
Figure 7: The effect of salinity and temperature on sperm count.....	137
Figure 8: The relationship between body size and sperm number.....	138
Figure 9: The effect of salinity and temperature on sperm velocity.....	138

Chapter 1: General Introduction

“The evolutionary interests of the two sexes are almost never identical.” — **Richard**

Dawkins, The Selfish Gene

Reproduction and sexual conflict

In sexually reproducing organisms, mating is sometimes described as a harmonious event, where during mating both males and females contribute equally to producing offspring. However, although both sexes have the same target, they differ in their way of reaching their destination due to their divergent fitness interests. The divergent interests of individuals of different sexes can cause evolution through sexual selection to act in opposing directions which creates conflict. This conflict is known as sexual conflict. The theory of sexual conflict was first outlined by Parker (Parker, 1979) in the 1970's. Sexual conflict has now become a widely important component of selection to consider when studying male-female interactions. Although sexual reproduction requires some level of coordination between males and females, sexual conflict can still occur due to the lack of alignment in their fitness interest. In addition, despite sharing the same genome, conflict over which alleles are favoured in males and females is common as the benefits of some genes when expressed in one sex conflict with costs when expressed in the other sex (Arnqvist & Rowe, 2005).

Sexual conflict is driven by divergent evolutionary interests of each sex which affects the mating tactics of males and females (Arnqvist & Nilsson, 2000; Chapman, 2006). Conflict can arise due to sex differences over mating, fertilization, parental care and reproductive investment (Parker, 1979). It can drive the coevolution of sexually antagonistic traits which can result in ever greater divergence in the fitness interests of each sex (Arnqvist & Rowe, 2005). These divergent interests initially arise due to variation in gamete size, which is known as anisogamy. Males need to invest less energy to produce tiny sperm than

females who need to invest more energy to produce eggs which are much larger than sperm. Females tend to have a lower optimal mating rate than males because of this higher reproductive investment per gamete which means they are less often in a state where they have usable gametes.

Sexual conflict can occur when traits of one sex increase the reproductive success of individuals of that sex but reduce the fitness of the other sex. Male fitness usually increases steadily with the number of matings, whereas for females often only one or two matings are sufficient to maximize reproductive success for a given breeding bout (Arnqvist & Nilsson, 2000). If females mate above their optimal mating rate, then their lifetime reproductive success will be lowered if there are costs associated with multiple mating (Arnqvist & Rowe, 2002, 2005; Parker, 2006). Although longer and repeated copulations can increase a male's fertilization success (Lev et al., 2025), longer copulations and a higher mating frequency can also lower the fitness of a female due to cost associated with intense male harassment to induce mating, disease transmissions while mating, transfer of toxic substances in semen, and genital tract injury (Arnqvist & Rowe, 2005; Dadda, 2015; Plath et al., 2007; Reinhardt et al., 2003). For example, in bedbugs, multiple mating is beneficial for males, but due to traumatic insemination through the female's body it can reduce a female's lifespan (Reinhardt et al., 2003). Studies have shown that mating above the optimal rate can actually lower a female's lifetime reproductive success (i.e. fitness) (Cummings, 2018).

Types of sexual conflict

Sexual conflict has been described as an evolutionary conflict that occurs between males and females because of their divergent fitness interests in reproduction, and where a trait that is favoured by one sex is costly to the other sex which can ultimately lead to distinct roles. There are two types of sexual conflict; when a genetic locus carries an allele that

has two opposite effects on the fitness of each sex, such as if one allele enhances the fitness of one sex at the expense of another sex, then this is referred to as **intra-locus conflict**. For example, in broad-horned flour beetles, males develop enlarged mandibles which make them more fit, but the daughters of those males with large mandibles have lower fitness, which means the allele that encodes a high quality male tends to produce lower quality females (Archer et al., 2018). On the other hand, when conflict occurs due to the interaction of a set of antagonistic alleles at two different loci (in other words when there is conflict over the outcome of male–female interactions), then that is called **inter-locus conflict**. For example, in *Drosophila melanogaster*, the seminal fluid proteins from the male accessory gland reduce the desire of females to re-mate, shorten their lifespan and thereby reduce the fitness of females (Chapman et al., 1995). Such conflicts can occur over when, where and how often to mate, fertilization, relative parental effort, female reproductive rate and clutch size (Chapman et al., 2003). These conflicts set up an evolutionary “tug-of-war” between the sexes due to their different evolutionary interest which shapes the behaviour, physiology, and can ultimately force the process of speciation (Parker, 1979). Selection on alleles for traits that increase male (or female) fitness are countered by selection on alleles for traits that counter the effect of these male (or female) traits and thereby increase female (or male) fitness.

Physical environmental stress and sexual conflict

Environmental factors such as temperature, salinity, turbidity, resource availability, predation risk, and habitat stability can strongly influence the strength and consequences of sexual conflict (Fricke et al., 2009; García-Roa et al., 2019; Lymbery, 2019; Plesnar-Bielak & Łukasiewicz, 2021; Sundin et al., 2017; Vincent et al., 2020). There is some direct evidence that sexual conflict is highly context dependent. For example, in *Drosophila melanogaster*, at higher temperature female suffered less from sexual conflict (García-Roa et al., 2019). In fish, changes in turbidity can affect different aspects

of sexual selection and reproduction (Engström-Öst & Candolin, 2007; Järvenpää & Lindström, 2004; Sundin et al., 2017). The level of salinity can also influence sexual conflict by changing the life history, mating behaviour and breeding performance of each sex as is seen in *Gambusia holbrooki* and *Eriocheir sinensis* (Chung et al., 2025; Huang et al., 2025). There are some other indirect lines of evidence which show that under different contexts, organisms alter their reproductive investments which can ultimately lead to sexual conflict. For instance, in guppies, *Poecilia reticulata* different sized oocytes are produced in response to variation in the food supply (Hester, 1964), and in *G. holbrooki*, males with a restricted diet had lower sperm reserves and slower sperm replenishment rate (Vega-Trejo et al., 2016). Likewise, male fruit flies, *Drosophila melanogaster*, in low-density conditions tend to be larger and healthier and produced larger ejaculates, whereas in high-density condition they became smaller and produced smaller ejaculates (Wigby et al., 2016). These findings show that both males and females often adjust their resource investment according to the surrounding environment. Specially the changes in male investment will then affect the selection they impose on females to respond in ways that reduce the costs of greater male investment in traits that create sexual conflict.

Poikilotherms and environmental stress

Fishes are poikilothermic animals, which means that their body temperature is directly regulated by the environmental temperature. Any ongoing changes in the water therefore directly influence the quality of their lives. For example, changes in temperature, salinity and turbidity can directly influence the behaviour and physiology of fishes. Fish reproduction is largely dependent on environmental cues which trigger the rate of maturation and breeding (Pankhurst & Porter, 2003). Consequently, any rapid changes in the environment directly influence their mating success. For instance, higher temperature can alter secretion of the gonadotrophin releasing hormone (GnRH) and steroidogenesis (Servili et al., 2020). Salinity is another factor which can impact the breeding and

reproductive success of fish. Changes in salinity can affect fish spermatogenesis and testicular homeostasis which then affects gonadal cell proliferation and apoptosis (Servili et al., 2020). The clarity or turbidity of water can also affect how fish behaviour in both positive and negative ways for their fitness. Human activities such as agriculture, deforestation, urbanization, cause sediment and nutrient runoff into water bodies, which increase the turbidity of the water. High concentration of suspended solid in the water can themselves cause physiological and behavioural stress to fish (Utne-Palm, 2002), while reduces visibility because of greater turbidity and alter their behaviour (Newport et al., 2021).

Study species

Among the vertebrates, poeciliids have the highest mating rates. Among the over 200 poeciliids, more than half of the species have a mating system dominated by male coercion (sneaking up to a female from behind and then engaging in gonopodial thrusts as they attempt forced copulations), where the other species show both courtship and coercion (Cummings, 2018). In poeciliids, male continuously attempt to mate with females, up to a rate of one/minute (Wilson, 2005). Whereas females only need to mate once to fertilise their eggs as they can store sperm for long periods of time (Constanz, 1989). To reduce the cost of male harassment female poeciliids need to prioritize foraging over mating (Dadda et al., 2005; Pilastro et al., 2003) as their fecundity is closely related to their body size (Booksmythe et al., 2016). Due to the disparity in their optimum mating rate, there is likely to be intense sexual conflict over mating.

Among the poeciliids, two species of *Gambusia* (*holbrooki* and *affinis*) are widely distributed throughout the world. These species reproduce quickly, grow fast, and are easy to maintain in captivity. They have been introduced to control mosquitoes and from there they got their common name of the “mosquitofish” (Pyke, 2008).

The **eastern mosquitofish** (*Gambusia holbrooki*) is an ideal species to test how environmental factors affect adult reproductive behaviour and sexual conflict. They show quite distinct sexual dimorphism. Females are much bigger than males and are identified by a black spot around their belly. The smaller males are identified by their modified anal fin (gonopodium), which they use to transfer sperm to the female. After sexual maturity males grow very little whereas females continue to grow (Pyke, 2005). In *Gambusia*, courtship/mating involves three distinct phases: association (where male and female move together, and males showed aggression towards other males), 'courtship' (sexually aggressive approach to the female-which include swinging the gonopodium forward) and mating. Copulation involves a very brief moment when the gonopodium tip makes contact with the oviduct opening (Bisazza & Marin, 1991). The speed of the copulation is very fast which makes it difficult to distinguish between successful and unsuccessful mating attempts. They are well suited to experiments because male always chase females, whereas females constantly try to avoid encounters with males to reduce the direct costs of mating; the hooked and barbed male gonopodium can cause injury to females and male harassment interferes with the ability of females to forage. They can also tolerate a wide range of habitats. All of these things together make Mosquitofish an ideal organism to answer all of my research questions.

My thesis

Sexual conflict is a broad and complex topic which extends across a wide range of biological disciplines. This thesis adopts a comprehensive approach to understand different aspects of sexual conflict in both a male and female perspective, looking at both behavioural and physiological traits and looking at effects of both social environment and physical environment. Together, these approaches provide a multifaceted understanding of how sexual conflict works in different contexts, what are the potential outcomes, and its role in shaping the biology of mosquitofish. Furthermore, this research contributes to

a deeper understanding of the evolutionary dynamics of this species which can arise from the conflict between the sexes. Together, these approaches provide a multifaceted understanding of how sexual conflict works in different contexts, what are the potential outcomes, and its role in shaping the biology of mosquitofish. Furthermore, this research contributes to a deeper understanding of the evolutionary dynamics of this species which can arise from the conflict between the sexes.

Experiment overview

For my PhD thesis, I have written four data chapters:

Chapter 2: Teasing apart the cost of sexual harassment and multiple mating in females

Chapter 3: Disentangling the life-history costs of direct male-male competition and sperm production

Chapter 4: Reduced water turbidity rather than male harassment elevates female physiological stress in a fish with intense sexual conflict

Chapter 5: How do temperature and salinity affect male reproductive traits?- An experimental test in mosquitofish

In the first two chapters (Chapter 2 & 3), I look at how the number of males per female affects the behaviour and fitness of each sex. I investigate the costs and benefits of multiple mating for females (Chapter 2), and the costs of fighting with rivals and increased sperm production for males (Chapter 3).

In the fourth and fifth chapters, I focus on environmental factors that might affect sexual conflict or investment in sexual traits. I test how water turbidity affects the strength of male sexual harassment experienced by females and the consequences of this for the physiological stress experienced by females (Chapter 4); and I also test how the water

temperature and salinity that males experience during their growth to adulthood affect their subsequent investment into sperm production (Chapter 5).

REFERENCES

- Archer, C. R., Recker, M., Duffy, E., & Hosken, D. J. (2018). Intralocus sexual conflict can resolve the male-female health-survival paradox. *Nature Communications*, 9(1), 5048. <https://doi.org/10.1038/s41467-018-07541-y>
- Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: Multiple mating and female fitness in insects. *Animal Behaviour*, 60(2), 145–164. <https://doi.org/10.1006/anbe.2000.1446>
- Arnqvist, G., & Rowe, L. (2002). Antagonistic coevolution between the sexes in a group of insects. *Nature*, 415(6873), 787–789. <https://doi.org/10.1038/415787a>
- Arnqvist, G., & Rowe, L. (2005). *Sexual conflict*. Princeton University Press.
- Bisazza, A., & Marin, G. (1991). Male size and female mate choice in the eastern mosquitofish (*Gambusia holbrooki*: Poeciliidae). *Copeia*, 1991(3), 730–735. <https://doi.org/10.2307/1446400>
- Booksmythe, I., Head, M. L., Keogh, J. S., & Jennions, M. D. (2016). Fitness consequences of artificial selection on relative male genital size. *Nature Communications*, 7, 11597. <https://doi.org/10.1038/ncomms11597>
- Chapman, T. (2006). Evolutionary conflicts of interest between males and females. *Current Biology*, 16(17), R744–R754. <https://doi.org/10.1016/j.cub.2006.08.020>
- Chapman, T., Arnqvist, G., Bangham, J., & Rowe, L. (2003). Sexual conflict. *Trends in Ecology & Evolution*, 18(1), 41–47. [https://doi.org/10.1016/S0169-5347\(02\)00004-6](https://doi.org/10.1016/S0169-5347(02)00004-6)
- Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, 373(6511), 241–244. <https://doi.org/10.1038/373241a0>

- Chung, M.-H. J., Noble, D. W. A., Fox, R. J., Harrison, L. M., & Jennions, M. D. (2025). Fluctuating salinity during development impacts fish life histories. *Journal of Animal Ecology*, 94(9), 1848–1865. <https://doi.org/10.1111/1365-2656.70095>
- Cummings, M. E. (2018). Sexual conflict and sexually dimorphic cognition—Reviewing their relationship in poeciliid fishes. *Behavioral Ecology and Sociobiology*, 72(4), 73. <https://doi.org/10.1007/s00265-018-2483-9>
- Constanz, G. D. (1989). Reproductive biology of poeciliid fishes. In G. K. Meffe & F. F. Snelson (Eds.), *Ecology and evolution of livebearing fishes (Poeciliidae)* (pp. 33–49). Prentice Hall.
- Dadda, M. (2015). Female social response to male sexual harassment in poeciliid fish: A comparison of six species. *Frontiers in Psychology*, 6, 1453. <https://doi.org/10.3389/fpsyg.2015.01453>
- Dadda, M., Pilastro, A., & Bisazza, A. (2005). Male sexual harassment and female schooling behaviour in the eastern mosquitofish. *Animal Behaviour*, 70(2), 463–471. <https://doi.org/10.1016/j.anbehav.2004.12.010>
- Engström-Öst, J., & Candolin, U. (2007). Human-induced water turbidity alters selection on sexual displays in sticklebacks. *Behavioral Ecology*, 18(2), 393–398. <https://doi.org/10.1093/beheco/arl097>
- Fricke, C., Perry, J., Chapman, T., & Rowe, L. (2009). The conditional economics of sexual conflict. *Biology Letters*, 5(5), 671–674. <https://doi.org/10.1098/rsbl.2009.0433>
- García-Roa, R., Chirinos, V., & Carazo, P. (2019). The ecology of sexual conflict: Temperature variation in the social environment can drastically modulate male

harm to females. *Functional Ecology*, 33(4), 681–692.

<https://doi.org/10.1111/1365-2435.13275>

Hester, F. J. (1964). Effects of food supply on fecundity in the female guppy, *Lebistes reticulatus* (Peters). *Journal of the Fisheries Research Board of Canada*, 21(4), 757–764. <https://doi.org/10.1139/f64-068>

Huang, X., He, L., Feng, G., Geng, Z., Zhang, T., Yang, G., Wang, S., Zhuang, P., & Zhao, F. (2025). Mating behavior and the effects of low salinity on the reproductive performance of *Eriocheir sinensis* H. Milne-Edwards, 1853 (Decapoda: Brachyura: Varunidae). *Journal of Crustacean Biology*, 45(3), ruaf055. <https://doi.org/10.1093/jcbiol/ruaf055>

Järvenpää, M., & Lindström, K. (2004). Water turbidity by algal blooms causes mating system breakdown in a shallow-water fish, the sand goby *Pomatoschistus minutus*. *Proceedings of the Royal Society B: Biological Sciences*, 271(1555), 2361–2365. <https://doi.org/10.1098/rspb.2004.2870>

Lev, A., Martinez, I. G., & Pischedda, A. (2025). Longer matings increase male competitive fertilization success in *Drosophila melanogaster*. *Biology Letters*, 21(12), 20250435. <https://doi.org/10.1098/rsbl.2025.0435>

Lymbery, S. (2019). *The outcome of sexual conflict depends on the social environment* [Doctoral thesis]. <https://doi.org/10.26182/5d4b9a321f073>

Meffe, G. K. (1992). Plasticity of life-history characters in eastern mosquitofish (*Gambusia holbrooki*: Poeciliidae) in response to thermal stress. *Copeia*, 1992(1), 94–98. <https://doi.org/10.2307/1446539>

Meffe, G. K., & Snelson, F. F. (1989). *Ecology and evolution of livebearing fishes (Poeciliidae)*. Prentice Hall.

- Newport, C., Padgett, O., & de Perera, T. B. (2021). High turbidity levels alter coral reef fish movement in a foraging task. *Scientific Reports*, 11(1), 5413.
<https://doi.org/10.1038/s41598-021-84814-5>
- Pankhurst, N. W., & Porter, M. J. R. (2003). Cold and dark or warm and light: Variations on the theme of environmental control of reproduction. *Fish Physiology and Biochemistry*, 28(1), 385–389.
<https://doi.org/10.1023/B:FISH.0000030602.51939.50>
- Parker, G. A. (1979). Sexual selection and sexual conflict. In M. S. Blum & N. A. Blum (Eds.), *Sexual selection and reproductive competition in insects* (pp. 123–166). Academic Press.
- Parker, G. A. (2006). Sexual conflict over mating and fertilization: An overview. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361(1466), 235–259. <https://doi.org/10.1098/rstb.2005.1785>
- Pilastro, A., Benetton, S., & Bisazza, A. (2003). Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour*, 65(6), 1161–1167.
<https://doi.org/10.1006/anbe.2003.2118>
- Plath, M., Makowicz, A. M., Schlupp, I., & Tobler, M. (2007). Sexual harassment in live-bearing fishes (Poeciliidae): Comparing courting and noncourting species. *Behavioral Ecology*, 18(4), 680–688. <https://doi.org/10.1093/beheco/arm030>
- Plesnar-Bielak, A., & Łukasiewicz, A. (2021). Sexual conflict in a changing environment. *Biological Reviews*, 96(5), 1854–1867.
<https://doi.org/10.1111/brv.12728>

Pyke, G. H. (2005). A review of the biology of *Gambusia affinis* and *G. holbrooki*.

Reviews in Fish Biology and Fisheries, 15(4), 339–365.

<https://doi.org/10.1007/s11160-006-6394-x>

Pyke, G. H. (2008). Plague minnow or mosquito fish? A review of the biology and

impacts of introduced *Gambusia* species. *Annual Review of Ecology, Evolution, and Systematics*, 39, 171–191.

<https://doi.org/10.1146/annurev.ecolsys.39.110707.173451>

Reinhardt, K., Naylor, R., & Siva-Jothy, M. T. (2003). Reducing a cost of traumatic

insemination: Female bedbugs evolve a unique organ. *Proceedings of the Royal Society B: Biological Sciences*, 270(1531), 2371–2375.

<https://doi.org/10.1098/rspb.2003.2515>

Servili, A., Canario, A. V. M., Mouchel, O., & Muñoz-Cueto, J. A. (2020). Climate

change impacts on fish reproduction are mediated at multiple levels of the brain-pituitary-gonad axis. *General and Comparative Endocrinology*, 291, 113439.

<https://doi.org/10.1016/j.ygcen.2020.113439>

Stearns, S. C., & Sage, R. D. (1980). Maladaptation in a marginal population of the

mosquito fish, *Gambusia affinis*. *Evolution*, 34(1), 65–75.

<https://doi.org/10.2307/2408315>

Sundin, J., Aronsen, T., Rosenqvist, G., & Berglund, A. (2017). Sex in murky waters:

Algal-induced turbidity increases sexual selection in pipefish. *Behavioral*

Ecology and Sociobiology, 71(5), 78. [https://doi.org/10.1007/s00265-017-2310-](https://doi.org/10.1007/s00265-017-2310-8)

[8](https://doi.org/10.1007/s00265-017-2310-8)

- Utne-Palm, A. C. (2002). Visual feeding of fish in a turbid environment: Physical and behavioural aspects. *Marine and Freshwater Behaviour and Physiology*, 35(1–2), 111–128. <https://doi.org/10.1080/10236240290025644>
- Vega-Trejo, R., Jennions, M. D., & Head, M. L. (2016). Are sexually selected traits affected by a poor environment early in life? *BMC Evolutionary Biology*, 16(1), 263. <https://doi.org/10.1186/s12862-016-0838-2>
- Vincent, A., Head, M. L., & Iglesias-Carrasco, M. (2020). Sexual conflict and the environment: Teasing apart effects arising via males and females. *Animal Behaviour*, 162, 57–66. <https://doi.org/10.1016/j.anbehav.2020.01.010>
- Wigby, S., Perry, J. C., Kim, Y.-H., & Sirot, L. K. (2016). Developmental environment mediates male seminal protein investment in *Drosophila melanogaster*. *Functional Ecology*, 30(3), 410–419. <https://doi.org/10.1111/1365-2435.12515>
- Wilson, R. S. (2005). Temperature influences the coercive mating and swimming performance of male eastern mosquitofish. *Animal Behaviour*, 70(6), 1387–1394. <https://doi.org/10.1016/j.anbehav.2004.12.024>

Chapter 2: Teasing apart the cost of sexual harassment and multiple mating in females

“What is good for a male's reproductive success is often detrimental to a female's.”— **Göran Arnqvist**

Introduction

Sexual conflict is the evolutionary conflict that occurs between males and females because of their divergent fitness interests in reproduction (Hosken et al., 2019). In general, males and females have a shared fitness interest because they both want to produce offspring, but they differ in their strategies about how to reach this goal (Arnqvist & Rowe, 2005; Hosken et al., 2019). The sexes have divergent interest which are associated with differences in the cost and benefits of mating, and each sex therefore follows a different path to maximize lifetime reproductive success. By definition, males produce smaller gametes and females produce larger gametes (Arnqvist & Rowe, 2005). As a consequence of this difference, females tend to invest more energy per gamete than males which then affects their optimal reproductive strategy (Cummings, 2018). One of the main differences is that a high mating rate is beneficial for males (more mates equates to more offspring), while females only require one or a few matings to ensure all of their eggs are fertilised (Wedell et al., 2006).

Females have a slower rate of reproduction than males due to their higher investment in each reproductive event. This is due to both larger gamete size, which slows down the rate of gamete production, and greater post-fertilization parental investment (i.e. parental care) that delay re-entry to the mating pool (Fromhage & Jennions, 2016). In contrast, males can quickly replenish their sperm supply and, especially when they do not provide parental care, they can soon re-enter the mating pool to sire more offspring with another female. A single mating is often enough to fertilize all of a female's eggs which is then

followed by the period of time taken to raise offspring and generate a new set of eggs, while a male's reproductive success depends on the number of mates he fertilises over the same period of time (Janicke et al., 2016). Also, in some species females can store sperm for long periods which can further reduce their need to re-enter the mating pool to mate again (e.g. if they have spermatheca; Olivera-Tlahuel et al., 2017). Males therefore have a higher optimal mating rate. These differences in gamete and parental investment and the resultant difference in the optimal mating rate between the sexes has led to different reproductive strategies, which ultimately causes sexual conflict.

Longer copulations and repeated mating with the same female often increase the fitness of males as they result in greater sperm transfer and higher fertilization success under sperm competition (reviewed in Edward et al., 2015). In contrast, females often incur costs of mating associated with intense male harassment, the transfer of toxic substances within semen, disease transmission and genital tract injury (Edward et al., 2015). These costs can lower the reproductive output and lifespan of females (Gay et al., 2009; Kawagoe et al., 2001; Le Galliard et al., 2008; McNamara et al., 2008; Rossi et al., 2010; Sakurai & Kasuya, 2008; Takahashi & Watanabe, 2010). There are also costs associated with mating for males (Chung et al., 2021), but the potential gain (i.e. number of offspring sired) relative to these costs is usually higher in males than females. For example, in mosquitofish (*Gambusia holbrooki*), males constantly chase females and attempt to forcibly inseminate them using their modified anal fin ("gonopodium") at rates of up to one attempt/minute (Wilson, 2005). The distal end of the gonopodia is characterized by spines that probably assist in sperm transfer but can damage the female's genital tract. Males provide only sperm during reproductive events and have no other resources to provide to females (Endler, 1987; Houde, 1997). Males increase their own reproductive output, seemingly at the expense of females, by harassing and forcibly inseminating females. In theory, the optimal mating rate for males is many times a day (i.e. as often as

they have viable sperm to transfer); whereas for females it is less than one mating event per breeding cycle (which can take up to a month as gestation lasts up to 21-40 days) and as the female can store the sperm, so they require fewer copulation to fertilize ger eggs (Kim et al., 2021; Wu et al., 2025). This sex difference gives rise to intense conflict between the sexes over whether to mate. Although females in a high harassment situation often suffer the cost of reduced survival, they can show a plastic evolutionary response of greater allocation of resources to eggs to maximize fitness given a shorter expected lifespan. This occurs, for example, in the ruby-spotted damselfly *Hetaerina americana*, where higher male harassment lowers female survival but females lay more eggs, and bigger eggs (Cordoba-Aguilar, 2009).

The rate of polyandry in nature is high, and monogamy is rare (e.g. it occurs in only a few taxa in insects, see (Arnqvist, 1998)). Although in many species female can fertilize her egg using sperm from only one male, but in nature this does not happen quite often as lots of males try to mate with her. One possible reason for a lack of monogamy is that females benefit from mating multiply. The benefits of multiple mating or polyandry have some strong theoretical and experimental support. Several studies have examined the consequences of polyandry and showed substantial direct ‘material’ benefits of polyandry because multiple mating increases female lifetime reproductive success (e.g. increasing egg production, growth or longevity via transferred nutrients in sperm or spermatophore) (Fromonteil et al., 2023; Jennions & Petrie, 2000). For instance, in green-veined white butterfly, *Pieris napi*, polyandrous females had higher lifetime fecundity, longevity and offspring survival (Karlsson, 1998; Wiklund et al., 1993).

In some species, multiple-mating females may also increase their reproductive output by producing more viable and sexually attractive offspring, possibly due to inheritance of beneficial genes from preferred males (Jennions & Petrie, 2000; Slatyer et al., 2012). This might explain why polyandrous females sometimes have higher reproductive output and

greater offspring viability than monandrous females (e.g., Eizaguirre et al., 2007; Fisher et al., 2006; Gowaty et al., 2010). If a polyandrous female can choose the best sperm to fertilize her eggs this could provide indirect genetic benefits to her offspring (meta-analysis: (Slatyer et al., 2012)).

Although many studies have shown the benefits of polyandry, it is not always advantageous: the net effect depends on both the costs and benefits of mating with several males. Indeed, multiple mating by females has also been shown to reduce a female's reproductive success and can significantly decrease offspring performance measures such as growth rate, survival and adult size. For example, in the windmill butterfly, *Atrophaneura alcinous*, multiply mated females have lower longevity than singly mated females, but with no compensatory increase in their fecundity (Kawagoe et al., 2001); similarly, in almond moth, *Cadra cautella*, no fitness benefit of multiple mating has been found as it decreases female longevity (McNamara et al., 2008). It is worth noting, however, that the fitness consequences of polyandry may vary across environments because the costs (or benefits) might change (Ivy et al., 1999).

Sexual conflict is common in poeciliid fishes due to the difference in optimal reproductive strategies between males and females. Among vertebrates, poeciliids have some of the highest mating rates documented, as well as some of the greatest relative differences in sex-specific optimal mating rates (Cummings, 2018). These fish are viviparous with internal fertilization and females give birth to live offspring. More than over half of the poeciliids species, the males only use gonopodial thrusts, where they swing their gonopodia forward and try to inseminate the female, as a mating tactic (sometimes referred to as 'forced copulation attempts') (Bisazza, 1993). If the only benefit of mating is fertilization, then the optimum mating rates for females is one successful mating per breeding cycle (and a cycle takes about a month), whereas in males it is as many times a day as they have viable sperm to transfer. Female poeciliids use a range of strategies to

avoid unwanted male mating attempts. For example, females will change their body orientation, slap the male using their fins and show greater shoaling tendencies (Bisazza, 1993). These actions reduce the rate of successful copulatory attempts by males. The fundamental process of responding to conflict over mating leads to a variety of behavioural differences between the sexes in poeciliid fishes (Magurran & Garcia, 2000). This makes Poeciliids an ideal model system for research in the fields of reproductive ecology and evolution.

The eastern mosquitofish, *Gambusia holbrooki* is a poeciliid that has been of particular interest to researchers studying sexual selection, mate choice, and the consequences of multiple mating. Males spend most of their time chasing and attempting to mate with females, while females try to avoid encounters with, or harassment by, males to reduce the direct costs of excess mating (Agrillo et al., 2006; Dadda et al., 2005; Pilastro et al., 2003). Males have a hooked and barbed gonopodium that can injure females (Sommer-Trembo et al., 2017). Excessive sexual harassment can also increase physiological stress levels in female mosquitofish which can negatively affect their reproductive physiology, potentially leading to reduced reproductive success (Iglesias-Carrasco et al., 2019). Constant harassment also requires females to allocate more energy towards evading or resisting unwanted mating attempts. This could potentially lower overall fitness by diverting energetic resources away from growth, immune function, and reproduction. Prolonged sexual harassment can alter a female's behaviour and activity patterns as she tries to reduce the costs of harassment. For instance, female mosquitofish may spend more time hiding or avoiding males, which could lower their foraging opportunities and overall fitness. As already noted, however, studies have also shown that multiple mating can be a source of variation in reproductive success among females. Some females may benefit from multiple mating, while others might experience negative effects due to costs associated with repeated mating, such as increased energy expenditure and risk of injury.

In mosquitofish, females who experience sexual harassment give birth sooner, have a shorter gestation period and produce larger broods (Fox et al., 2019). The exact trade-off between the benefits of multiple mating and the costs of increased harassment due to the presence of several males may vary among females (Fox et al., 2019).

My aim in the current study was first to explore how variation in the number of males per female changes the reproductive behaviour of females. Next, I investigated the independent effects of: (a) the level of sexual harassment and (b) the number of mates on the behaviour and reproductive success of females. To do this, I manipulated the level of sexual conflict by varying the number of males per female (either one or three males). But I also wanted to measure the independent effect on females of the number of mates. The key challenge is that if polyandry does not provide benefits to females this might be because of the confounding cost of being housed with several males that increases sexual harassment. The level of male harassment and polyandry can, however, be experimentally separated by cutting the gonopodia of some males (ablation) so that several males harass a female but only one of them can transfer sperm.

In this study, I cut the gonopodia of some males and set up three treatments. I put (a) one female and one intact male in a tank (low harassment and monogamy, M); (b) one female and two ablated males and one intact male in a tank (high harassment and monogamy, H); and (c) one female and three intact males in a tank (high harassment and polygamy, P). This experimental design allowed me to measure the effects of polyandry while controlling for the level of sexual conflict (H vs P); and to measure the effects of sexual harassment while controlling for the number of mates (H vs M) as ablated males could harass the female but were unable to inseminate her. My experimental design therefore allowed me to distinguish how harassment and polyandry affect females.

Materials and Method

Study Species: Origin and maintenance of individuals

The eastern mosquitofish (*Gambusia holbrooki*) is a freshwater poeciliid fish native to North America, which was introduced into Australia in the 1920s to control mosquito populations. Mosquitofish are livebearers with internal fertilization. Males almost exclusively employ a coercive strategy to achieve copulation. Males continually chase females, pursuing them from behind, and then insert the tip of their gonopodium (intromittent organ) into a female's gonopore to transfer sperm. The tip of the gonopodium has claw and hook structures which are assumed to prolong copulatory duration (Chung et al., 2020; Kwan et al., 2013) but can also harm the female by damaging her reproductive tract (Sommer-Trembo et al., 2017). Females often reject mating attempts by swimming away from males, or by actively attacking them. The high degree of sexual conflict over mating makes *G. holbrooki* an ideal species for my experiment.

To generate experimental fish, I collected juvenile mosquitofish from Sullivan's Creek, Canberra, Australia in 2022. I then transferred the fish to 90-L stock aquaria (up to 50 individuals per aquarium) at the Australian National University. I inspected the fish twice a week to determine their sex, identifying females by the presence of visible eggs and a round anal fin, and identifying males by an elongated anal fin (i.e., gonopodium). Immature females and males were then transferred to single-sex aquaria (90-L) to ensure they were initially virgins when used in subsequent experiments. All fish were housed at 26 ± 1 °C under a 14 h light:10 h dark cycle and fed commercial fish flakes and brine shrimp twice daily.

Experimental design

To test for the effect of sexual harassment and multiple mating on females, I randomly selected three size-matched virgin females (Linear Model, $F_{2,384} = 0.296$; $P = 0.744$) from stock tanks and housed them with either one intact male (i.e., presumed to be low sexual harassment) or three males (i.e., two ablated and one intact or three intact males; presumed to be high sexual harassment) in an individual 7L tank (Fig 1).

I investigated the relative contribution of harassment (after controlling for the number of males that can mate); and polyandry (controlling for the number of males attempting to mate) by manipulating the ability of some males to transfer sperm. To prevent some males from inseminating females, I surgically removed the tip of their gonopodium (hereafter termed ‘ablated males’). Ablated males are unable to receive sensory cues from the tip of their gonopodium that trigger ejaculation. Notably, the ablation surgery only affects the process of ejaculation. It does not affect the mating behaviour of males, such as their propensity to chase females or to swing the gonopodium forward to try to mate (Chung et al., 2020, 2024). I had three treatment groups:

a. Low harassment and monogamy (M): A virgin female was housed with a non-ablated male (intact gonopodium) for 8 weeks. The female was only harassed by one male (i.e., low harassment) and could only be inseminated by him (i.e., monogamy). (N = 43)

b. High harassment and monogamy (H): A virgin female was housed with two ablated males and a non-ablated male for 8 weeks. The female was therefore harassed by three males (i.e., high harassment) but could only be inseminated by the one non-ablated male (i.e., monogamy). (N = 43)

c. High harassment and polygamy (P): A virgin female was housed with three non-ablated males for 8 weeks. The female was harassed by three males (i.e., high harassment) and could also be inseminated by all three males (i.e., polygamy). (N = 43)

I compared behavioural, reproductive and life-history traits of females with a non-ablated male (M) to that of females with two ablated and a non-ablated male (H) to evaluate the effect of sexual harassment (i.e., pre-copulatory sexual processes) while controlling for the number of mates (Fig 1). I compared the performance of females with two ablated males and a non-ablated male (H) to that of females with three non-ablated males (P) to investigate the effect of polyandry (i.e., post-copulatory sexual processes following insemination that might alter sperm-egg interactions) while controlling for the amount of sexual harassment (i.e., the number of males) (Fig 1).

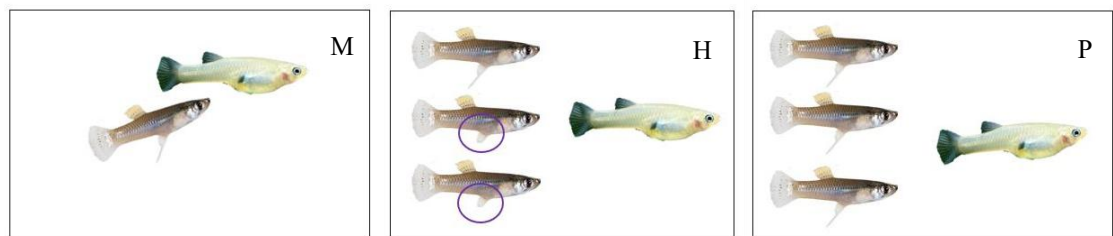


Figure 1: Experimental design with three treatments (M= low harassment and monogamy, H= high harassment and monogamy and P= high harassment and polygamy). Violet circle indicates ablated gonopodia.

Size of fish

Once fish reached sexual maturity, I photographed each female to measure her body length. I placed the fish in AQUI-S solution (20 μ l dissolved in 200ml water) for less than one minute and then put it on a glass slide alongside a size scale (1mm grid paper). I later used *ImageJ* to measure their standard length (SL: the snout tip to the base of caudal fin).

Ablation surgery

The process of ablation was carried out by anesthetizing males in AQUI-S solution. The anesthetized male was then placed on a glass slide and put under a dissecting microscope. I swung the gonopodia forward and removed the tip using a sterile scalpel blade (Diplomat Blades, Victoria, Australia). Males were then transferred into individual 4-L

tanks and given three to five days to recover from the surgery. There was no mortality due to the surgical procedure.

Behavioural observations

After placing the fish in their respective treatments, I observed the mating behaviour of females at week 1 (on the day a female was first placed with males), then again at week 4 and a third time at week 8. At week 1, I introduced the focal female and the male(s) (i.e., one male for treatment “M”, and three males for treatments “H” and “P”) into two chambers within a 7L tank, separated by an opaque barrier. After a 10-minute acclimation period, I carefully removed the barrier and recorded the following behaviours for 10 minutes: (a) the number of rapid approaches: how many times the female rapidly approached and attempted to attack a male that approached within two body lengths; (b) the number of rejections: how many times the female changed her swimming direction or pressed herself against the gravel substrate to avoid mating; (c) the number of mating attempts: how many times the female received a mating attempt (male swung his gonopodium forward and lunged towards the female’s gonopore). The observation tank was surrounded by black paper on three sides during observations to minimize any potential external disturbances. On weeks 4 and 8, there was no acclimation period as the fish were already together.

Female reproductive output

After 21 days, I began to check the tanks twice a day and removed newborn offspring. I then took photographs of the offspring by placing them in a square plastic container with a shallow layer of water. Later I measured their total length (snout to the end of the caudal peduncle) using *Image J*. I recorded: (a) the gestation period, the time between putting the male(s) and female together until the female gave birth to her first brood; (b) the number of offspring in the first brood; and (c) the size of each offspring in the first brood.

At the end of the 8-week period, after checking a female's immune response (see below), I euthanized her and dissected her to: (d) count the number of eggs; and (e) calculate the total mass of the eggs. In addition, I recorded the female's somatic mass after removing her eggs to calculate (f) relative reproductive investment (total egg mass relative to somatic mass).

Growth and body condition

To test if the treatments affected female growth, I measured female size at week 1 and week 8 (i.e. first and last day of the mating treatment). As there was no significant difference in initial size among the treatments, I used their size at week 8 as a measure of their growth. I assume that differences in the rate of growth among treatments is an index of the amount of energy that was spent in response to a higher level of sexual conflict and/or being inseminated by one or several males. To test whether the body condition of females differed among the three treatments, I measured their weight at week 8; the body condition of a female was then calculated by dividing her final body weight by her final standard length at week 8. Body condition was defined as the overall health and well-being of an animal as it provides a quick and simple way to assess the animal's health condition.

Female immune response

To test if a higher level of sexual conflict and/or being inseminated by multiple males had any effect on a female's immune response, I measured the immune response at the end of the mating treatment. I used a phytohaemagglutinin injection assay (PHA test) to measure cell-mediated immunity. After anesthetizing the female in a solution of AQUI-S (20 μ l dissolved in 200ml water) for 30 seconds, I measured her body thickness at the posterior end of the dorsal fin using a pressure-sensitive spessimeter (Mitutoyo 547-301, accuracy: 0.01 mm; five measurements per fish, the intra-class correlation coefficient value was r_1

= 0.909). I then injected 0.01 mg of PHA dissolved in 0.01 ml of PBS into the right side of the fish at the location where the thickness was initially measured. The females were then housed individually for 24 hr, after which I re-measured the thickness five times (r_1 = 0.928). I used the difference between the average pre- and post-injection measurement to estimate the cell-mediated immune response.

Statistical analysis

Each response trait was analysed using a separate linear mixed model (LMM). I included treatment (3 levels: M, H, P) as a fixed factor and included zero-centred initial body size (Week 1; mean SL = 0) as a covariate to account for allometry and any initial female size differences among the three treatments. For the immune response, I used the current body size (Week 8) as a covariate, because body thickness might covary with body size at the time of measurement. In all models that compared the treatments I included block identity as a random factor. I always included the interaction between treatment and body size in the initial models.

I used linear models (LMs) for growth, immunity, body condition, and gestation period because their residuals fulfilled the assumptions of normality, homoscedasticity, and linearity. For the total number of offspring, egg number, egg mass, and relative reproductive investment, I first log-transformed the data before analysing these traits using LMs.

For offspring size, I used a linear mixed model (LMM), with the same terms as the other models, but added female ID as a random factor to control for multiple offspring being produced by the same female. Only two females gave birth twice, so I only used data from the first brood. For the likelihood of giving birth, I used a generalised linear mixed model (GLMM) with binomial error.

As mating behaviours were measured three times over the experimental period, I included

day of testing (3 levels: Week 1, Week 4, Week 8) as a fixed factor and included female ID as a random factor to account for repeated measurements. I analysed behavioural traits using generalized linear mixed models (GLMMs) with the appropriate error distribution: number of rapid approaches (negative binomial), number of rejections (quasi-Poisson), and number of mating attempts (quasi-Poisson). I included the treatment-by-day and treatment-by-body size interactions in the initial models for behavioural traits.

I used Wald chi-square tests to calculate p values (*Anova* function in the *car* package), with type III sum of squares for models with interactions, and type II sum of squares for models without interactions. If there was a significant treatment by day interaction, I ran Tukey's post hoc pairwise tests (*emmeans* package) to assess the treatment effect on behaviour on each testing day. I removed any non-significant interactions from the final models to interpret the main effect of treatment (Engqvist, 2005). If there was a significant treatment effect, I ran a Tukey's post hoc pairwise tests to evaluate the source of differences between treatments.

Summary statistics are presented as mean $\pm$ SE, and the significance level is set at $\alpha = 0.05$ (two-tailed). The outputs of both initial and final models are provided in the Supplementary material. All analyses were conducted in R (version 4.3.1).

Results

Behavioural traits

There was a significant interaction between treatment and day of testing (all $\chi^2 > 12.39$; $P < 0.015$) that affected all female behaviours, but there was no significant interaction between treatment and body size for any of the behavioural traits (all $\chi^2 < 5.85$; $P > 0.054$). I therefore compared treatments in each of the three weeks (Weeks 1, 4 and 8).

Number of rapid approaches

At week 1, all females made a similar number of rapid approaches towards all males regardless of whether there were one or three males present; or whether one or three males could potentially inseminate the female (pairwise: all $P > 0.555$) (Fig 2, see supplementary material Table S1). At week 4, however, females with one male made significantly fewer rapid approaches to males than females with three non-ablated males (M vs P, $P = 0.037$). Interestingly, neither polyandry (controlling for the level of harassment, i.e., number of males present) (H vs P, $P = 0.733$), nor the levels of harassment (controlling for the number of mates) (M vs H, $P = 0.202$) affected the number of rapid approaches. At week 8, female with one male made significantly fewer approaches towards males than females with three males (M vs P or H, both $P < 0.001$). Whether or not all three males could inseminate the female did not affect the number of rapid approaches (P vs H, $P = 0.815$) (Fig 2).

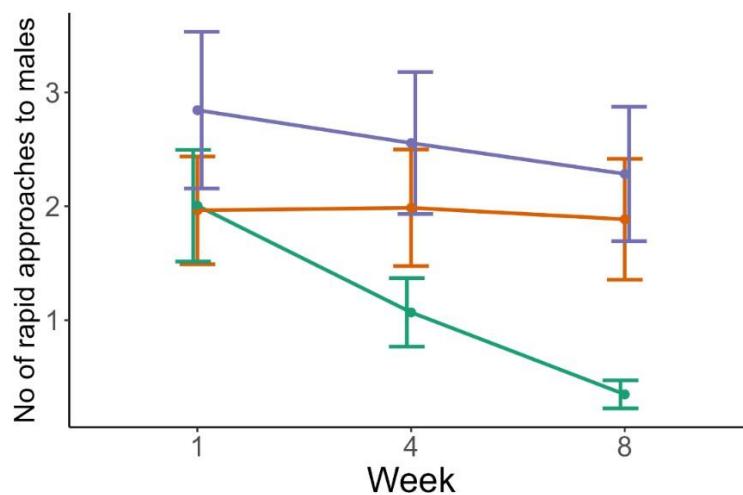


Figure 2: Effects of sexual harassment and polyandry on the number of rapid approaches by female *G. holbrooki* towards males. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P). Data are presented as mean $\pm$ SE.

Number of rejections

At week 1, females who could receive sperm from three males (P) rejected males significantly more often than females who could receive sperm from only a single male (M or H) (both $P < 0.001$) (Fig 3). A higher level of sexual harassment also significantly increased the number of rejections (M vs H; $P = 0.003$). In contrast, at weeks 4 and 8, how many of the three males could inseminate the female did not affect the number of rejections (P vs H, $P > 0.461$), whereas greater sexual harassment (i.e., M vs H or P) increased the number of rejections (both $P < 0.001$) (Fig 3, see supplementary material Table S2).

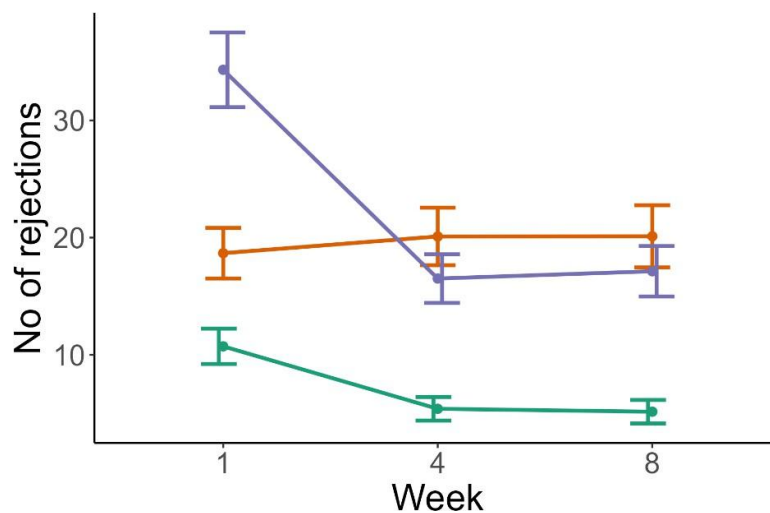


Figure 3: Effects of sexual harassment and polyandry on the number of times that female *G. holbrooki* rejected males. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P). Data are presented as mean $\pm$ SE.

Number of mating attempts

At week 1, females with three males received significantly more mating attempts than females with a single male (pairwise: H vs M, $P = 0.005$; P vs M, $P < 0.001$) (Fig 4). Controlling for the number of males, the number of mating attempts significantly increased when all three males could inseminate the female (P vs H, $P = 0.006$). On both

week 4 and week 8, females with three males again received significantly more mating attempts than females with a single male (M versus H or P, both $P < 0.001$, see supplementary material Table S3). However, whether all three males could inseminate the female did not affect the number of mating attempts that she received (H vs P, $P > 0.448$) (Fig 4).

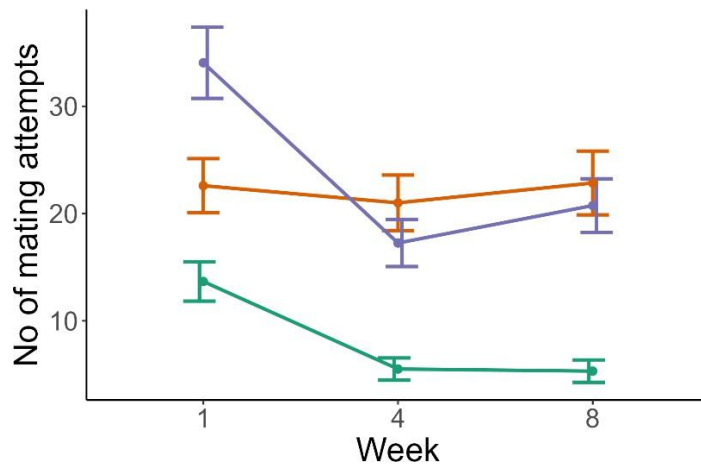


Figure 4: Effects of sexual harassment and polyandry on the number of times that female *G. holbrooki* received gonopodial thrusts from males. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P). Data are presented as mean $\pm$ SE.

Reproductive output

There was no treatment effect on the likelihood of giving birth, the gestation period, the number of offspring, or the size of offspring ($\chi^2 < 5.067$, all $P > 0.362$, Fig 5, see supplementary material Table S4-S7).

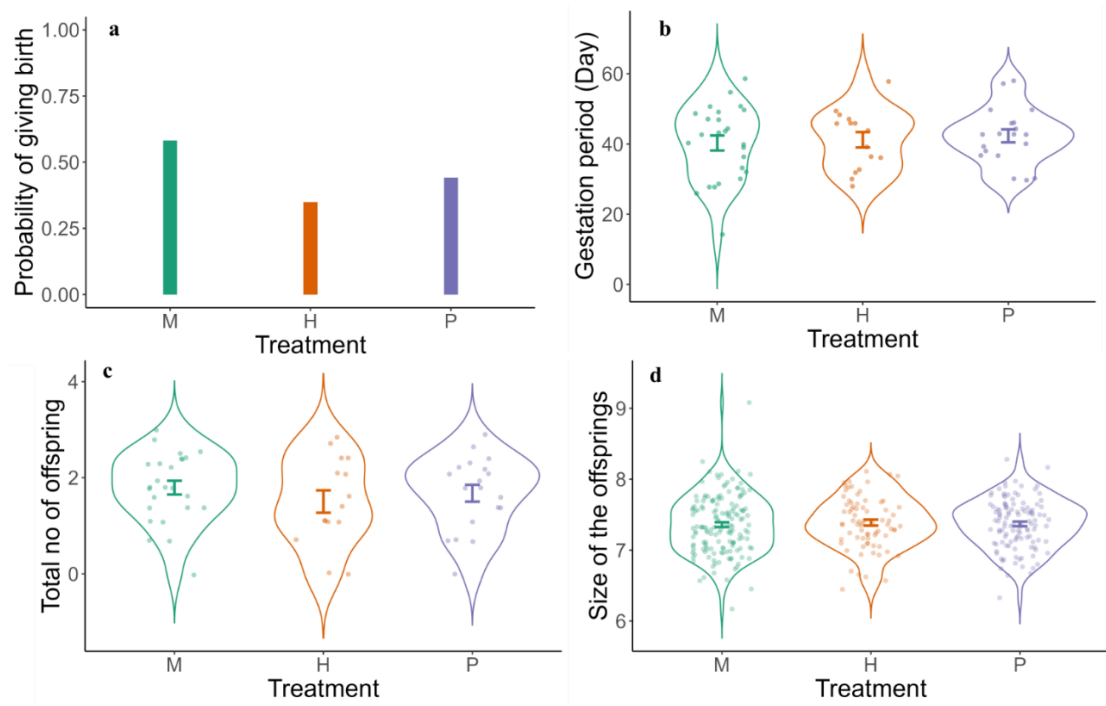


Figure 5: Plots showing the effect of sexual harassment and polyandry on a female's reproductive performance: (a) likelihood of giving birth, (b) gestation period, (c) total number of offsprings, and (d) size of offsprings. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P). Data are presented as mean $\pm$ SE.

Treatment had a significant effect on relative reproductive investment ($\chi^2 = 7.20$, $P = 0.027$, see supplementary material Table S8): females with one male invested significantly less in their egg mass than females with three non-ablated males (pairwise; P vs M, $P = 0.047$). Whereas, neither polyandry (controlling for the level of harassment, i.e., number of males present) (H vs P, $P = 0.106$), nor the levels of harassment (controlling for the number of mates) (M vs H, $P = 0.935$) significantly affected investment in egg mass (Fig 6). Egg mass was strongly related to somatic mass: bigger females produced larger eggs ($\chi^2 = 27.98$, $P < 0.001$). There was no significant difference among treatments in the relationship between female somatic mass and egg mass.

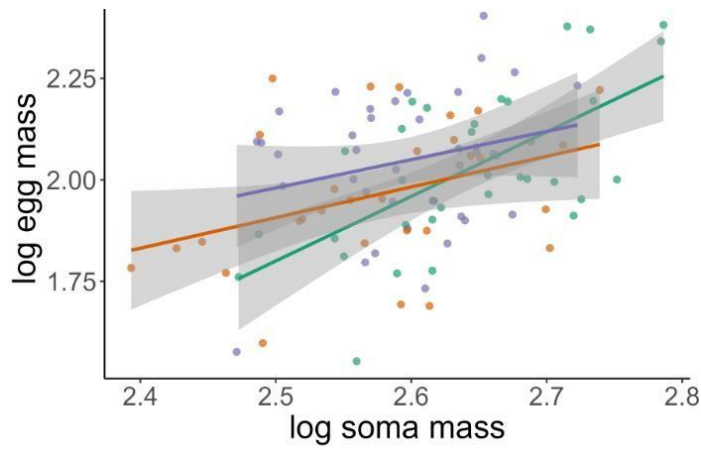


Figure 6: Plots showing the effect of sexual harassment and polyandry on a female's relative reproductive investment. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P).

There was a significant treatment effect on egg number ($\chi^2 = 7.590$; $P = 0.022$, see supplementary material Table S9) (Fig 7). Harassment affected females: females with a single male produced significantly more eggs than females with two ablated and a non-ablated male (pairwise: M vs H, $P = 0.027$). After controlling for the number of males, how many males could inseminate the female also affected the number of eggs as females with three non-ablated males produced more eggs than females with two ablated and a non-ablated male (P vs H, $P = 0.04$). Interestingly, there was no difference in egg number between females with one (M) or three non-ablated males (P) (P vs M, $P = 0.978$) (Fig 7).

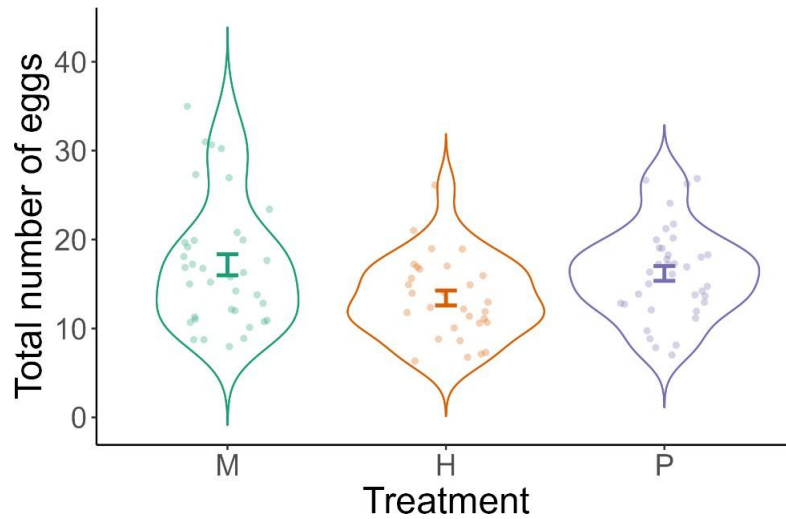


Figure 7: Violin plot showing the effect of sexual harassment and polyandry on the total number of eggs. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P). Data are presented as mean $\pm$ SE.

Life history traits

Treatment significantly affected female growth ($\chi^2 = 21.549$, $P < 0.001$, see supplementary material Table S10), but it had no effect on immune response or body condition (all $\chi^2 < 5.891$; $P > 0.053$, see supplementary materials Table S11-S12) (Fig 8). Females with one male (M) grew significantly larger than females with three males (H or P) (pairwise: both $P < 0.008$). There was therefore an effect of harassment. Whether or not all three males could inseminate the female did not affect her growth (H vs P, $P = 0.222$) (Fig 8).

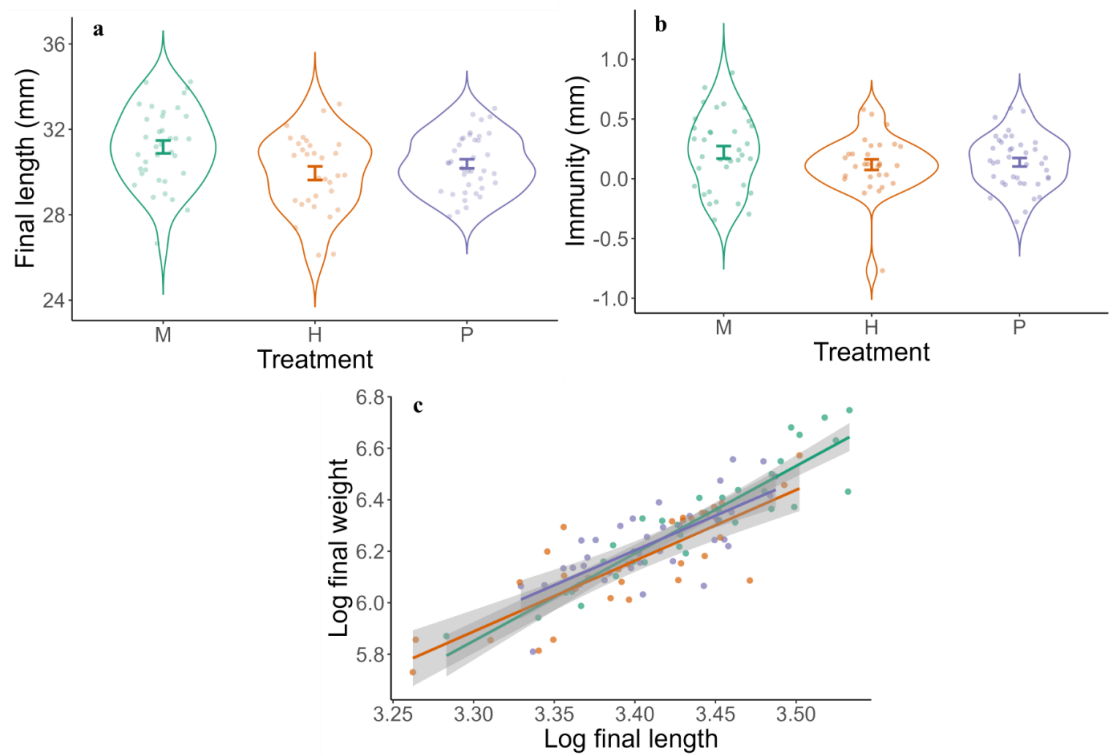


Figure 8: Plots showing the effect of sexual harassment and polyandry on female life history traits: (a) growth (measured as size at week 8 given there was no initial difference in female size, see Methods), (b) immunity, and (c) body condition. Colour indicates the three treatments: green: low harassment and monogamy (M), orange: high harassment and monogamy (H), purple: high harassment and polygamy (P). Data are presented as mean $\pm$ SE.

Discussion

In this study, I teased apart the costs associated with sexual harassment and the potential benefits of multiple mating (polyandry) for female *G. holbrooki*. I controlled the level of harassment by varying the number of males the female interacted with, and I controlled the number of potential mates by ablating the gonopodium tip of some males. Females exposed to three males experienced significantly more mating attempts, rejected males more frequently, and engaged in more chases than females housed with a single male.

These three differences confirm the expected outcome of a change in the level of sexual harassment due to variation in the number of males present.

I then examined the effects of sexual harassment and multiple mating on a female's reproductive investment. There were very few detectable costs to females associated with either the level of male sexual harassment or benefits associated with whether or not a female could mate polyandrously. Most traits were unaffected by sexual harassment (tested for by a comparison of the M vs H treatments where all females mated monogamously). There were only two traits affected after experimentally controlling for any effect of polyandry: females that experienced a lower level of sexual harassment because they only interacted with one male had significantly more eggs and also grew significantly faster than females that experienced sexual harassment by three males.

Likewise, most traits were unaffected by polyandry (tested for by a comparison of P vs H treatments where all females were housed with three males). There was only one trait affected. After controlling experimentally for the level of male harassment, females that could mate polygamously had significantly more eggs than females that could only mate with a single male. The effects of polyandry and sexual harassment are summarised in Table 1.

Cost of harassment (M vs H comparison)

This study shows that sexual harassment imposes measurable costs on female *G. holbrooki*. Females exposed to persistent male harassment grew significantly more slowly and produced significantly fewer eggs than those housed with one male, demonstrating that harassment can directly reduce fecundity (see Table 1). In contrast, a similar study on *G. holbrooki* showed that females who experienced harassment were significantly more likely to give birth, had shorter gestation periods and produce a bigger brood than females who did not experience male harassment (Fox et al., 2019). One possible

explanation for this difference in outcome between the two studies is a difference in the experimental design. Fox et al., 2019 compared females exposed to harassment by three males to females housed without any males, whereas I compared females who were continuously housed with either one or three males. The constant presence of a male in my low harassment treatment might explain the reduced effect of a higher level of harassment on fecundity that I observed in my study. Another study on bruchid beetles (*Callosobruchus maculatus*) showed some similarity to my results (Gay et al., 2009): male sexual harassment reduced the number of eggs produced, although the authors were not sure whether this was due to sexual harassment or costs of mating as their experimental design did not allow them to differentiate between these two factors. In my study, however, I could differentiate between the cost of sexual harassment and multiple mating, so I could show that females produced fewer egg due to the cost of sexual harassment, although harassment did not then affect my measure of offspring fitness (i.e. offspring size).

While harassment did not affect the number of offspring a female gave birth to, it did reduce female growth. This decline in growth is likely to have occurred because harassed females spend less time foraging and more time evading male attention. Previous studies have reported that harassment diminishes foraging efficiency and feeding opportunities (Magurran & Seghers, 1994; Pilastro et al., 2003), leading to reduced somatic investment. To avoid unwanted harassment by males, female may invest less energy in searching for foods and adopt different strategies which lower their foraging efficiency. One study on *Gambusia holbrooki* showed that female foraging efficiency was halved due to male sexual harassment (Pilastro et al., 2003). Sexual harassment therefore seems to be costly for fecundity because it results in smaller body size for females.

The fecundity of fish is largely determined by their size. This could explain why female in the low harassment treatment produced more eggs because they grew to be bigger

than female in the high harassment treatment. In addition to effects of sexual harassment on female foraging, interactions with males might increase injuries (Worthington & Kelly, 2016), which could further reduce female growth. It is noteworthy, however, that the offspring of females that experienced low sexual harassment were no bigger than the offspring of females that experienced high sexual harassment. A study on another poeciliid, the guppy, *Poecilia reticulata*, supports this finding: despite females housed with more males producing bigger eggs, the offspring produced from those eggs were not larger (Barbosa et al., 2012). In sum, in *G. holbrooki*, sexual harassment primarily imposes costs by lowering growth which then appears to affect egg production.

Although female housed with three males received more mating attempts than those housed with one male there was no significant differences in the likelihood of giving birth, the gestation period, the number of offspring or the size of these offspring. This proves that a higher rate of mating (assuming that the actual mating rate is correlated with the rate of mating attempts) does not significantly increase a female's reproductive output and is not beneficial.

Benefits of polyandry (P vs H comparison)

None of the potential benefits of polyandry I measured were observed to occur, except that polyandrous females produced more eggs, demonstrating an immediate benefit of multiple mating. Mating with multiple males did not increase a female's reproductive output or improve other life history traits such as her immunocompetence or growth (see Table 1).

In some species polyandrous females can benefit from higher fecundity when male provide direct material resources or services, such as protection from predator, defence against harassing males, assistance in defending a territory, male parental care, nutritional gifts, hydration, or essential ejaculate-derived compounds (Wagner, 2011). But male *G.*

holbrooki do not appear to provide any of these materials or services to females, which might be why I found few fecundity benefits of polyandry for females. My results also disagree with those from another study on *G. holbrooki*, where another fecundity benefit of polyandry was detected: polyandrous females were more likely to give birth (Fox et al., 2019). However, in this earlier study, monandrous and polyandrous females were created using artificial insemination, while in my study females were with males for the whole experimental period and mating occurred naturally. Repeated mating with a single male might be more likely to ensure sperm transfer than a single artificial insemination attempt.

I did not find any benefits of polyandry/multiple mating for offspring performance (offspring, and number born or the likelihood of producing offspring, which might reflect early offspring mortality). This finding is in contrast to several other studies where offspring performance is positively correlated with multiple mating (Fedorka & Mousseau, 2002; Fox et al., 2019). However, even studies that find benefits of multiple mating report relatively small benefits. There are also many studies that have found no detectable benefits of multiple mating on offspring fitness (Dibattista et al., 2008; McNamara et al., 2008). Indeed, a meta-analysis confirms that the mean advantage of polyandry due to genetic benefits that elevate offspring performance is very small and usually does not differ from zero (Slatyer et al., 2012). In sum, my findings suggest that in *G. holbrooki*, females probably mate with multiple males either unwillingly, or to avoid the cost of harassment (Dibattista et al., 2008). Moreover, my result shows that females who repeatedly mate monandrously with the same male probably gain as much in fitness as females that mate polyandrously (Bybee et al., 2005; House et al., 2009). The only difference being that polyandrous females had more eggs after 8 weeks. This finding aligns with some other studies, such as one on the damselfly *Hetaerina americana*, where

female produced more eggs when they mated with multiple males (Cordoba-Aguilar, 2009).

Finally, my findings about the effect of polyandry on immunity do not align with other studies (e.g. Gershman, 2008; Gordon et al., 2022; Pauletto et al., 2020). These studies showed that polyandry negatively affects a female's immune response. However, another study on lizards that looked at the effects of polyandry on body condition, which is often linked to immune function, also found no significant difference in body condition between monandrous and polyandrous females (Noble et al., 2013).

Interactive effect of polyandry and harassment

Controlling for the number of mates, when females suffered more sexual harassment, they produced significantly fewer eggs ($H < M$); and when controlling for the amount of sexual harassment, polyandrous females produced significantly more eggs than monandrous females ($P > H$). However, females that experienced both multiple mating and higher harassment did not differ in how many eggs they produced compared to females that mated monogamously with low harassment ($P = M$). This suggests that the benefits of multiple mating and the costs of increased harassment tend to be equal and opposite (see Table 1). A study on guppies aligns with my finding as they found no significant difference in fecundity changes due to the effects of multiple mating and sexual harassment (Gasparini et al., 2012).

Relative investment in eggs (i.e. egg mass controlling for soma mass) showed a significant interaction between polyandry and sexual harassment. Controlling for the level of sexual harassment, polyandrous and monandrous females invested equally ($P = H$). Likewise, controlling for the number of mates, females that suffered high or low sexual harassment invested equally ($H = M$). However, females that experienced both multiple mating and higher harassment invested significantly more than females that

mated monogamously with low harassment ($P > M$). This finding is intriguing given that in poeciliids, female significantly spent less time in feeding in the presence of male which should reduce the amount they can invest in eggs (e.g. Pilastro et al., 2003; Plath et al., 2007); and that polyandry seems more likely to be associated with energetic costs to females, such as increased risk of sexually transmitted diseases, (even if there are benefits for their offspring). Instead, the combination of multiple mating and high harassment *increased* the investment in eggs relative to soma. Further studies are required to resolve this puzzle.

Table 1: A summary of tests for the potential costs of harassment and the potential benefit of polyandry. M: Low harassment and monogamy, H: high harassment and monogamy, and P: high harassment and polygamy. P-values for the tests for the main effect of polyandry or harassment are provided in the main text.

Measured traits	Polyandry (P vs H)	Harassment (H vs M)	Interactive effect of polyandry and harassment?	P value
Birth of brood (yes/no)	P = H	H = M	No (i.e. P = H = M)	0.079
Gestation period	P = H	H = M	No (i.e. P = H = M)	0.635
Number of offspring	P = H	H = M	No (i.e. P = H = M)	0.362
Offspring size	P = H	H = M	No (i.e. P = H = M)	0.929
Number of eggs	P > H	H < M	No, P > H < M, but it is curious that M = P	0.022
Total mass of eggs relative to soma mass	P = H	H = M	Yes, P = H and H = M, but, crucially, P > M, so polyandry + harassment leads to a bigger investment!	0.027
Growth	P = H	H < M	No, P = H, and H < M, but also P < M	<0.001
Immune response	P = H	H = M	No (i.e. P = H = M)	0.053

Conclusion

In natural populations it is often difficult to differentiate the cost of continuous male harassment from the cost of multiple mating because the number of mates and level of harassment tend to covary. However, my experimental study provides insight that help to distinguish these two sources of costs from one another. In terms of sexual harassment,

females spent more time avoiding males and consequently presumably had less time for feeding, resulting in slower growth and fewer eggs production. As harassment did not significantly affect other measured traits, my results suggest that its effects are only limited to female condition, rather than exerting any direct effect on offspring quality. Similarly, in terms of polyandry, my study identified only one measurable benefit: polyandrous females produced more eggs, which is somewhat surprising given the fact that male do not provide any direct benefits to the females except sperm. This result suggests that when polyandry and harassment occur simultaneously then the negative effects of harassment can be partially mitigated by polyandry. My study opens several doors for future research. Future studies can be done using the same experimental design to test how harassment and polyandry interact to affect offspring viability and growth rates, which would allow us to determine whether polyandry can confer indirect genetic benefits to females that mate multiply.

REFERENCES

- Agrillo, C., Dadda, M., & Bisazza, A. (2006). Sexual harassment influences group choice in female mosquitofish. *Ethology*, 112(6), 592–598.
<https://doi.org/10.1111/j.1439-0310.2006.01188.x>
- Arnqvist, G. (1998). Comparative evidence for the evolution of genitalia by sexual selection. *Nature*, 393(6687), 784–786. <https://doi.org/10.1038/31689>
- Arnqvist, G., & Rowe, L. (2005). *Sexual conflict*. Princeton University Press.
- Barbosa, M., Connolly, S. R., Hisano, M., Dornelas, M., & Magurran, A. E. (2012). Fitness consequences of female multiple mating: A direct test of indirect benefits. *BMC Evolutionary Biology*, 12(1), 185. <https://doi.org/10.1186/1471-2148-12-185>
- Bisazza, A. (1993). Male competition, female mate choice and sexual size dimorphism in poeciliid fishes. *Marine and Freshwater Behaviour and Physiology*, 23, 257–286. <https://doi.org/10.1080/10236249309378869>
- Bybee, L. F., Millar, J. G., Paine, T. D., & Hanlon, C. C. (2005). Effects of single versus multiple mates: Monogamy results in increased fecundity for the beetle *Phoracantha semipunctata*. *Journal of Insect Behavior*, 18(4), 513–527.
<https://doi.org/10.1007/s10905-005-5609-7>
- Chung, M. H. J., Fox, R. J., & Jennions, M. D. (2020). Fine-scale genital morphology affects male ejaculation success: An experimental test. *Biology Letters*, 16(6), 20200251. <https://doi.org/10.1098/rsbl.2020.0251>
- Chung, M. H. J., Fox, R. J., & Jennions, M. D. (2024). Male allocation to ejaculation and mating effort imposes different life history trade-offs. *PLOS Biology*, 22(5), e3002519. <https://doi.org/10.1371/journal.pbio.3002519>

- Chung, M. H. J., Jennions, M. D., & Fox, R. J. (2021). Quantifying the costs of pre- and postcopulatory traits for males: Evidence that costs of ejaculation are minor relative to mating effort. *Evolution Letters*, 5(4), 315–327.
<https://doi.org/10.1002/evl3.228>
- Cordoba-Aguilar, A. (2009). A female evolutionary response when survival is at risk: Male harassment mediates early reallocation of resources to increase egg number and size. *Behavioral Ecology and Sociobiology*, 63(5), 751–763.
<https://doi.org/10.1007/s00265-009-0709-6>
- Cummings, M. E. (2018). Sexual conflict and sexually dimorphic cognition—Reviewing their relationship in poeciliid fishes. *Behavioral Ecology and Sociobiology*, 72(4), 73. <https://doi.org/10.1007/s00265-018-2483-9>
- Dadda, M., Pilastro, A., & Bisazza, A. (2005). Male sexual harassment and female schooling behaviour in the eastern mosquitofish. *Animal Behaviour*, 70(2), 463–471. <https://doi.org/10.1016/j.anbehav.2004.12.010>
- DiBattista, J. D., Feldheim, K. A., Gruber, S. H., & Hendry, A. P. (2008). Are indirect genetic benefits associated with polyandry? Testing predictions in a natural population of lemon sharks. *Molecular Ecology*, 17(3), 783–795.
<https://doi.org/10.1111/j.1365-294X.2007.03623.x>
- Edward, D. A., Stockley, P., & Hosken, D. J. (2015). Sexual conflict and sperm competition. *Cold Spring Harbor Perspectives in Biology*, 7(4), a017707.
<https://doi.org/10.1101/cshperspect.a017707>
- Eizaguirre, C., Laloi, D., Massot, M., Richard, M., Federici, P., & Clobert, J. (2007). Condition dependence of reproductive strategy and the benefits of polyandry in

- a viviparous lizard. *Proceedings of the Royal Society B: Biological Sciences*, 274(1608), 425–430. <https://doi.org/10.1098/rspb.2006.3740>
- Endler, J. A. (1987). Predation, light intensity and courtship behaviour in *Poecilia reticulata* (Pisces: Poeciliidae). *Animal Behaviour*, 35(5), 1376–1385. [https://doi.org/10.1016/S0003-3472\(87\)80010-6](https://doi.org/10.1016/S0003-3472(87)80010-6)
- Engqvist, L. (2005). The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Animal Behaviour*, 70, 967–971. <https://doi.org/10.1016/j.anbehav.2005.01.016>
- Fedorka, K. M., & Mousseau, T. A. (2002). Material and genetic benefits of female multiple mating and polyandry. *Animal Behaviour*, 64(3), 361–367. <https://doi.org/10.1006/anbe.2002.3052>
- Fisher, D. O., Double, M. C., Blomberg, S. P., Jennions, M. D., & Cockburn, A. (2006). Post-mating sexual selection increases lifetime fitness of polyandrous females in the wild. *Nature*, 444(7115), 89–92. <https://doi.org/10.1038/nature05206>
- Fox, R. J., Head, M. L., & Jennions, M. D. (2019). Disentangling the costs of male harassment and the benefits of polyandry for females. *Behavioral Ecology*, 30(3), 872–881. <https://doi.org/10.1093/beheco/arz024>
- Fromhage, L., & Jennions, M. D. (2016). Coevolution of parental investment and sexually selected traits drives sex-role divergence. *Nature Communications*, 7(1), 12517. <https://doi.org/10.1038/ncomms12517>
- Fromonteil, S., Marie-Orleach, L., Winkler, L., & Janicke, T. (2023). Sexual selection in females and the evolution of polyandry. *PLOS Biology*, 21(1), e3001916. <https://doi.org/10.1371/journal.pbio.3001916>

- Gasparini, C., Devigili, A., & Pilastro, A. (2012). Cross-generational effects of sexual harassment on female fitness in the guppy. *Evolution*, 66(2), 532–543.
<https://doi.org/10.1111/j.1558-5646.2011.01455.x>
- Gay, L., Eady, P. E., Vasudev, R., Hosken, D. J., & Tregenza, T. (2009). Costly sexual harassment in a beetle. *Physiological Entomology*, 34(1), 86–92.
<https://doi.org/10.1111/j.1365-3032.2008.00656.x>
- Gershman, S. N. (2008). Sex-specific differences in immunological costs of multiple mating in *Gryllus vocalis* field crickets. *Behavioral Ecology*, 19(4), 810–815.
<https://doi.org/10.1093/beheco/arn040>
- Gordon, K. E., Wolfner, M. F., & Lazzaro, B. P. (2022). A single mating is sufficient to induce persistent reduction of immune defense in mated female *Drosophila melanogaster*. *Journal of Insect Physiology*, 140, 104414.
<https://doi.org/10.1016/j.jinsphys.2022.104414>
- Gowaty, P. A., Kim, Y.-K., Rawlings, J., & Anderson, W. W. (2010). Polyandry increases offspring viability and mother productivity but does not decrease mother survival in *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences*, 107(31), 13771–13776.
<https://doi.org/10.1073/pnas.1006174107>
- Hosken, D. J., Archer, C. R., & Mank, J. E. (2019). Sexual conflict. *Current Biology*, 29(11), R451–R455. <https://doi.org/10.1016/j.cub.2019.03.052>
- Houde, A. E. (1997). *Sex, color and mate choice in guppies*. Princeton University Press.
- House, C. M., Walling, C. A., Stamper, C. E., & Moore, A. J. (2009). Females benefit from multiple mating but not multiple mates in the burying beetle *Nicrophorus*

vespilloides. *Journal of Evolutionary Biology*, 22(9), 1961–1966.

<https://doi.org/10.1111/j.1420-9101.2009.01800.x>

Iglesias-Carrasco, M., Fox, R. J., Vega-Trejo, R., Jennions, M. D., & Head, M. L.

(2019). An experimental test for body size-dependent effects of male harassment and an elevated copulation rate on female lifetime fecundity and offspring performance. *Journal of Evolutionary Biology*, 32(11), 1262–1273.

<https://doi.org/10.1111/jeb.13526>

Ivy, T. M., Johnson, J. C., & Sakaluk, S. K. (1999). Hydration benefits to courtship feeding in crickets. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 266(1428), 1523–1527.

<https://doi.org/10.1098/rspb.1999.0810>

Janicke, T., Häderer, I. K., Lajeunesse, M. J., & Anthes, N. (2016). Darwinian sex roles confirmed across the animal kingdom. *Science Advances*, 2(2), e1500983.

<https://doi.org/10.1126/sciadv.1500983>

Jennions, M. D., & Petrie, M. (2000). Why do females mate multiply? A review of the genetic benefits. *Biological Reviews*, 75(1), 21–64.

<https://doi.org/10.1017/S0006323199005423>

Karlsson, B. (1998). Nuptial gifts, resource budgets, and reproductive output in a polyandrous butterfly. *Ecology*, 79(8), 2931–2940. [https://doi.org/10.1890/0012-9658\(1998\)079\[2931:NGRBAR\]2.0.CO;2](https://doi.org/10.1890/0012-9658(1998)079[2931:NGRBAR]2.0.CO;2)

Kawagoe, T., Suzuki, N., & Matsumoto, K. (2001). Multiple mating reduces longevity of females of the windmill butterfly *Atrophaneura alcinous*. *Ecological Entomology*, 26(3), 258–262. <https://doi.org/10.1046/j.1365-2311.2001.00326.x>

- Kim, B., Moran, N. P., Reinhold, K., & Sánchez-Tójar, A. (2021). Male size and reproductive performance in three species of livebearing fishes (*Gambusia* spp.): A systematic review and meta-analysis. *Journal of Animal Ecology*, 90(10), 2431–2445. <https://doi.org/10.1111/1365-2656.13554>
- Kwan, L., Cheng, Y. Y., Rodd, F. H., & Rowe, L. (2013). Sexual conflict and the function of genitalic claws in guppies (*Poecilia reticulata*). *Biology Letters*, 9(5), 20130267. <https://doi.org/10.1098/rsbl.2013.0267>
- Le Galliard, J.-F., Cote, J., & Fitze, P. S. (2008). Lifetime and intergenerational fitness consequences of harmful male interactions for female lizards. *Ecology*, 89(1), 56–64. <https://doi.org/10.1890/06-2076.1>
- Magurran, A. E., & Garcia, C. M. (2000). Sex differences in behaviour as an indirect consequence of mating system. *Journal of Fish Biology*, 57(4), 839–857. <https://doi.org/10.1111/j.1095-8649.2000.tb02196.x>
- Magurran, A. E., & Seghers, B. H. (1994). Sexual conflict as a consequence of ecology: Evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proceedings of the Royal Society B: Biological Sciences*, 255(1342), 31–36. <https://doi.org/10.1098/rspb.1994.0005>
- McNamara, K. B., Elgar, M. A., & Jones, T. M. (2008). A longevity cost of re-mating but no benefits of polyandry in the almond moth, *Cadra cautella*. *Behavioral Ecology and Sociobiology*, 62(9), 1433–1440. <https://doi.org/10.1007/s00265-008-0573-9>
- Noble, D. W. A., Keogh, J. S., & Whiting, M. J. (2013). Multiple mating in a lizard increases fecundity but provides no evidence for genetic benefits. *Behavioral Ecology*, 24(5), 1128–1137. <https://doi.org/10.1093/beheco/art040>

- Olivera-Tlahuel, C., Villagrán-Santa Cruz, M., Moreno-Mendoza, N. A., & Zúñiga-Vega, J. J. (2017). Morphological structures for potential sperm storage in poeciliid fishes: Does superfetation matter? *Journal of Morphology*, 278(7), 907–918. <https://doi.org/10.1002/jmor.20684>
- Pauletto, M., Cattelan, S., Pilastro, A., Babbucci, M., Bargelloni, L., & Gasparini, C. (2020). Molecular insights into post-mating immune response in a fish with internal fertilization. *Journal of Evolutionary Biology*, 33(6), 751–761. <https://doi.org/10.1111/jeb.13614>
- Pilastro, A., Benetton, S., & Bisazza, A. (2003). Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour*, 65(6), 1161–1167. <https://doi.org/10.1006/anbe.2003.2118>
- Plath, M., Makowicz, A. M., Schlupp, I., & Tobler, M. (2007). Sexual harassment in live-bearing fishes (Poeciliidae): Comparing courting and noncourting species. *Behavioral Ecology*, 18(4), 680–688. <https://doi.org/10.1093/beheco/arm030>
- Rossi, B. H., Nonacs, P., & Pitts-Singer, T. L. (2010). Sexual harassment by males reduces female fecundity in the alfalfa leafcutting bee, *Megachile rotundata*. *Animal Behaviour*, 79(1), 165–171. <https://doi.org/10.1016/j.anbehav.2009.10.023>
- Sakurai, G., & Kasuya, E. (2008). The costs of harassment in the adzuki bean beetle. *Animal Behaviour*, 75(4), 1367–1373. <https://doi.org/10.1016/j.anbehav.2007.09.010>
- Slatyer, R. A., Mautz, B. S., Backwell, P. R. Y., & Jennions, M. D. (2012). Estimating genetic benefits of polyandry from experimental studies: A meta-analysis.

Biological Reviews, 87(1), 1–33. <https://doi.org/10.1111/j.1469-185X.2011.00182.x>

Sommer-Trembo, C., Plath, M., Gismann, J., Helfrich, C., & Bierbach, D. (2017). Context-dependent female mate choice maintains variation in male sexual activity. *Royal Society Open Science*, 4(7), 170303. <https://doi.org/10.1098/rsos.170303>

Takahashi, Y., & Watanabe, M. (2010). Female reproductive success is affected by selective male harassment in the damselfly *Ischnura senegalensis*. *Animal Behaviour*, 79(1), 211–216. <https://doi.org/10.1016/j.anbehav.2009.10.032>

Wagner, W. E. (2011). Direct benefits and the evolution of female mating preferences: Conceptual problems, potential solutions, and a field cricket. In H. J. Brockmann, T. J. Roper, M. Naguib, J. C. Mitani, & L. W. Simmons (Eds.), *Advances in the Study of Behavior*, 43, 273–319). Academic Press. <https://doi.org/10.1016/B978-0-12-380896-7.00006-X>

Wedell, N., Kvarnemo, C., Lessells, C. M., & Tregenza, T. (2006). Sexual conflict and life histories. *Animal Behaviour*, 71(5), 999–1011. <https://doi.org/10.1016/j.anbehav.2005.06.023>

Wiklund, C., Kaitala, A., Lindfors, V., & Abenius, J. (1993). Polyandry and its effect on female reproduction in the green-veined white butterfly (*Pieris napi* L.). *Behavioral Ecology and Sociobiology*, 33(1), 25–33. <https://doi.org/10.1007/BF00164343>

Wilson, R. S. (2005). Temperature influences the coercive mating and swimming performance of male eastern mosquitofish. *Animal Behaviour*, 70(6), 1387–1394. <https://doi.org/10.1016/j.anbehav.2004.12.024>

- Worthington, A. M., & Kelly, C. D. (2016). Direct costs and benefits of multiple mating: Are high female mating rates due to ejaculate replenishment? *Behavioural Processes*, 124, 115–122. <https://doi.org/10.1016/j.beproc.2015.12.009>
- Wu, T., Li, P., Wu, Z., Wang, Y., Li, S., Shao, F., & Peng, Z. (2025). The morphogenesis of sperm storage micropockets in the western mosquitofish (*Gambusia affinis*). *Animals*, 15(5), 707. <https://doi.org/10.3390/ani15050707>

Chapter 3: Disentangling the life-history costs of direct male-male competition and sperm production

“Our doubts are traitors, and make us lose the good we oft might win, by fearing to attempt.” - **William Shakespeare**

Fatema Akhter ^{1,2}, Megan L. Head ¹, Michael D. Jennions ¹, Meng Han Joseph Chung ^{1,3}

¹ Division of Ecology and Evolution, Research School of Biology, The Australian National University, Acton 2600, Australian Capital Territory, Australia

² Department of Fish Biology and Biotechnology, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University, Khulshi, Chattogram, Bangladesh

³ Centre for Conservation Ecology and Genomics, Institute for Applied Ecology, University of Canberra, Bruce, Australian Capital Territory, Australia

Chapter published in Evolutionary Ecology: <https://doi.org/10.1007/s10682-026-10383-6>

Abstract

To achieve reproductive success, males typically invest more than their rivals to win fights and secure mating opportunities. Male-male competition can then continue after copulation as sperm competition if females mate multiply. While male reproduction often comes at the expense of other fitness-related traits (e.g., growth, immunity), the relative costs of different components of male reproduction (e.g., fighting rivals or sperm production) are unclear. Addressing this oversight is challenging due to the inherent correlation between different reproductive activities: a male must fight and mate successfully before investing in sperm competition. Here, we conducted an experiment in *Gambusia holbrooki* to estimate the life-history costs of different components of

reproduction. To quantify the cost of male-male fighting, we compared life-history traits of males in the presence and absence of rivals. To quantify the cost of sperm investment, we compared life-history traits in males that either could or could not release sperm (following ablation surgery) when in the presence of rivals. Males that fought with rivals for eight weeks did not show reduced growth, immune response, sperm count or sperm velocity than males that had not fought rivals. Interestingly, males that fought rivals spent significantly less time following females but still made the same number of mating attempts, suggesting increased mating efficiency. Conversely, males that repeatedly ejaculated over eight weeks produced significantly fewer sperm than those that could not ejaculate because of ablation of the tip of their gonopodium. Greater past sperm investment due to the inability to ejaculate increased the time spent following females, but did not affect the number of mating attempts. Our findings show that sperm investment in the presence of rivals reduces a male's future ability to produce sperm, whereas no life-history costs of physical fighting were detected.

Keywords

Sperm competition, combat, trade-off, reproductive costs

Introduction

Male reproduction in the presence of rivals is a zero-sum game. To achieve reproductive success, a male often needs to invest more than his rivals. For example, males with larger weapons are more likely to win fights in the bug *Narnia femorata* (Emberts et al. 2018), while greater courtship intensity in the wolf spider *Schizocosa uetzi* is associated with increased attractiveness and higher mating success (Shamble et al. 2009). In species without courtship, males that spend more time pursuing females often make more mating attempts (Head et al. 2015). Male-male competition continues after copulation when females mate multiply. In this case, males that invest more in ejaculate traits (e.g., larger sperm reserves or higher-quality sperm) are more likely to achieve greater fertilisation success (Boschetto et al. 2011). Male reproductive success often requires resource allocation to fighting rivals, acquiring mates and/or producing sperm.

Increases in reproductive investment can however divert resources away from other important body functions (e.g., growth and immune function), potentially creating life-history trade-offs (Flatt and Heyland 2011). Such resource competition may also occur within reproduction, such as trade-offs between different sexually selected traits (e.g., testes size vs. ornaments; Parker et al. 2013; Simmons et al. 2017) or trade-offs within the same traits over time (e.g., current vs. future offspring production) (Scharf et al. 2013; Lüpold et al. 2019). There is a well-developed theory surrounding the life-history costs of reproductive investment (Höglund and Sheldon 1998; Stearns 1989), but it is still unclear how the different components of male reproductive investment contribute to life history trade-offs that lower performance later in life. For example, does effort expended to fight rivals or acquire mates affect future sperm production, and *vice versa*? And what are the relative costs of fighting, mating effort and sperm production on naturally selected traits (e.g., growth, immune function)? To better understand the life-history costs of male reproduction, it is necessary to tease apart the effect of investment in fighting rivals, effort

to acquire mates (by courting, ornament production or sexual coercion) and investment in ejaculates. Such experiments are rare, however, partly because it is difficult to independently manipulate one reproductive component without affecting the others given the inherent correlations among reproductive activities.

The total cost of reproduction due to sexual selection is likely to be higher when many males compete for a mate. When there are more rivals, males often increase their investment in pre-copulatory sexual traits to win fights and secure mating opportunities (Emery Thompson and Georgiev 2014); and/or they invest more in post-copulatory sexual traits that increase the ability to fertilize eggs under sperm competition (Kelly and Jennions 2011). There is evidence that males that consistently compete with rivals show a faster decline in fitness-enhancing traits ('senescence') than males that do not compete (Preston et al. 2011; South et al. 2009). This faster senescence is likely to arise because of the cumulative costs of competition-related investment. Few studies have, however, investigated the extent to which greater male-male competition increases the reproductive costs of greater expression of traits that are sexually selected due to their effect on fighting success, mate acquisition and sperm production.

The time and energy spent tracking females and repelling rivals can be costly. Direct male-male competition involves fights with rivals that can occur both before mating and after mating in the form of sperm competition. The fights may reduce foraging efficiency (Girard-Buttoz et al. 2014; Higham et al. 2011; Matsubara 2003) and cause injuries that lower body condition (Clutton-Brock et al. 1979; Drews 1996). As the number of rivals increases, we therefore expect to see stronger life history trade-offs because males spend more time fighting for access to and/or defending females.

When females mate multiply, indirect male-male competition usually continues after mating in the form of sperm competition. Sperm competition typically favours males that produce more and/or higher quality sperm because this increases fertilization success (Wedell 2010). Current evidence suggests, however, that greater sperm production is expensive. For example, sperm investment increases when there is greater access to food (Evans et al. 2023; Kahrl and Cox 2015), or when males are in better body condition (reviewed in Macartney et al. 2019). As the number of males inseminating females increases, we therefore expect stronger life history trade-offs because of greater investment in sperm production.

The individual costs of fighting with rivals, acquiring mates and producing sperm appear to be high. However, these costs are naturally confounded because males who fight and mate more often tend to invest more in sperm production to replenish depleted sperm reserves. Only a few species provide an opportunity to distinguish between the cost of sperm production and those of acquiring mates because sperm production and mating occur at different times. Researchers can thereby compare changes in body condition before and after sperm production to estimate the relevant cost. For example, male adders (*Vipera berus*) lose considerable body mass during early season sperm production (Olsson et al. 1997); male grasshoppers (*Isophya kraussi*) use about 20% of their total body energy to produce a spermatophore (Voigt et al. 2005); and male nematodes (*Caenorhabditis elegans*) that produce sperm have a 65% shorter lifespan than those that do not, even in the absence of mating (Van Voorhies 1992). These studies do not, however, estimate how the cost of sperm production changes in a more competitive environment; nor do they tease apart the costs of fighting rivals from those of acquiring mates. Experimental manipulations are needed to compare the life-history costs of fighting rivals, acquiring mates, and producing ejaculates under the risk of male-male competition.

Here we used the eastern mosquitofish (*Gambusia holbrooki*) to test how different components of male reproduction affect subsequent investment in fitness-related traits (thereby, testing for early-late life trade-offs driven by specific reproductive components). Male mosquitofish frequently fight for access to females and repel rivals from approaching a female (Harrison et al. 2023). Males rarely court and they lack ornamentation. Instead, males ‘sneak mate’ by stealthily approaching a female from behind and then darting forward at high speed to thrust their gonopodium (a modified anal fin used to transfer sperm) into her gonopore (Bisazza 1993; Bisazza and Marin, 1995). Males are highly persistent, constantly chase females, and can make up to one mating attempt per minute (Wilson 2005). Although females evade most mating attempts they are still inseminated by many males, which results in multiple paternity (Booksmythe et al. 2016; Gao et al. 2019; Head et al. 2017; Vinogradov et al. 2025). There is, therefore, intense sperm competition. Sperm production is condition-dependent and males require up to five days to replenish depleted sperm reserves (O’Dea et al. 2014). Fighting rivals, chasing females and sperm production each appear to be costly in this species.

To quantify the effect of a change in the level of direct male-male competition, we housed males either with or without rivals. To quantify the effect of a change in sperm production, we surgically ablated the tip of the gonopodium to prevent males from ejaculating when they mate. This ablation prevents males from receiving the stimuli that cause sperm release when the gonopodium tip enters a female (Chung et al. 2019, 2020). Ablated males have lower sperm production than non-ablated males because there is less demand to replenish depleted sperm reserves (Chung et al. 2020). Surgical ablation *per se* has otherwise been shown not to affect male mating or fighting behaviour (Chung et al. 2020, 2021, 2024; Fox et al. 2019). We therefore experimentally ablated a group of focal males

to reduce their sperm production without altering other traits that are under pre-copulatory sexual selection (i.e. fighting and mating success).

Materials and Method

Study species

The eastern mosquitofish is a small live-bearing fish that is a globally invasive pest (Pyke 2005). Males are much smaller than females. Males grow only slightly after maturation, while females continue to grow steadily (Chung et al. 2025). Males directly compete for mating opportunities by pursuing females and attacking rivals when they approach a female; and males indirectly compete to fertilise eggs under sperm competition (Evans et al. 2003).

We established fish stocks by collecting juveniles from streams in Canberra, Australia (35°16'46.1"S, 149°07'00.1" E). We reared juveniles in 90L aquaria (<50 fish/aquarium) in temperature-controlled rooms ($26 \pm 1^\circ\text{C}$) under a light: dark cycle of 14:10. Fish were fed commercial fish flakes (Aqua One Tropical Flakes) and live *Artemia* brine shrimp *ad libitum* twice daily. Immature males (still virgin) can be identified by the presence of a partially developed gonopodium. To ensure virginity, we placed all immature males and females individually in separate single-sex aquaria (<50 fish/aquarium). Once the male matured (after approximately 2-3 weeks in male-only aquaria), we transferred them to individual 7L plastic tanks. Mature females were used as stimulus fish to generate different social environments for each treatment (see below).

Experimental design

We aimed to test how fighting rivals versus producing sperm under male-male competition affects a male's subsequent mating behaviour, sperm production, growth and immune function (Fig. 1). We manipulated direct male-male competition by housing either 0 or 2 rivals with a focal male. We manipulated investment in sperm production by

surgically ablating the tip of the focal male's gonopodium (see below). Ablated males fail to release sperm when they attempt to mate, resulting in little investment in sperm production. There were three social environments in a 7 L experimental tank. A focal male was housed with a female with: (a) no rivals; (b) two intact (i.e., non-ablated) rivals; (c) two ablated rivals. Both rivals in (b) and one rival in (c) were marked with elastomer tags (orange; NorthWest Marine Technology, Washington, USA) for individual identification (Fig. 1). Orange-coloured tags do not affect male attractiveness (Aich et al. 2020).

This experimental set-up allowed us to test:

- (i) the effects of direct competition (e.g., male-male fighting) by comparing the intact focal male in environment (a) with the intact focal male in environment (b) or (c). We expected focal males in environment (a) to pay a lower life history cost as direct male-male competition is absent.
- (ii) the effect of rivals' sperm production ability (i.e., ablated or not) by comparing the intact focal males between environments (b) and (c). We did not expect a difference because ablation should not affect a rival's fighting or mating behaviour (Chung et al. 2021, 2024), meaning focal males would lack information about whether their rivals were transferring sperm.
- (iii) the effect of sperm production by comparing the intact focal males with the untagged ablated males in environment (c). We expected intact males to pay a higher cost of reproduction because they needed to replenish sperm more frequently after ejaculation and thus invest more in sperm production.

If test (ii) reveals no difference between being housed with intact and ablated rivals, we can treat test (iii) as equivalent to testing for a difference between an intact and ablated male when each is competing with two equivalent (i.e. intact) rivals.

We set up three 7L tanks (one of each treatment type) together as a block (total N = 129 tanks for 43 blocks). The tanks were placed next to each other in a different order for each

sequential block (i.e. all six possible orders). We first conducted the ablation surgery and allowed the males to recover for at least three days (see below). We then housed each focal male with a female and different number of rivals (Fig. 1) for 8 weeks during which they could freely mate and interact. To test the effects (i-iii), we measured male mating behaviour, growth, ejaculate traits (sperm number and sperm velocity) and immune response throughout the 8-week reproductive period. 8 weeks is approximately half the length of the breeding season in our source population (Kahn et al. 2013). Our previous work detected reproductive costs in males that had mated and fought for 8 weeks and showed that extending the treatment period for another 8 weeks did not amplify these costs (Chung et al. 2024).

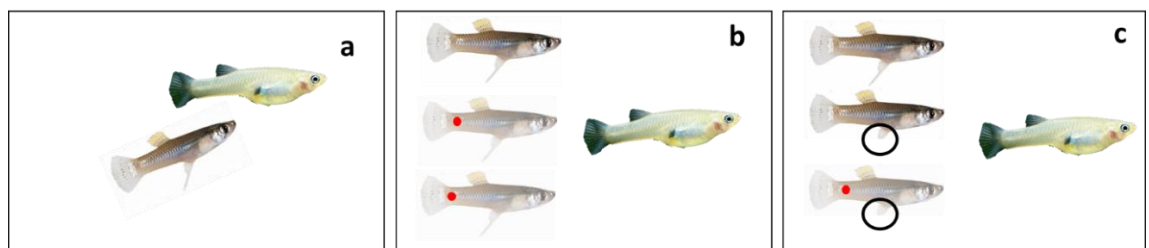


Figure 1: Experimental design manipulating the presence of rivals and the male's ability to release sperm. In each social environment, the intact focal male (untagged) was housed with a female and either: (a) no rivals, (b) two rivals with intact gonopodium tips (both tagged) or (c) two rivals with ablated gonopodium tips (one tagged and one untagged). A red dot represents an elastomer tag on the rivals that allows us to identify the focal male. A black circle indicates the gonopodium status (ablated) of the male. To test the long-term cost of sperm investment, we also tracked performance changes in one of the ablated rivals in treatment (c). To do this, we tagged only one ablated rival to distinguish him from the other rival. Traits of the untagged males were measured.

Ablation surgery

We surgically removed the distal tip of rival males in the environment (c) at least three days before the start of the experiment. Ablated males still compete for mating

opportunities, but they fail to release sperm when trying to mate, presumably because they do not receive sensory cues indicating that they have inserted their gonopodium into a female's gonopore (Chung et al. 2020). The failure of ablated males to inseminate females (Kwan et al. 2013) is assumed to lower their rate of sperm production because they do not have to ramp up sperm production to replenish sperm reserves after ejaculating. Chung et al. (2020) showed that ablation significantly reduces sperm release but does not otherwise alter male mating behaviour.

To surgically ablate the gonopodium tip, we anesthetized a male in AQUI-S solution (20 μ L in 200mL water) for 30s. The male was then put on a glass slide under a dissecting microscope. We swung his gonopodium forward and removed the tip using a scalpel blade. Males were then transferred to individual 4 L tanks and held for 3-5 days until the start of the experiment. There was no mortality.

Mating and fighting behaviour

Once fish were in the 7 L experimental tanks, we recorded each focal male's behaviour in weeks 1 (i.e. day 0-7), 4 (i.e. day 22-28), and 8 (i.e. day 50-56). In Week 1, we introduced all males from a treatment (either 1 or 3 males; see Fig. 1) to one side of the tank and a virgin female from the female-only stock tanks was placed on the other side. An opaque barrier prevented males and the female from seeing each other. After a 10-minute acclimation, we removed the barrier and recorded the following behaviours of the focal male for 10 minutes: **(a) following time**: the time the male spent following the female, defined as swimming below or behind her within two female body lengths; **(b) aggression toward rivals**: the number of aggressive acts directed by the focal male towards rivals; **(c) aggression from rivals**: the number of aggressive acts directed at the focal male by rivals; and **(d) mating attempts**: the number of occasions when the focal male approached the female from behind and thrust his gonopodium towards her

gonopore. To minimize external disturbances, we covered the tank on three sides with black paper while making observations.

The Week 1 behavioural observation occurred during the first 10 minutes of the 8-week reproductive period (i.e., when the focal males first encountered rivals and females). After this observation, we left the fish in the same tanks for the entire 8-week period. 4 and 8 weeks after the start of the experimental period (i.e., Weeks 4 and 8), we again set up a 7L tank (as above) and observed the same four behavioural traits for 10 minutes to assess changes in mating and fighting behaviours over time. All observation were made by eye, and the data were directly noted down by the same person (F. Akhter) observing one tank at a time.

Growth

We measured each focal male's standard length (from the snout tip to the end of the vertebral column) at the start (Week 1) and end (Week 8) of the experimental treatment. A male was anesthetized in AQUI-S solution (20 μ L in 200mL water) for 30s and then photographed alongside a 1 cm scale. The photos were later analysed using *ImageJ* software (Abràmoff et al. 2004) version 1.53t.

Sperm traits

At the end of the 8-week experimental period, we stripped focal males of sperm and transferred them to individual tanks. Then, 72 hours after this initial stripping, we again stripped their sperm to measure sperm velocity, and count sperm using a Computer-Assisted Sperm Analysis system (CEROS Sperm Tracker, Hamilton Thorne, USA). We followed the methods of Vega-Trejo et al. (2019), which are described below. The initial stripping ensured that the sperm samples that were collected 72 hours later had sperm of the same age, and that all males had the same amount of time to replenish sperm so that we could measure the rate of sperm production.

Sperm velocity: We anesthetized a male in AQUI-S solution and then placed him on a glass slide coated with a 1% polyvinyl alcohol solution (PVA) under a dissection microscope. We carefully positioned his gonopodium to face forward and perpendicular to his body. By gently pressing the abdomen (3-5 times per fish), we collected the ejaculate onto the PVA-coated slide. To keep the sperm hydrated, we added 100 μ l of Extender solution (mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, and 10 mM Tris (Cl); pH 7.5). From the ejaculate, we collected two sets of 5 sperm bundles per male using a micropipette. Five bundles were then each placed into separate microtubes with 3 μ L of Extender solution. To measure sperm velocity, we placed the 3 μ L sample on one cell of a 12-cell multi-test slide and activated it with 3 μ L of solution (125mM KCL and 2 mg/ml bovine serum albumin). We waited 30s before placing a cover slip on top. Next, we placed the slide under the microscope and recorded as many motile tracks as possible per sample (mean 15.8; range 1-108). We recorded the average curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). We only report VCL as all three measures were highly correlated (VSL-VCL: $r = 0.990$; VSL-VAP: $r = 0.998$; and VCL-VAP: $r = 0.993$; all $p < 0.001$). The same procedure was repeated for the second 3 μ L sample to determine the repeatability of our estimate of mean velocity across the two samples (repeatability: $r \pm SE = 0.247 \pm 0.086$, $p = 0.002$, $N = 126$ males).

Sperm count: After collecting 10 sperm bundles, we transferred the rest of the ejaculate from the PVA-coated slide to a 1.5 ml Eppendorf tube. Depending on the amount of ejaculate, we diluted the sample with an appropriate amount of extender medium (100-600 μ L). We vortexed the samples for about 1 minute to break up the sperm bundles, and we pipetted the solution up and down 20-30 times to ensure even distribution of the sperm. Next, we placed 3 μ L on a 20-micron capillary slide (Leja). Using the CEROS Sperm Tracker under 100 $\times$ magnification, we took five measurements per male from randomly

selected areas of the slide. The number of sperm per sample was highly repeatable across males (repeatability: $r \pm SE = 0.903 \pm 0.013$, $p < 0.001$, $N = 128$ males).

Immune response

We conducted a cell-mediated immunity assay using a phytohemagglutinin (PHA) injection test. The day after sperm measurements were made, we anesthetized the focal male and used a pressure-sensitive spessimeter (Mitutoyo 547-301, accuracy: 0.01 mm) to measure his caudal peduncle thickness at the posterior end of his dorsal fin. We measured the thickness five times (repeatability: $r \pm SE = 0.952 \pm 0.007$, $p < 0.001$, $N = 123$). Next, we injected 10 μ L of the PHA solution (0.01 mg of PHA dissolved in 0.01 ml of PBS) into the right side of the caudal peduncle at the same location. The males were then housed in individual 1L tanks. 24 hours after the PHA injection, we measured the caudal peduncle five times to quantify the amount of swelling (repeatability: $r \pm SE = 0.963 \pm 0.005$, $p < 0.001$, $N = 123$ males). We define the difference between the average post- and pre-injection thickness as the immune response.

All procedures were carried out with approval from the ANU Animal Ethics Committee (protocol number: A2021/04).

Predictions

We predicted that direct male-male competition (presence/absence of rivals) would increase the time the focal male spent fighting rivals but decrease the time he spent following a female and the number of mating attempts due to a time trade-off. We then predicted that the longer-term costs of fighting are: (i) decreased growth and lower immune responses (given that reproduction would divert resources away from somatic functions); (ii) a steeper decrease in the time spent following females, number of mating attempts and aggression towards rivals (due to an early-late life trade-off) – that is, there would be an interaction between the presence/absence of rivals and test week; but (iii) no

changes in sperm production or velocity. Predictions (ii) and (iii) are based on the findings of Chung et al. (2021, 2024), who showed that increased mating effort (which included both mating and fighting in the 2024 study) lowered future mating effort, but did not affect sperm traits.

We predicted that increased sperm production (i.e., the intact male relative to the ablated males in environment (c)) would: (i) decrease growth and lower immune responses; (ii) decrease the future rate of sperm replenishment; but (iii) not affect the time spent following females, number of mating attempts or aggression towards rivals. Predictions (ii) and (iii) are based on the findings of Chung et al. (2021, 2024), where increased sperm production lowered future sperm production, but did not affect future mating effort.

Statistical analysis

Each trait was analysed with a separate model. To test for an effect of male-male competition, we compared the performance of intact focal males without rivals [environment (a)] to those with two rivals [environments (b) and (c)]. We treated social environment (3 levels) as a fixed factor. To test for an effect of greater sperm production, we compared the intact and ablated focal males in environment (c). We treated ablation status (2 levels: intact or ablated) as a fixed factor.

For immunity and sperm traits, we included zero-centred body size at the time of measurement (Week 8) as a covariate because larger males often produce more sperm (O'Dea et al., 2014) and have a greater increase in body thickness after injection. For the other traits (growth, behaviour and sperm traits), we included zero-centred initial body size (i.e., standard length at week 1) as a covariate to account for pre-treatment variation in male size. Mating behaviours were measured three times, so we included the test week (3 levels: Weeks 1, 4 and 8) as a fixed factor and treated male ID as a random factor to account for repeated measurements. In all models that compared intact, focal males across the three environments we included block identity as a random factor. In models that

compared the intact and ablated focal males in environment (c), we included tank identity as a random factor to account for the block effect, as two focal males in a block were in the same tank. In all initial models, we included the interaction between environment and body size, or ablation status and body size. For the models of behavioural traits, we additionally include the two-way interaction between environment and test week, or ablation status and test week.

We analysed the behavioural traits using generalized linear mixed models with the appropriate error distribution: quasi-Poisson for (a) following time, (b) number of mating attempts, and (c) aggression towards rivals; negative binomial for (d) aggression from rivals. We used linear mixed models for (e) growth, (f) immune response, (g) sperm count (log-transformed) and (h) sperm velocity because their model residuals fulfilled the assumptions of normality, homoscedascity, and linearity (determined via Q-Q plots).

We used Wald chi-square tests to calculate p values (*Anova* function in the *car* package version v3.1.3; Fox et al. 2012), with type III sum of squares for models with interactions, and type II for models without interactions. If there was a significant interaction between a fixed factor and test week, we ran Tukey's post hoc pairwise tests (*emmeans* package version v1.10.7; Lenth and Lenth 2018) to assess the effect of the fixed factor on each test week. We removed non-significant interactions from final models to interpret the main effect of environment or ablation status (Engqvist 2005). If there was a significant environment effect, we ran Tukey's post hoc pairwise tests to evaluate the source of differences.

Summary statistics are presented as mean $\pm$ SE. We set $\alpha = 0.05$ (two-tailed). The outputs of all initial and final models are in the Supplementary Material. Analyses were run in R (version 4.3.1; R Core Team 2022).

Results

Life-history costs of direct male-male competition

We compared focal intact males from three social environments (Fig. 1) to test for an effect of direct male-male competition [no rivals in environment (a) versus two rivals in environment (b) or (c)] on behaviour traits, growth and immune response.

Mating and fighting behaviour: The presence/absence of rivals significantly affected the time the focal male spent following the female ($\chi^2_2 = 39.21$; $p < 0.001$). Intact focal males housed with rivals spent significantly less time near females (Tukey's test, a vs b: ratio = 2.21, $z = 5.77$, $p < 0.001$; a vs c: ratio = 1.90, $z = 4.69$, $p < 0.001$), but whether these rivals could release sperm (i.e. intact or ablated) had no effect (Tukey's test, b vs c: ratio = 0.86, $z = -1.02$, $p = 0.562$) (Fig. 2(i)). Despite the decrease in following time, the presence of rivals did not lower the number of mating attempts by the intact focal male ($\chi^2_2 = 0.083$; $p = 0.959$) (Fig. 2(ii)). Neither the week of testing nor pre-treatment body size moderated how the social environment affected the following time (environment*week: $\chi^2_4 = 1.408$, $p = 0.843$; environment*size: $\chi^2_2 = 0.842$, $p = 0.657$) or the number of mating attempts (environment*week: $\chi^2_4 = 5.522$, $p = 0.238$; environment*size: $\chi^2_2 = 2.090$, $p = 0.352$) (Supplementary Material Tables S1-S2). Larger males spent significantly more time following females ($\chi^2_1 = 8.716$; $p = 0.003$) and made more mating attempts ($\chi^2_1 = 11.186$; $p < 0.001$). Both following time ($\chi^2_2 = 60.526$; $p < 0.001$) and the number of mating attempts ($\chi^2_2 = 23.609$; $p < 0.001$) decreased significantly over the 8 weeks.

Because there were no rivals in environment (a), fighting could only be compared between environments (b) and (c). Thus, we tested the effect of rival sperm-releasing ability (ablated or not) on the focal male's fighting. The amount of aggression towards rivals by the intact focal male differed when the rivals were intact or ablated, but the effect depended on the week of testing (environment*week: $\chi^2_2 = 8.488$; $p = 0.014$) (Fig. 2 (iii);

Supplementary Material, Table S3). In week 1, focal males showed the same amount of aggression towards intact or ablated rivals (Tukey's test, b vs c: ratio = 1.07, $z = 0.26$, $p = 0.792$). However, focal males were significantly less aggressive towards intact rivals than towards ablated rivals in both week 4 (Tukey's test, b vs c: ratio = 0.58, $z = -2.28$, $p = 0.022$) and week 8 (Tukey's test, b vs c: ratio = 0.39, $z = -3.50$, $p < 0.001$). Focal males that had a larger pre-treatment body size were significantly more aggressive towards rivals, but the effect of body size was stronger in environment (b) with two intact rivals than in environment (c) with two ablated males (environment*size: $\chi^2_1 = 5.580$; $p = 0.018$). In addition, there was an environment*size interaction on how often the focal males received aggression from rivals ($\chi^2_1 = 4.108$; $p = 0.043$; Supplementary Material, Table S4). Larger focal males received more aggression from rivals, but the effect was stronger when the rivals were intact rather than ablated. There was, however, no interaction between week of testing and social environment ($\chi^2_2 = 4.96$; $p = 0.084$). The week of testing independently affected aggression from rivals ($\chi^2_2 = 6.668$; $p = 0.036$): the number of aggressive attacks directed at the focal male increased with time (Fig. 2 (iv)).

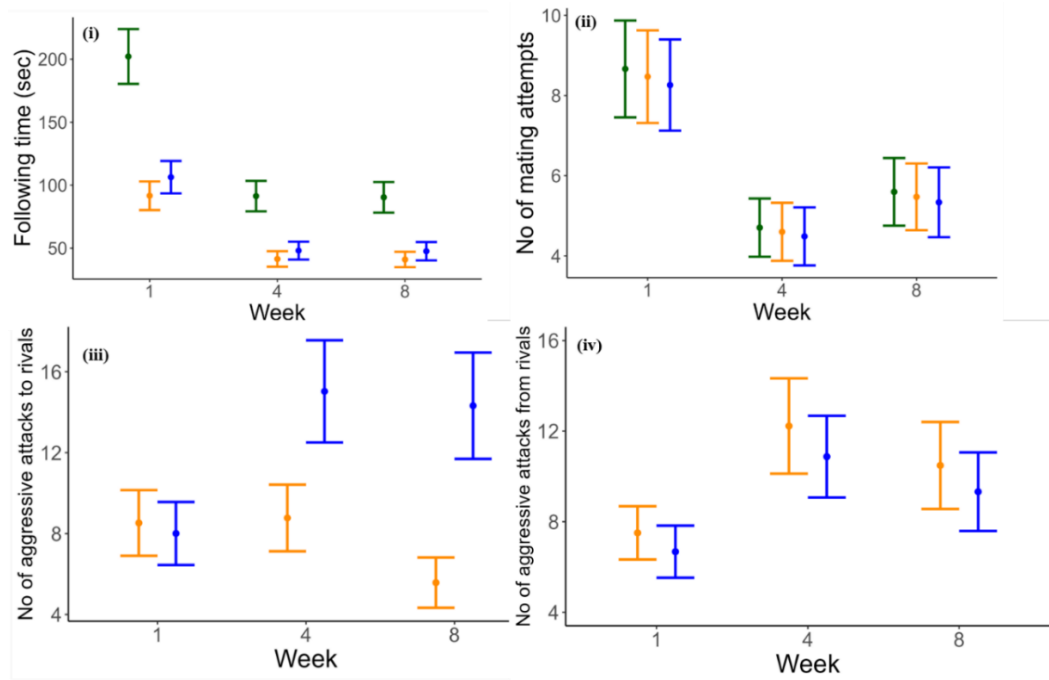


Figure 2: The effect of direct male-male competition on (i) the time focal males spent following females; (ii) the number of mating attempts; (iii) the number of aggressive attacks towards rivals; and (iv) the number of aggressive attacks received from rivals. Focal males in different social environments are shown by colour: Green = (a) focal male with a female. Orange = (b) focal male with a female and two intact (non-ablated) rivals. Blue = (c) focal male with a female and two ablated rivals. Data are presented as mean $\pm$ SE. As there were no rivals in environment (a), data for (iii) aggression towards rivals and (iv) aggression from rivals are shown only for environments (b) and (c).

Growth and immunity: The social environment did not significantly affect growth ($\chi^2_2 = 0.967$; $p = 0.617$) or immune response ($\chi^2_2 = 0.145$; $p = 0.930$) of the focal males (Fig. 3). Males with a larger pre-treatment size grew faster ($\chi^2_1 = 8808.632$; $p < 0.001$). Males that were larger at the time of the immune challenge exhibited a greater swelling after the PHA injection ($\chi^2_1 = 4.511$; $p = 0.034$). There was no environment*size interaction on growth ($\chi^2_2 = 0.564$; $p = 0.754$) or immune response ($\chi^2_2 = 1.377$; $p = 0.502$) (Supplementary Material Table S5, S6).

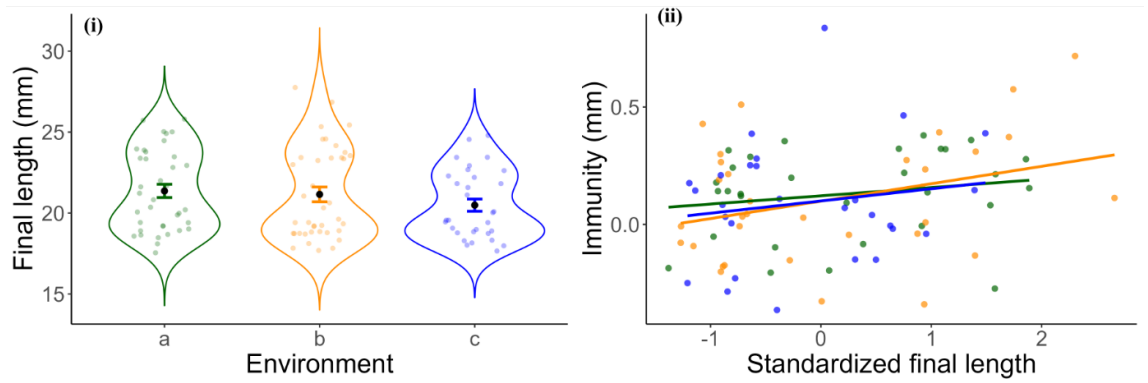


Figure 3: The effect of direct male-male competition on (i) body size and (ii) immune response (measured as swelling size) at the end of the 8-week mating period. Growth is represented by body size at week 8 because focal males across environments had similar pre-treatment body sizes. Green = (a) focal male with a female. Orange = (b) focal male with a female and two intact rivals. Blue = (c) focal male with a female and two ablated rivals. Data are presented as mean $\pm$ SE.

Sperm traits: Neither sperm count ($\chi^2_2 = 0.414$; $p = 0.813$) nor sperm velocity ($\chi^2_2 = 1.019$; $p = 0.601$) of the focal males were affected by the social environment; nor was there an interactive effect with male body size (sperm count: $\chi^2_2 = 2.404$; $p = 0.301$; sperm velocity: $\chi^2_2 = 1.077$; $p = 0.584$; Supplementary Material Tables S7,S8). Larger males produced significantly more sperm ($\chi^2_1 = 31.806$; $p < 0.001$), but male size did not affect sperm velocity ($\chi^2_1 = 1.700$; $p = 0.192$) (Fig. 4).

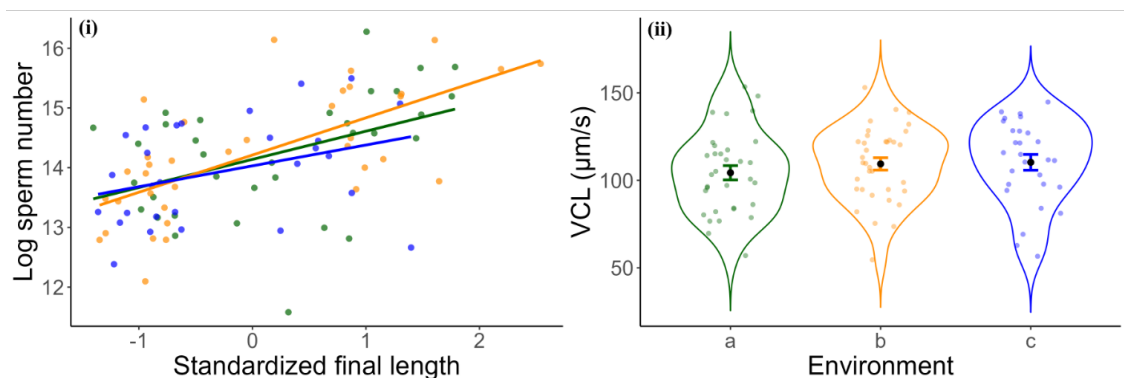


Figure 4: The effect of direct male-male competition on (i) sperm number (log-transformed) and (ii) sperm velocity. Sperm number is represented relative to Week 8

body size, given a significant body size effect on sperm number (see text). Green = (a) focal male with a female; Orange = (b) focal male with a female and two intact rivals; Blue = (c) focal male with a female and two ablated rivals. Data are presented as mean $\pm$ SE.

Life-history costs of greater sperm investment

To estimate the costs of greater sperm investment, we compared trait performance between the intact focal male and the untagged ablated male within environment (c), given their difference in sperm-release ability and hence the need to replenish sperm reserves.

Mating and fighting behaviour: Past sperm investment significantly affected future aggression towards rivals, but this effect depended on the week of testing (interaction: $\chi^2_2 = 11.801$; $p = 0.003$). In week 1, intact and ablated males showed a similar amount of aggression towards rivals (Tukey's test: ratio = 0.78, $z = -1.08$ $p = 0.282$), but intact males (who ejaculated and frequently replenished sperm) were significantly more aggressive towards rivals than their ablated counterparts (who could not ejaculate) in both week 4 (Tukey's test, ratio = 1.71, $z = 2.33$, $p = 0.020$) and week 8 (Tukey's tests ratio = 2.43, $z = 3.186$, $p = 0.001$) (Fig. 5). Apart from aggression towards rivals, there were no interactive effects of past sperm investment (with either week of testing or body size) on any of the behavioural traits namely, time spent following females (past sperm investment*week; $\chi^2_2 = 0.633$; $p = 0.729$; past sperm investment*size; $\chi^2_1 = 3.002$; $p = 0.083$), number of mating attempts (past sperm investment*week; $\chi^2_2 = 0.630$; $p = 0.730$; past sperm investment*size; $\chi^2_1 = 3.084$; $p = 0.079$), and aggression received from the rivals (past sperm investment*week; $\chi^2_2 = 0.870$; $p = 0.647$; past sperm investment*size; $\chi^2_1 = 0.549$; $p = 0.459$) (Supplementary Material, Tables S9-S12).

Past sperm investment independently affected the time spent following females ($\chi^2_1 = 8.412$; $p = 0.004$). Intact male (who ejaculated and frequently replenished sperm) spent significantly more time following females than the ablated males who could not ejaculate (Fig 5(i)). Past sperm investment had no main effect on any other behavioural traits (mating attempts; $\chi^2_1 = 2.941$; $p = 0.086$; aggression towards rivals; $\chi^2_1 = 1.159$; $p = 0.282$; and aggression from rivals; $\chi^2_1 = 0.447$; $p = 0.504$).

The week of testing independently affected following time ($\chi^2_2 = 18.358$; $p < 0.001$) and aggression from rivals ($\chi^2_2 = 19.199$; $p < 0.001$), irrespective of past sperm investment. Over the 8-week reproductive period, males decreased the time spent following females but received more aggression from their rivals (Fig. 5).

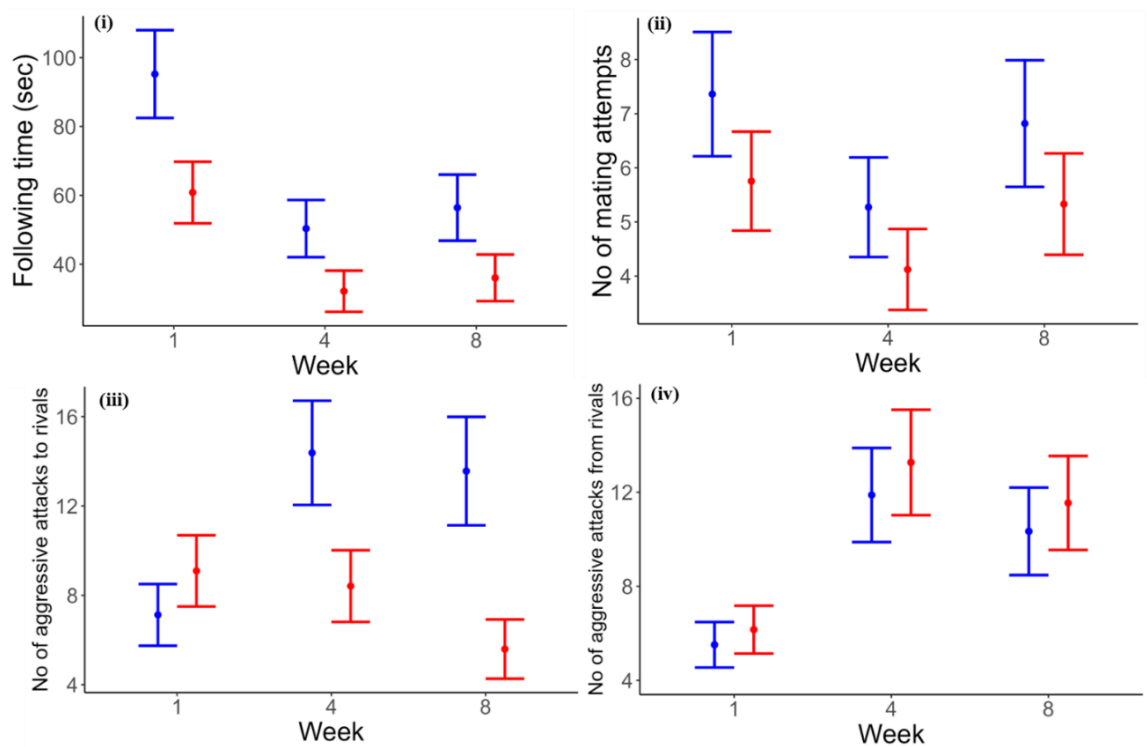


Figure 5: The effect of past sperm investment on (i) following time; (ii) number of mating attempts; (iii) the number of aggressive attacks towards rivals; and (iv) the number of aggressive attacks received from rivals. The focal male's ability to release sperm is indicated by colour: Blue = intact male that could ejaculate. Red = ablated male that could not ejaculate. Data are presented as mean $\pm$ SE.

Growth and immunity: Past sperm investment did not significantly affect growth or immune response, either as a main effect (growth: $\chi^2_1 = 0.561$; $p = 0.454$; immunity: $\chi^2_1 = 0.023$; $p = 0.879$) or through an interaction with body size (growth: $\chi^2_1 = 0.012$; $p = 0.914$; immunity: $\chi^2_1 = 0.224$; $p = 0.636$) (Fig. 6; Supplementary Material, Table S13, S14). Males with a larger pre-treatment size grew faster ($\chi^2_1 = 3030.506$; $p < 0.001$), but body size did not affect the size of swelling after the PHA inflammation challenge ($\chi^2_1 = 0.826$; $p = 0.363$).

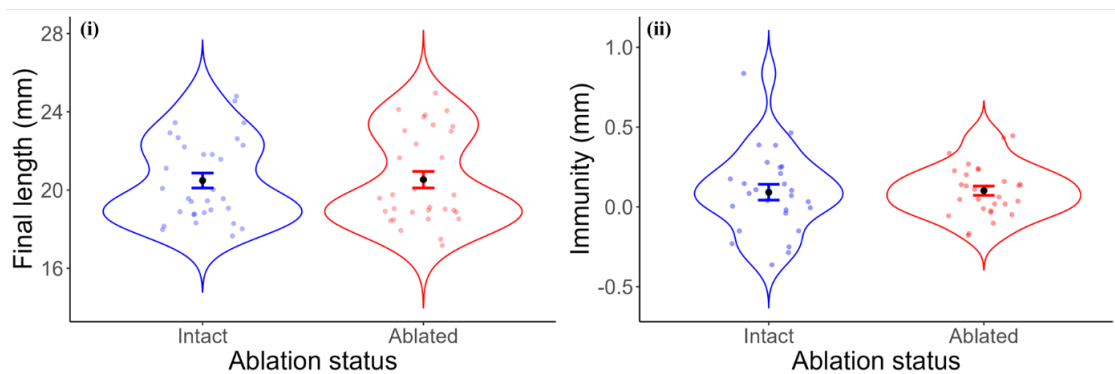


Figure 6: The effect of past sperm investment on (i) growth and (ii) immune response at the end of the experiment. Blue = intact male that could ejaculate. Red = ablated male that could not ejaculate. Data are presented as mean $\pm$ SE.

Sperm traits: Past sperm investment significantly affected future sperm count ($\chi^2_1 = 33.865$; $p < 0.001$): intact males (who ejaculated and frequently replenished sperm) produced fewer sperm at the end of the experiment (Week 8) than ablated males who could not ejaculate (Fig. 7(i)). Past sperm investment did not affect future sperm velocity ($\chi^2_1 = 0.010$; $p = 0.920$; Fig 7(ii)). Larger males produced significantly more sperm ($\chi^2_1 = 4.195$; $p = 0.041$), despite no body size effect on sperm velocity ($\chi^2_1 = 3.578$; $p = 0.059$). There was no interaction between past sperm investment and male size on sperm count ($\chi^2_1 = 0.909$; $p = 0.340$) or sperm velocity ($\chi^2_1 = 0.386$; $p = 0.534$) (Supplementary Material, Tables S15, S16).

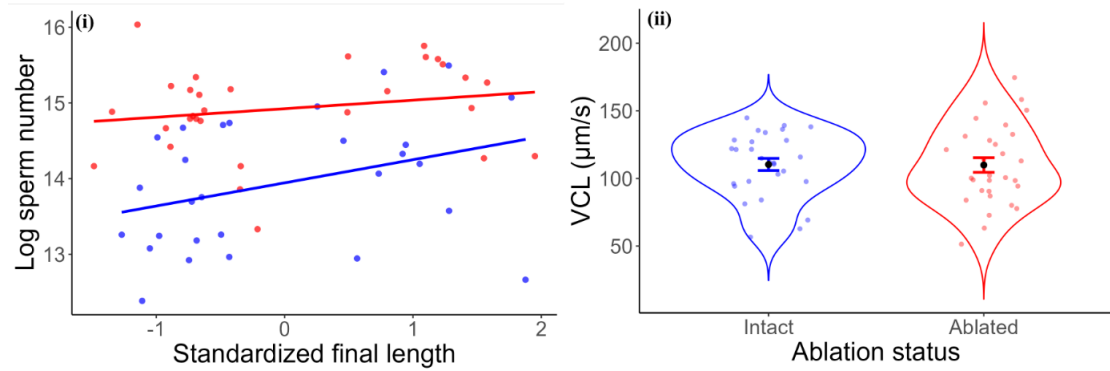


Figure 7: The effect of greater sperm investment on (i) sperm count (log-transformed) and (ii) sperm velocity. Sperm number is represented relative to Week 8 body size, given a significant body size effect on sperm number (see text). Blue = intact male that could ejaculated; Red = ablated male that could not ejaculate. Data are presented as mean $\pm$ SE.

Discussion

We used a novel surgical technique to tease apart the life-history costs of direct male-male competition and greater sperm production in male *G. holbrooki*. We tested the effect of greater sperm production by comparing males that either had the tip of their gonopodium intact or surgically ablated. Ablated males are unable to ejaculate (Chung et al. 2020) or transfer sperm during intromission (Kwan et al. 2013), so they invest less in ejaculates. We tested how fighting for access to a female trades off with future trait performance by housing a focal male with either 0 or 2 rivals when they could freely compete and mate with a female for 8 weeks.

Our results showed that fighting with rivals did not lower a male's sperm count or sperm velocity. There was therefore no detectable trade-off between current pre-copulatory investment and future post-copulatory investment. However, for a male with greater past sperm investment (i.e. intact rather than ablated), his total sperm count decreased at the end of the experiment. This indicates a trade-off between early and late life sperm investment. Interestingly, greater past sperm investment significantly *increased* the time a male spent following the female. This increase is inconsistent with a trade-off between

sperm production and mating effort. There were otherwise no detectable costs associated with either fighting or sperm investment on a male's future rate of attempting to mate, adult growth or immune response. Overall, our results suggest that when mating in the presence of rivals, any costs associated with physical fighting are less detectable than those associated with sperm investment.

Life-history costs of direct male-male competition

Mating and fighting behaviour: As predicted, males housed with rivals spent significantly less time following females than did males without rivals. This likely occurred because males spent some of their time fighting with rivals (Chung et al. 2024). Despite this decrease in following time, however, focal males with two rivals did not make fewer mating attempts than those without rivals. This result suggests no cumulative costs of fighting rivals on future copulatory attempts, which contrasts with our prediction. There was therefore no clear evidence for a trade-off between past investment in fighting and future mating effort, as their opportunity to mate remained unchanged. In contrast, Chung et al. (2024) reported that male *G. holbrooki* that interacted with two rivals showed a significant decrease in future mating effort (including a reduced number of future mating attempts) than males without rivals (i.e., those in their 'naïve' treatment, where rivals were behind a mesh barrier; Fig 8). Their results suggest a trade-off between past and current mating effort (i.e., fighting rivals *plus* interacting with females). Importantly, focal males in their 'naïve' treatment lacked physical access to females, which differs from our design. Our study can specifically quantify the costs of fighting rivals (by manipulating the number of rivals) without altering the presence/absence of females – that is, focal males in all three environments always had full access to females (Fig 1). This difference between our results and those in Chung et al. (2024) suggests that past effort spent fighting rivals (but not past effort spent interacting with females) reduced the

time males spent following females without affecting their number of mating attempts. In other words, males in a more competitive environment increased their mating efficiency: they spent less time following females yet managed to approach females closely enough to perform the same number of mating attempt. The unaffected mating attempts (independent of the costs of fighting rivals) may reflect strong selection in our source population of *G. holbrooki*, where males must achieve mating success despite dramatic seasonal fluctuations in the number of rivals per female (Kahn et al. 2013).

In addition, both the time spent following females and the number of mating attempts decreased over the 8-week reproductive period. These declines could reflect a decreased motivation to mate as the males accumulated more copulatory experience over time and repeatedly mated with the same female i.e., the “Coolidge effect” (Steiger et al. 2008). In contrast, it is less likely that the decrease in mating effort was due to age-related senescence, because virgin male mosquitofish has been reported to exhibit similar levels of mating effort (i.e., remained eager to mate) at both 8 and 16 weeks after maturation (Chung et al. 2024).

Sperm number and sperm velocity: As predicted, being housed with rivals affected neither sperm count nor sperm velocity. This corroborates the findings of Chung et al. (2024), who also showed that physical access to rivals did not affect sperm traits. Again, however, it is worth noting that in their study, males without rivals also lacked physical access to a female – that is, the effect of the presence/absence of rivals was always confounded with the effect of the presence/absence of females. Together, these findings suggest that there were no costs of past effort fighting rivals, or the combined costs of fighting rivals *and* interacting with females (Chung et al. 2024), on future sperm production.

The absence of an effect of direct male-male competition on sperm traits is, however, surprising because physical combat often results in lower body condition (Archie 2013; Emberts et al. 2021), and lower body condition can subsequently reduce sperm production (Macartney et al. 2019; O’Dea et al. 2014). There are three possible explanations for the lack of a trade-off between fighting and sperm. First, variation in resource acquisition can affect the detection of trade-offs (Van Noordwijk and De Jong 1986; Reznick et al. 2000). For example, the trade-off between survival and reproduction is often undetectable in zoos because captive animals have sufficient food/resources to support both functions (Ricklefs and Cadena 2007). For our males, the energetic cost of fighting rivals might similarly be less apparent given they had ready access to food in the laboratory. Second, hierarchical allocation structures can weaken trade-offs between specific traits (Johnson and Nassrullah 2024). For example, sperm investment is reduced with increased expression of sexual ornaments but not with increased expression of weapons in primates (Lüpold et al. 2019). In *Teleogryllus oceanicus*, postcopulatory traits strongly trade off with immunity function, but not with precopulatory traits (Winkler et al. 2025). Third, sperm production and fighting effort might genuinely not trade off because they involve different physiological mechanisms, such as meiosis for sperm production versus mitosis for the production of muscles for physical combat (Maklakov and Immler 2016).

Growth and immune response: Contrary to our prediction, the presence or absence of rivals in a social environment affected neither male growth nor immune response. This finding is at odds with general claims that fighting is costly (Emery Thompson and Georgiev 2014; Girard-Buttoz et al. 2014). It should be noted that immunity response was measured as the changes in body thickness following the PHA-injection challenge. Because our males were small and showed weak inflammation, measurement errors occasionally produced negative swelling values (Figure 3.2). However, our findings align well with previous findings in *G. holbrooki*. A cross-study comparison between

environments with rivals (Chung et al. 2024) versus without rivals (Chung et al. 2021) (Fig 8) suggested that the cost of male reproduction on growth and immune response did not depend on the presence of rivals (i.e. no interaction; see Discussion in Chung et al. 2024). In addition, male mosquitofish greatly reduce their growth after maturation (i.e., determinate growth), so resource allocation trade-offs between reproduction and body size may be inherently hard to detect. The costs of reproduction may therefore be better captured using other proxies of somatic condition (e.g., body mass, fat content).

In *G. holbrooki*, males continue to feed during the reproductive period, so they might compensate for the energetic loss of fighting through increased food intake. In contrast, for species that stop feeding during reproduction, the resource allocation trade-offs tend to be more apparent (Grone et al. 2012; Olsson et al. 1997). This might explain why we did not detect a trade-off between fighting and growth or immune response. Another explanation is to take our results at face value. Male-male contests in *G. holbrooki* are often brief and might impose minimal energetic or physical costs, not least because males without rivals spend more time pursuing females so that the net energy spent on locomotion might be similar in the presence and absence of rivals.

Life-history costs of greater sperm production

Mating and fighting behaviour: Contrary to our prediction, intact males (who ejaculated and thus invested more in sperm replenishment) spent significantly *more* time than ablated males following females. This is inconsistent with the general prediction of a trade-off between pre- and post-copulatory traits (Parker et al. 2013). Another intriguing, and unexpected, finding was that intact focal males were more aggressive towards ablated than intact rivals; and intact rivals were more aggressive than ablated rivals. One possible explanation for these results is that males can adjust their mating performance based on body condition and/or past copulatory success (Saleem et al. 2014; Schütz et al. 2017).

If ablated males perceive themselves to have low insemination success, they might be less likely to follow females or initiate fights for access to mates. However, we consider this explanation unlikely because it has been well established that ablation surgery itself does not affect mating or fighting behaviour (Chung et al. 2020, 2021, 2024). Our findings therefore contrast with those of these previous studies, which did not find any effect of past sperm investment on subsequent mating behaviours. The factors contributing to the behavioural changes seen in our study remain unclear.

Poeciliid fishes often adjust their reproductive strategy according to predation risk (Dzikowski et al. 2004) or the sperm competition risk they experience early in life (Guevara-Fiore et al. 2012). However, neither factor can explain our results because all males were reared in the absence of predators and experienced the same early-life social environment (male-only aquaria). It would be interesting in a future study to investigate whether juvenile social contexts (e.g. different sex ratios) and predation risk modify the costs of reproduction later in life.

Sperm number and sperm velocity: As predicted, ablated males (who could not ejaculate for 8 weeks) produced sperm at a faster rate than intact males (who repeatedly ejaculated and replenish their sperm). This finding indicates a trade-off between past and future sperm production. Frequent sperm release and replenishment might reduce future sperm production because the resources, physical apparatus, or energy required to produce sperm is limited. A similar trade-off has been reported in the two other *G. holbrooki* studies (Chung et al. 2021, 2024). More generally, several studies have shown that males are often sperm-limited (Preston et al. 2001; Schütz et al. 2017), implying that sperm is costly to produce (Thomsen et al. 2006). In contrast, past sperm investment did not affect future sperm velocity, aligning well the findings of a meta-analysis showing that sperm quality is less sensitive to changes in male body condition (in our case, the

remaining resources after the 8-week sperm investment) than sperm quantity (Macartney et al. 2019).

Growth and immune response: Greater sperm investment during the 8-week reproductive period had no detectable effect on subsequent growth or immune function, consistent with the findings in Chung et al. (2021, 2024). These findings, however, contrast with growing evidence that ‘sperm is not cheap’ (Morbiato et al. 2023; Tuni et al. 2018), although not all studies find a cost of sperm production (Cordts and Partridge 1996). The conflicting evidence regarding the costs of sperm production may again reflect variation in resource allocation strategies across species. For capital breeders that build energy reserves prior to reproduction, the total energy budget is fixed during the reproductive event (e.g. *V. berus*; Olsson et al. 1997), making resource allocation to gametes more likely to trade off against somatic function. In contrast, income breeders like *G. holbrooki* continuously ingest food and may therefore be able to maintain their somatic function while investing in sperm traits (Soulsbury 2019). On the other hand, sperm may be cheaper to produce in *G. holbrooki* than in other species whose ejaculates contain nutrient-rich seminal gifts that enhance fertilisation success (Lewis et al. 2011).

Conclusion

In this study, we individually quantified the cost of direct male-male competition by manipulating the number of rivals in the social environment; and the cost of increased sperm investment by using ablation surgery to alter a male’s ability to release sperm. We found no detectable cost of fighting rivals (when access to females remained unchanged), except for a reduction in time spent following females – likely reflecting an immediate trade-off between the time spent fighting rivals versus pursuing females. In contrast, increased sperm investment significantly reduced future sperm count. Our results suggest that the costs of sperm production are more readily detectable than those of fighting rivals.

This contrasts with a previous study that found the costs of sperm production to be minor relative to those of mating effort (Chung et al. 2021). The absence of detectable costs of past fighting effort on other traits (growth, immunity, mating attempt and sperm traits) is intriguing, given that male-male competition is energetically expensive in other species (e.g., developing weapons and repairing injuries after combat: Somjee et al. 2018; Feder et al. 2019). A future direction of research would be to repeat our experiment under condition of low food availability. This would help clarify the role (if any) of compensatory feeding in mitigating the costs of past reproductive investment on traits that otherwise showed no measurable decline in response to increased male-male competition. Finally, while our study detected a decline in sperm production due to past sperm investment, we were unable to test whether this decline reduced male fitness (e.g., the number of offspring sired). This remains a future challenge.

Data accessibility

Raw data and R code can be downloaded from Zenodo:

<https://doi.org/10.5281/zenodo.17626234>

Acknowledgement

We thank Ben Durant, Michelle Stephens, Ivan Vinogradov, Md Mahmud Al Hasan, Diego De Moura Campos and Chenke Zang for helping in the fish maintenance. The study was partly funded by the Australian Research Council (DP190100279).

REFERENCES

- Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics International*, 11, 36–42.
- Aich, U., Bonnet, T., Fox, R. J., & Jennions, M. D. (2020). An experimental test to separate the effects of male age and mating history on female mate choice. *Behavioral Ecology*, 31, 1353–1360. <https://doi.org/10.1093/beheco/araa092>
- Archie, E. A. (2013). Wound healing in the wild: Stress, sociality and energetic costs affect wound healing in natural populations. *Parasite Immunology*, 35, 374–385. <https://doi.org/10.1111/pim.12048>
- Bisazza, A. (1993). Male competition, female mate choice and sexual size dimorphism in poeciliid fishes. *Marine and Freshwater Behaviour and Physiology*, 23, 257–286. <https://doi.org/10.1080/10236249309378869>
- Bisazza, A., & Marin, G. (1995). Sexual selection and sexual size dimorphism in the eastern mosquitofish *Gambusia holbrooki* (Pisces Poeciliidae). *Ethology Ecology & Evolution*, 7, 169–183. <https://doi.org/10.1080/08927014.1995.9522963>
- Booksmythe, I., Head, M. L., Keogh, J. S., & Jennions, M. D. (2016). Fitness consequences of artificial selection on relative male genital size. *Nature Communications*, 7, 11597. <https://doi.org/10.1038/ncomms11597>
- Boschetto, C., Gasparini, C., & Pilastro, A. (2011). Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, 65, 813–821. <https://doi.org/10.1007/s00265-010-1085-y>
- Chung, M. H. J., Fox, R. J., & Jennions, M. D. (2020). Fine-scale genital morphology affects male ejaculation success: An experimental test. *Biology Letters*, 16, 20200251. <https://doi.org/10.1098/rsbl.2020.0251>

- Chung, M. H. J., Fox, R. J., & Jennions, M. D. (2024). Male allocation to ejaculation and mating effort imposes different life history trade-offs. *PLOS Biology*, 22, e3002519. <https://doi.org/10.1371/journal.pbio.3002519>
- Chung, M. H. J., Jennions, M. D., & Fox, R. J. (2019). Novel ablation technique shows no sperm priming response by male eastern mosquitofish to cues of female availability. *Behavioral Ecology and Sociobiology*, 73, 167. <https://doi.org/10.1007/s00265-019-2779-4>
- Chung, M. H. J., Jennions, M. D., & Fox, R. J. (2021). Quantifying the costs of pre- and postcopulatory traits for males: Evidence that costs of ejaculation are minor relative to mating effort. *Evolution Letters*, 5, 315–327. <https://doi.org/10.1002/evl3.228>
- Chung, M. H. J., Noble, D. W. A., Fox, R. J., Harrison, L. M., et al. (2025). Fluctuating salinity during development impacts fish life histories. *Journal of Animal Ecology*. <https://doi.org/10.1111/1365-2656.70095>
- Clutton-Brock, T. H., Albon, S. D., Gibson, R. M., & Guinness, F. E. (1979). The logical stag: Adaptive aspects of fighting in red deer (*Cervus elaphus* L.). *Animal Behaviour*, 27, 211–225. [https://doi.org/10.1016/0003-3472\(79\)90141-6](https://doi.org/10.1016/0003-3472(79)90141-6)
- Cordts, R., & Partridge, L. (1996). Courtship reduces longevity of male *Drosophila melanogaster*. *Animal Behaviour*, 52, 269–278. <https://doi.org/10.1006/anbe.1996.0172>
- Drews, C. (1996). Contexts and patterns of injuries in free-ranging male baboons (*Papio cynocephalus*). *Behaviour*, 133, 443–474. <https://doi.org/10.1163/156853996X00530>
- Dzikowski, R., Hulata, G., Harpaz, S., & Karplus, I. (2004). Inducible reproductive plasticity of the guppy *Poecilia reticulata* in response to predation cues. *Journal of Experimental Zoology Part A*, 301, 776–782. <https://doi.org/10.1002/jez.a.61>

- Emberts, Z., Somjee, U., & Wiens, J. J. (2021). Damage from intraspecific combat is costly. *Behavioral Ecology*, 32, 1240–1245.
<https://doi.org/10.1093/beheco/arab090>
- Emberts, Z., St Mary, C. M., Herrington, T. J., & Miller, C. W. (2018). Males missing their sexually selected weapon have decreased fighting ability and mating success in a competitive environment. *Behavioral Ecology and Sociobiology*, 72, 81. <https://doi.org/10.1007/s00265-018-2494-6>
- Emery Thompson, M., & Georgiev, A. V. (2014). The high price of success: Costs of mating effort in male primates. *International Journal of Primatology*, 35, 609–627. <https://doi.org/10.1007/s10764-014-9790-4>
- Engqvist, L. (2005). The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Animal Behaviour*, 70, 967–971. <https://doi.org/10.1016/j.anbehav.2005.01.016>
- Evans, J. P. (2003). Male mating behavior and ejaculate expenditure under sperm competition risk in the eastern mosquitofish. *Behavioral Ecology*, 14, 268–273.
<https://doi.org/10.1093/beheco/14.2.268>
- Evans, J. P., Turnbull, E. J., & Lymbery, R. A. (2023). Testing for age-dependent effects of dietary restriction on the strength of condition dependence in ejaculate traits in the guppy (*Poecilia reticulata*). *Royal Society Open Science*, 10, 230805. <https://doi.org/10.1098/rsos.230805>
- Feder, J. A., Lu, A., Koenig, A., & Borries, C. (2019). The costs of competition: Injury patterns in two Asian colobine monkeys. *Behavioral Ecology*, 30, 1242–1253.
<https://doi.org/10.1093/beheco/arz070>
- Flatt, T., & Heyland, A. (2011). *Mechanisms of life history evolution: The genetics and physiology of life history traits and trade-offs*. Oxford University Press.

- Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., et al. (2012).
Package 'car'. R Foundation for Statistical Computing.
- Fox, R. J., Head, M. L., & Jennions, M. D. (2019). Disentangling the costs of male harassment and the benefits of polyandry for females. *Behavioral Ecology*, 30, 872–881. <https://doi.org/10.1093/beheco/arz024>
- Gao, J., Santi, F., Zhou, L., et al. (2019). Geographical and temporal variation of multiple paternity in invasive mosquitofish (*Gambusia holbrooki*, *Gambusia affinis*). *Molecular Ecology*, 28, 5315–5329. <https://doi.org/10.1111/mec.15294>
- Girard-Buttoz, C., Heistermann, M., Rahmi, E., et al. (2014). Mate-guarding constrains feeding activity but not energetic status of wild male long-tailed macaques (*Macaca fascicularis*). *Behavioral Ecology and Sociobiology*, 68, 583–595. <https://doi.org/10.1007/s00265-013-1673-8>
- Grone, B. P., Carpenter, R. E., Lee, et al. (2012). Food deprivation explains effects of mouthbrooding on ovaries and steroid hormones, but not brain neuropeptide and receptor mRNAs, in an African cichlid fish. *Hormones and Behavior*, 62, 18–26. <https://doi.org/10.1016/j.yhbeh.2012.04.012>
- Guevara-Fiore, P., Svensson, P. A., & Endler, J. A. (2012). Sex as moderator of early life experience: Interaction between rearing environment and sexual experience in male guppies. *Animal Behaviour*, 84, 1023–1029. <https://doi.org/10.1016/j.anbehav.2012.07.028>
- Harrison, L. M., Vega-Trejo, R., & Jennions, M. D. (2023). The effect of brief or prolonged bouts of winning or losing male-male contests on plasticity in sexually selected traits. *American Naturalist*, 201, 442–459. <https://doi.org/10.1086/722829>
- Head, M. L., Kahn, A. T., Henshaw, J. M., et al. (2017). Sexual selection on male body size, genital length and heterozygosity: Consistency across habitats and social

settings. *Journal of Animal Ecology*, 86, 1458–1468.

<https://doi.org/10.1111/1365-2656.12742>

Head, M. L., Vega-Trejo, R., Jacomb, F., & Jennions, M. D. (2015). Predictors of male insemination success in the mosquitofish (*Gambusia holbrooki*). *Ecology and Evolution*, 5, 4999–5006. <https://doi.org/10.1002/ece3.1775>

Higham, J. P., Heistermann, M., & Maestripieri, D. (2011). The energetics of male–male endurance rivalry in free-ranging rhesus macaques, *Macaca mulatta*. *Animal Behaviour*, 81, 1001–1007.

<https://doi.org/10.1016/j.anbehav.2011.02.001>

Höglund, J., & Sheldon, B. C. (1998). The cost of reproduction and sexual selection. *Oikos*, 83, 478–483. <https://doi.org/10.2307/3546675>

Johnson, M. T. J., & Nassrullah, Z. (2024). The improbability of detecting trade-offs and some practical solutions. *Journal of Evolutionary Biology*, 37, 1205–1214.

<https://doi.org/10.1093/jeb/voae096>

Kahn, A. T., Kokko, H., & Jennions, M. D. (2013). Adaptive sex allocation in anticipation of changes in offspring mating opportunities. *Nature Communications*, 4, 1603. <https://doi.org/10.1038/ncomms2634>

Kahrl, A. F., & Cox, R. M. (2015). Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology*, 26, 1502–1511.

<https://doi.org/10.1093/beheco/arv105>

Kelly, C. D., & Jennions, M. D. (2011). Sexual selection and sperm quantity: Meta-analyses of strategic ejaculation. *Biological Reviews*, 86, 863–884.

<https://doi.org/10.1111/j.1469-185X.2011.00175.x>

Kwan, L., Cheng, Y. Y., Rodd, F. H., & Rowe, L. (2013). Sexual conflict and the function of genitalic claws in guppies (*Poecilia reticulata*). *Biology Letters*, 9, 20130267. <https://doi.org/10.1098/rsbl.2013.0267>

- Lenth, R., & Lenth, M. R. (2018). Package 'lsmeans.' *American Statistician*, 34, 216–221.
- Lewis, S., South, A., Burns, R., & Al-Wathiqui, N. (2011). Nuptial gifts. *Current Biology*, 21, R644–R645. <https://doi.org/10.1016/j.cub.2011.05.046>
- Lüpold, S., Simmons, L. W., & Grueter, C. C. (2019). Sexual ornaments but not weapons trade off against testes size in primates. *Proceedings of the Royal Society B*, 286, 20182542. <https://doi.org/10.1098/rspb.2018.2542>
- Macartney, E. L., Crean, A. J., Nakagawa, S., & Bonduriansky, R. (2019). Effects of nutrient limitation on sperm and seminal fluid: A systematic review and meta-analysis. *Biological Reviews*, 94, 1722–1739. <https://doi.org/10.1111/brv.12524>
- Maklakov, A. A., & Immler, S. (2016). The expensive germline and the evolution of ageing. *Current Biology*, 26, R577–R586. <https://doi.org/10.1016/j.cub.2016.04.012>
- Matsubara, M. (2003). Costs of mate guarding and opportunistic mating among wild male Japanese macaques. *International Journal of Primatology*, 24, 1057–1075. <https://doi.org/10.1023/A:1026228312706>
- Morbiato, E., Cattelan, S., Pilastro, A., & Grapputo, A. (2023). Sperm production is negatively associated with muscle and sperm telomere length in a species subjected to strong sperm competition. *Molecular Ecology*, 32, 5812–5822. <https://doi.org/10.1111/mec.17158>
- O'Dea, R. E., Jennions, M. D., & Head, M. L. (2014). Male body size and condition affects sperm number and production rates in mosquitofish, *Gambusia holbrooki*. *Journal of Evolutionary Biology*, 27, 2739–2744. <https://doi.org/10.1111/jeb.12534>

- Olsson, M., Madsen, T., & Shine, R. (1997). Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society B*, 264, 455–459. <https://doi.org/10.1098/rspb.1997.0065>
- Parker, G. A., Lessells, C. M., & Simmons, L. W. (2013). Sperm competition games: A general model for precopulatory male–male competition. *Evolution*, 67(1), 95–109. <https://doi.org/10.1111/j.1558-5646.2012.01741.x>
- Preston, B. T., Jalme, M. S., Hingrat, Y., et al. (2011). Sexually extravagant males age more rapidly. *Ecology Letters*, 14, 1017–1024. <https://doi.org/10.1111/j.1461-0248.2011.01668.x>
- Preston, B. T., Stevenson, I. R., Pemberton, J. M., & Wilson, K. (2001). Dominant rams lose out by sperm depletion. *Nature*, 409, 681–682. <https://doi.org/10.1038/35055617>
- Pyke, G. H. (2005). A review of the biology of *Gambusia affinis* and *G. holbrooki*. *Reviews in Fish Biology and Fisheries*, 15, 339–365. <https://doi.org/10.1007/s11160-006-6394-x>
- R Core Team. (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. <https://www.R-project.org>
- Reznick, D., Nunney, L., & Tessier, A. (2000). Big houses, big cars, superfleas and the costs of reproduction. *Trends in Ecology & Evolution*, 15, 421–425. [https://doi.org/10.1016/S0169-5347\(00\)01941-8](https://doi.org/10.1016/S0169-5347(00)01941-8)
- Ricklefs, R. E., & Cadena, C. D. (2007). Lifespan is unrelated to investment in reproduction in populations of mammals and birds in captivity. *Ecology Letters*, 10, 867–872. <https://doi.org/10.1111/j.1461-0248.2007.01085.x>
- Saleem, S., Ruggles, P. H., Abbott, W. K., & Carney, G. E. (2014). Sexual experience enhances *Drosophila melanogaster* male mating behavior and success. *PLOS ONE*, 9, e96639. <https://doi.org/10.1371/journal.pone.0096639>

- Scharf, I., Peter, F., & Martin, O. Y. (2013). Reproductive trade-offs and direct costs for males in arthropods. *Evolutionary Biology*, 40, 169–184.
<https://doi.org/10.1007/s11692-012-9213-4>
- Schütz, D., Tschirren, L., Pachler, G., et al. (2017). Sperm-limited males save ejaculates for future matings when competing with superior rivals. *Animal Behaviour*, 125, 3–12. <https://doi.org/10.1016/j.anbehav.2016.12.016>
- Shamble, P. S., Wilgers, D. J., Swoboda, K. A., & Hebets, E. A. (2009). Courtship effort is a better predictor of mating success than ornamentation for male wolf spiders. *Behavioral Ecology*, 20, 1242–1251.
<https://doi.org/10.1093/beheco/arp116>
- Simmons, L. W., Lüpold, S., & Fitzpatrick, J. L. (2017). Evolutionary trade-off between secondary sexual traits and ejaculates. *Trends in Ecology & Evolution*, 32, 964–976. <https://doi.org/10.1016/j.tree.2017.09.011>
- Somjee, U., Miller, C. W., Tatarnic, N. J., & Simmons, L. W. (2018). Experimental manipulation reveals a trade-off between weapons and testes. *Journal of Evolutionary Biology*, 31, 57–65. <https://doi.org/10.1111/jeb.13193>
- Soulsbury, C. D. (2019). Income and capital breeding in males: Energetic and physiological limitations on male mating strategies. *Journal of Experimental Biology*, 222, jeb184895. <https://doi.org/10.1242/jeb.184895>
- South, S. H., Steiner, D., & Arnqvist, G. (2009). Male mating costs in a polygynous mosquito with ornaments expressed in both sexes. *Proceedings of the Royal Society B*, 276, 3671–3678. <https://doi.org/10.1098/rspb.2009.0991>
- Stearns, S. C. (1989). Trade-offs in life-history evolution. *Functional Ecology*, 3, 259–268. <https://doi.org/10.2307/2389364>
- Steiger, S., Franz, R., Eggert, A.-K., & Müller, J. K. (2008). The Coolidge effect, individual recognition and selection for distinctive cuticular signatures in a

- burying beetle. *Proceedings of the Royal Society B*, 275, 1831–1838.
<https://doi.org/10.1098/rspb.2008.0375>
- Thomsen, R., Soltis, J., Matsubara, M., et al. (2006). How costly are ejaculates for Japanese macaques? *Primates*, 47, 272–274. <https://doi.org/10.1007/s10329-005-0171-7>
- Tuni, C., Han, C. S., & Dingemanse, N. J. (2018). Multiple biological mechanisms result in correlations between pre- and post-mating traits that differ among versus within individuals and genotypes. *Proceedings of the Royal Society B*, 285, 20180951. <https://doi.org/10.1098/rspb.2018.0951>
- Van Noordwijk, A. J., & De Jong, G. (1986). Acquisition and allocation of resources: Their influence on variation in life history tactics. *American Naturalist*, 128, 137–142. <https://doi.org/10.1086/284547>
- Van Voorhies, W. A. (1992). Production of sperm reduces nematode lifespan. *Nature*, 360, 456–458. <https://doi.org/10.1038/360456a0>
- Vega-Trejo, R., Fox, R. J., Iglesias-Carrasco, M., et al. (2019). The effects of male age, sperm age and mating history on ejaculate senescence. *Functional Ecology*, 33, 1267–1279. <https://doi.org/10.1111/1365-2435.13305>
- Vinogradov, I. M., Fox, R. J., Fichtel, C., et al. (2025). Paternity analysis reveals sexual selection on cognitive performance in mosquitofish. *Nature Ecology & Evolution*. <https://doi.org/10.1038/s41559-025-02645-3>
- Voigt, C. C., Michener, R., & Kunz, T. H. (2005). The energetics of trading nuptial gifts for copulations in katydids. *Physiological and Biochemical Zoology*, 78, 417–423. <https://doi.org/10.1086/430224>
- Wedell, N. (2010). Sperm competition. In M. D. Breed & J. Moore (Eds.), *Encyclopedia of animal behavior* (pp. 322–328). Academic Press.
<https://doi.org/10.1016/B978-0-08-045337-8.00190-X>

- Wilson, R. S. (2005). Temperature influences the coercive mating and swimming performance of male eastern mosquitofish. *Animal Behaviour*, 70, 1387–1394. <https://doi.org/10.1016/j.anbehav.2004.12.024>
- Winkler, L., McNamara, K. B., Lovegrove, M., Fitzpatrick, J. L., & Simmons, L. W. (2025). How resource acquisition influences the detection of trade-offs. *Evolution*, 79, 2133–2143. <https://doi.org/10.1093/evolut/qpaf139>

Chapter 4: Reduced water turbidity rather than male harassment elevates female physiological stress in a fish with intense sexual conflict

“The eyes are the window to your soul.”- **William Shakespeare**

Submitted to Proceeding of the Royal Society B (under review, see Appendix A)

Introduction

Deforestation, agriculture, and urbanization cause eutrophication and sedimentation of waterways, which tends to increase water turbidity (Davies-Colley & Smith, 2001; Mi et al., 2019). Turbidity is caused by suspended particles that scatter and reduce the amount of light, and can also change its wavelength, which ultimately reduces visibility (Utne-Palm, 2002). Turbidity can limit the ability of animals to obtain information either from the environment or from other individuals (Ioannou et al., 2011). This is especially relevant for fish whose main channel of communication is vision (Guthrie, 1986; Hain & Neff, 2007). Greater turbidity might bring important changes such as a reduced ability to detect and identify predators (Day et al., 2001; Kimbell & Morrell, 2015; Sohel & Lindström, 2015; Swanbrow Becker & Gabor, 2012). This, in turn, could affect their shoaling and foraging behaviour (Hoare et al., 2000; Nieman & Gray, 2020; Utne-Palm, 2002; Wishingrad et al., 2015). For example, juvenile cod (*Gadus morhua*) that are in turbid water, increase their activity to maintain their foraging rate (Meager & Batty, 2007); and pike (*Esox Lucius*), in turbid water more often use open areas rather than hiding in the vegetation when predators are present (Lehtiniemi et al., 2005). Ultimately, greater turbidity could lower the evolutionary fitness of fish if it increases the costs of performing fitness-enhancing behaviours, and this then reduces their reproductive output (Chapman et al., 2009; Endler, 1992; Engström-Öst & Candolin, 2007; Michelangeli et al., 2015; Sundin et al., 2010; Van Der Sluijs et al., 2011).

In many freshwater fishes, females use the conspicuous coloration and courtship displays of males to assess them as potential mates (Houde, 1997). For example, in guppies (*Poecilia reticulata*), female mate choice depends on assessment of the amount of orange and other coloration of males (Devigili et al., 2013; Evans et al., 2011). Female mate choice is often sensitive to environmental changes that affect the costs and benefits of being choosy (Heuschele et al., 2009; Järvenpää & Lindström, 2004; Sundin et al., 2010). More specifically, high levels of turbidity can alter mate choice when females visually assess potential mates (Sundin et al., 2010). When there is increased turbidity, it is more difficult for females to assess how males differ in their colouration or courtship behaviour. A reduced ability to assess mates can alter the robustness or trajectory of sexual selection (Heuschele et al., 2009; Järvenpää & Lindström, 2004; Sundin et al., 2010; Wong et al., 2007). For example, in three-spined stickleback (*Gasterosteus aculeatus*) and in guppies (*Poecilia reticulata*), males spent less time with females in turbid water than clear water, but they then increased their rate of mating behaviours (e.g., courtship displays) when they encountered females (Ehlman et al., 2018; Engström-Öst & Candolin, 2007; Venkatesan et al., 2024). Turbidity can therefore increase the cost of mating for males by increasing the time and energy spent on courtship (Candolin et al., 2007). Turbidity could also increase the costs for females if there is sexual conflict over mating and greater mate attention is costly.

In many species, the optimal number of matings is lower for females than males (Bateman, 1948). This can lead to the evolution of male reproductive tactics that involve sexual harassment (e.g., chasing females, persistent courtship) because males continuously try to mate with females who are often unwilling to mate (Clutton-Brock & Parker, 1995). Females therefore adopt various strategies to reduce the costs of harassment, such as forming female-only groups, changing their movement patterns, and hiding or fleeing (Bisazza, 1993; Brewster & Houde, 2003; Condon & Wilson, 2006).

There is some evidence that the strength of sexual conflict, hence the costs imposed on females, varies in different environments. For example, in low-food environment mating is especially costly for female water striders, as it interferes with their foraging activity (Perry & Rowe, 2018). It seems likely that changes in turbidity will affect the level of sexual conflict and the intensity of sexual harassment in fishes, because males rely heavily on vision to locate potential mates; and females need to detect males from a distance to evade their mating attempts. To date, however, there have been very few studies that test how turbidity affects rates of male harassment in fishes.

Vertebrates respond to stressors through activation of the neuroendocrine pathway of the hypothalamic-pituitary-adrenal (HPA) axis in birds and mammals (Romero, 2004), or the hypothalamic-pituitary-inter-renal (HPI) axis in fish (Mommsen et al., 1999). Environmental challenges and disturbances increase HPI-axis activity that elevate production of cortisol (the main glucocorticoid ‘stress’ hormone in fish) (Sapolsky et al., 2000; Wingfield & Kitaysky, 2002). Elevated level of glucocorticoids can inhibit reproduction via suppression of the hypothalamic-pituitary-gonadal (HPG) axis which regulates the secretion of sex steroids (Crino et al., 2017). Cortisol mobilizes energy reserves, allowing fish to cope with a greater energy demand after exposure to stressors (Sapolsky et al., 2000).

Turbidity could increase stress for fish in at least two ways. First, turbidity lowers visibility which reduces the ease with which predators and conspecifics are detected and assessed. Second, when the concentration of suspended solids in water is high then it might interfere with gill function and reduce blood oxygenation (Hess et al., 2017). There are many studies on stress physiology and changes in glucocorticoid hormones levels in teleost fishes (Barton & Iwama, 1991; Pankhurst, 2011). Some of the studies have looked at the effect of turbidity. For example, in Coho Salmon (*Oncorhynchus kisutch*) and Steelhead (*Salmo gairdneri*), cortisol level increased when fish were exposed to higher

level of suspended materials in the water (Redding et al., 1987). To my knowledge, however, there is currently no study that tests whether turbidity affects female physiology because of its effect on the rate of sexual harassment.

Given the lack of suitable studies, I conducted an experiment to test how turbidity affects the level of sexual harassment experienced by females and whether this then affects their physiological stress levels. I used the mosquitofish (*Gambusia holbrooki*), which is a small freshwater poeciliid fish. As with many poeciliids, in mosquitofish there is a high level of sexual harassment because males constantly chase females and try to mate with them (Agrillo et al., 2006; Pilastro et al., 2003). Mosquitofish live in both clear water and highly turbid habitats (they can tolerate up to 275 Jackson Turbidity Units (Cherry et al., 1976)). I set up a 2x2 factorial experiment. In each trial a female was placed with either one or three males to create a high or low harassment scenario. This was done for females in either clear or turbid water. For each female, I noted the number of mating attempts, and the amount of time she was closely followed by males. I then quantified the level of cortisol as a measure of the female's primary response to stress.

I tested three predictions.

First, that females will experience more mating attempts, spend more time being followed by males, and therefore have greater stress (measured as cortisol level) when there are three males rather than one male present.

Second, that females in clear water will experience more mating attempts if greater visibility allows males to detect and approach females more often than it allows females to detect and evade approaching males. It is, of course, also possible that females will experience more mating attempts, spend more time being followed by males, and therefore have greater stress in turbid than clear water. Our chosen prediction is based on males being able to approach a female before she is aware of their presence.

Three, that females in clear water with three males will experience a disproportionately high level of harassment, hence have a disproportionately high level of stress (i.e. there will be a synergistic interaction between turbidity and the number of males).

This experiment is designed to differentiate the cost of male sexual harassment and the cost of living in turbid water, to better understand how changing environmental conditions in the wild might affect the stress level of female mosquitofish that could then affect their reproductive success.

Materials and Method

Origin and maintenance of individuals

To generate our adult fish stock, I collected juvenile mosquito fish from Sullivan's Creek, Canberra, Australia in 2023. I then transferred them to our aquarium facilities at the Australian National University and kept them in 90-L aquaria (up to 50 fish per aquarium). I checked the fish twice a week to identify their sex: males were identified by their elongated anal fin ('gonopodium'), and females were identified by yellow eggs being visible through the body wall. Immature males and females were then transferred to single sex 90L aquaria (up to 50 individuals/tank) to ensure the virginity of all experimental fishes. All fish were housed at 26°C (± 1 °C) under a 14 h light:10 h dark cycle and fed commercial fish flakes and *Artemia* brine shrimp twice daily.

Experimental setup and protocols

The experimental design involves a single female who was housed with either one or three males in either clear or turbid water. Clear water was aged tap water with a turbidity level of < 5 NTU (Nephelometric Turbidity unit), while to generate turbid water (~ 50 NTU, measured using a turbidity tube), I added 70 mg of algal (*Spirulina*) paste (algal dry weight = 23.8mg) to 1L of conditioned (Seachem, Madison, USA) tap water. I briefly

stirred the water every 5-6 hours during the 14 h light cycle to maintain the turbidity level to keep the algae in suspension. Each experimental block consisted of four treatment groups, with one female per group. I set up 43 blocks, resulting in a total of 172 tanks (N = 172 females and 344 males). In each block/replicate, individual females were randomly assigned to a tank containing either one or three males (Fig 1). The fish were kept in the aquaria for 30 hours. The four treatments were:

- a. Clear water and low male density (CL): A female with one male in clear water.
- b. Clear water and high male density (CH): A female with three males in clear water.
- c. Turbid water and low male density (TL): A female with one male in turbid water.
- d. Turbid water and high male density (TH): A female with three males in turbid water.

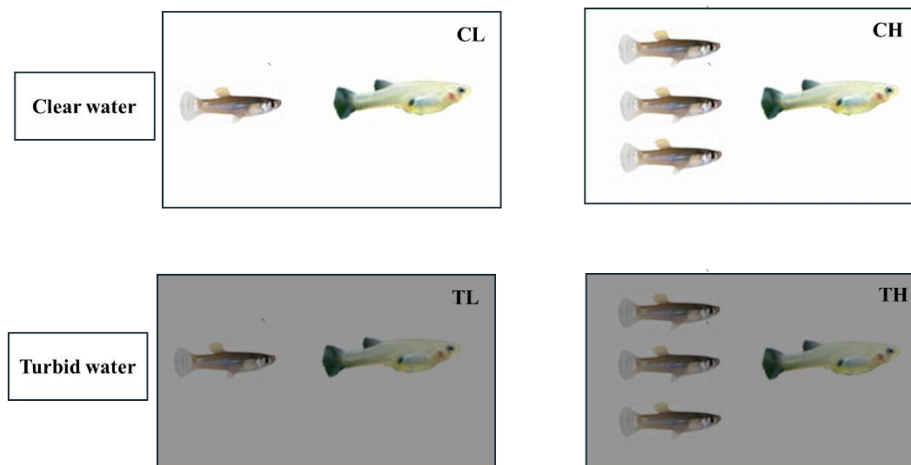


Figure 1: Experimental design with four treatments (CL= clear water and low male density, CH= clear water and high male density, TL= turbid water and low male density and TH= turbid water and high male density).

Ablation surgery

To prevent sperm transfer during the experiment, I ablated the males' gonopodia prior at least one week to them being placed in treatment tanks. All females in this experiment were therefore virgins. Ablated males still chase females and attempt to mate but they cannot inseminate females (Chung et al. 2019).

To ablate the gonopodia tip, I first anesthetizing males in an AQUI-S solution (20 μ l dissolved in 200ml water) for 30 seconds. The male was then placed on a glass slide under a dissecting microscope. His gonopodia was swung forward and the tip was removed using a sterile scalpel blade (Diplomat Blades, Victoria, Australia). After ablation I put the males back into the stock tanks for at least three days before using them in the experiment. This is sufficient time to ensure full recovery from the surgery (Chung et al., 2019). Removing the gonopodium tip prevents sperm release, but it does not affect male reproductive behaviour (see Chapter 3; see also Chung et al., 2019).

Behavioural observations

On the morning of the first day of the experiment, I placed a single female with either one or three ablated males into either clean or turbid water in a plastic tank (37cm X 26cm X 12cm) with a water depth of 7.5cm (Fig 1). To avoid any external disturbances, I covered all four sides of the tank with white paper. I initially placed the female in a plastic cylinder in the middle of the tank so that she was separated from the males. After a 10-minute acclimatization period, I removed the cylinder and left the fish to interact. I set up a digital camera (Model: NCDOTIR21-SCAT500MA) above the tank to record their behaviour.

I recorded the fishes' behaviour on two occasions: on Day 1 immediately after acclimatization, and on Day 2 one hour after the lights in the room came on in the morning. Each time I recorded behaviour for 15 minutes. Later I analysed the recorded video and noted the following: (a) how many times the female received a gonopodial

thrust from a male (hereafter ‘mating attempts’); and (b) the total amount of time she was followed by males (<2 body lengths behind the female, hereafter ‘time followed’). Following is defined as a male chasing the female. Chasing behaviour was readily visible in the videos, even with turbid water, as the camera was installed overhead and the water was shallow. It should be noted, however, that horizontal visibility within the tank was low, so the fish are unlikely to have been able to see beyond 12 cm.

Measuring whole-body cortisol of female

After a female had been housed with males for 30 hours (Fig 2), each female was euthanised using AQUI-S (0.05% v/v) in under five minutes (the average time to death was 201.63 ± 37.174) and then stored at -80 °C to later measure the glucocorticoid hormone level of females. After controlling for the turbidity and harassment treatment, I confirmed that there was no correlation between the time taken to kill the female and the estimated cortisol level ($P = 0.836$, see supplementary materials Table S5). Cortisol levels were measured from whole fish using Arbor Assay Enzyme Immunoassay (EIA) kits (Cat. No. K003) with methods adapted from (Sarma et al., 2020). To extract cortisol, fish were first thawed on ice, patted dry using Kimwipes, and weighed on an analytical balance to the nearest ± 0.1 mg. Fish were homogenized with an electric tissue homogenizer (LK Lab, HGD-10) in 2.20 – 4.40 mL of chilled assay buffer to achieve a tissue homogenate concentration of ca. 20 mg/mL. Cortisol was extracted from 1 mL aliquots of tissue homogenates using a double wash of dichloromethane. For each wash, homogenates were combined with 4 mL of dichloromethane, vortexed briefly, and mixed on a test tube shaker for 20 minutes. I centrifuged homogenates (at 3000 rpm, 4°C for 3 minutes) to isolate the organic layer, and then combined the organic layers from each wash and dried the samples under nitrogen gas at 35°C. The dehydrated samples were stored at -20°C.

To determine the extraction efficiency, I stripped cortisol from samples of pooled tissue homogenate using a 1% Dextran coated charcoal solution (Sigma, Cat. No. C6241). I spiked aliquots of stripped homogenate with cortisol (320 pg/mL) and extracted cortisol from spiked and control aliquots (methods as above). I assayed a spiked and control sample on each EIA plate and compared the percentage of cortisol recovered from the spiked sample relative to the control sample to determine extraction efficiency (methods as per Crino et al., 2024; Laven et al., 2025). The average extraction efficiency was 90.8% (std dev = 11.0). I tested assay parallelism for the cortisol assay by serially diluting a pooled extracted sample to six dilutions ranging from 1:4 – 1:128 and comparing the slopes of antibody binding relative to the cortisol concentrate of the serially diluted samples to the standard curve (see supplementary material, Fig 1). I found no evidence of slope heterogeneity ($P = 0.63$, $F_{1,9} = 0.23$; see supplementary material Table S1).

Extracted samples were reconstituted in 1 mL of assay buffer prior to conducting EIA assays. I assayed all samples and standards in duplicate across five microplates. Plates were read on a FLUOstar Omega microplate reader at 450 nm. I calculated cortisol levels from a four parameter seven-point curve ranging from 50 – 3,200 pg/mL. I normalized cortisol levels to fish mass and used mass corrected cortisol levels (pg/mg) in statistical analyses. An external standard of 400 pg/mL was assayed on every plate to estimate inter-plate variation. Intra- and inter-plate variation was 4.64% and 2.94%, respectively.



Figure 2: Timeline of the experimental protocol. Fish were put in one of two turbidity treatments (clear or turbid water). After 10 minutes of acclimatization the first behavioral observations (time followed and mating attempts) were made; after 24 hours a second behavioral observations were made. Finally, after 30 hours, the females were euthanized and preserved at -80°C for cortisol analysis.

Statistical analysis

Each response variable was analysed using a separate model. For the two behavioural measures, I included turbidity (clear or turbid), day of testing (day 1 or 2) and male density (high or low) as fixed factors; and I treated Block ID (the date a group of tanks were set up) and Female ID (because the same female was observed twice) as random factors. In the initial model, I tested for the three-way interaction of turbidity, day of testing and harassment, as well as all two-way interactions. There was no significant three-way interaction (see Results). I then ran a reduced model with only the two-way interaction. Again, there were no significant two-way interactions, so I ran a final model that exclude the two-way interactions to quantify the main effects of turbidity, day of testing and male density (high or low) (Engqvist, 2005). Exclusion of non-significant interactions did not significantly reduce model fit between the consecutively reduced models (see supplementary material).

For the analysis of cortisol level, in the initial model, I included turbidity, male density and their two-way interaction as fixed factors; and I treated Block ID and plate ID as random factors. There was no significant two-way interaction, so I ran a final model with only the main effects of turbidity and harassment (Engqvist, 2005). Again, exclusion of the non-significant interaction did not significantly reduce model fit between the full and reduced models (see supplementary data).

I analysed the behavioural traits using generalized linear mixed models (GLMMs) with the appropriate error distribution: time followed (quasi-Poisson) and mating attempts received (quasi-Poisson). I analysed the cortisol levels using a linear mixed model (LMMs). The residuals fulfilled the assumptions of normality, homoscedascity, and linearity.

I used Wald chi-square tests to calculate p values (*Anova* function in the *car* package), with type III and type II sum of squares for models with and without interactions, respectively. The alpha level of significance was set at 0.05 (two-tailed).

Results

The effect of male density and turbidity on reproductive behaviour

There was no three-way interaction between the turbidity of the water, male density and the day of testing that affected how long the female spent being followed by males, or how many mating attempts she received (both: $\chi^2 > 0.258$; $P > 0.212$, see supplementary materials, Table S2 & S3). There were also no two-way interactions between water turbidity, male density and day of testing that affected how long the female spent being followed by males, or how many mating attempts she received (all: $\chi^2 > 0.043$; $P > 0.131$, Fig 3).

As predicted, females experienced significantly more mating attempts and spent significantly longer being followed when there were three males rather than one male (both $\chi^2 > 45.83$; $P < 0.001$, see supplementary materials, Table S2 & S3).

Unexpectedly, however, females experienced significantly *more* mating attempts and spent significantly *more* time being followed when in turbid rather than clear water (followed time: $\chi^2 = 10.48$; $P = 0.001$; mating attempts: $\chi^2 = 10.96$; $P < 0.001$).

Finally, females experienced significantly more mating attempts and spent significantly more time being followed on day 1 than day 2 (followed time: $\chi^2 = 64.85$; $P = 0.001$; mating attempts: $\chi^2 = 52.32$; $P < 0.001$).

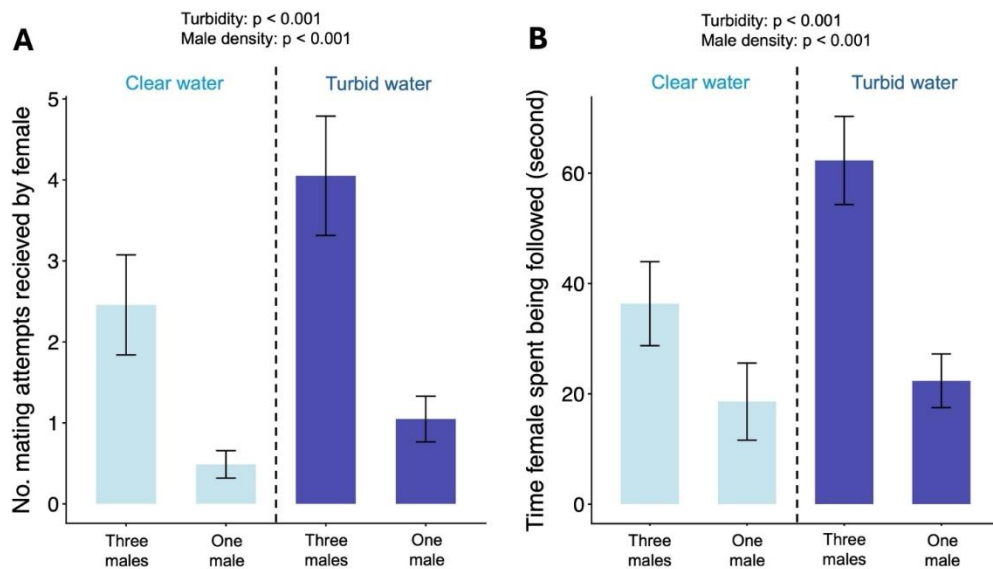


Figure 3: Effect of water turbidity and male density on the amount of sexual harassment received by females: (A) number of mating attempts and (B) time spent being followed. Data are presented as mean $\pm$ SE. The independent effect of water turbidity and the level of sexual harassment were both significant ($P < 0.001$).

Effects of male density and turbidity on cortisol levels

There was no interaction between water turbidity and male density that affected the level of cortisol of females ($\chi^2 = 0.1019$; $P = 0.750$). Females had significantly higher cortisol levels in clear than turbid water ($\chi^2 = 39.547$; $P < 0.001$), but there was no difference in their cortisol levels between the low and high male density treatment ($\chi^2 = 0.001$; $P = 0.975$, Fig 4; see also supplementary materials Table S4).

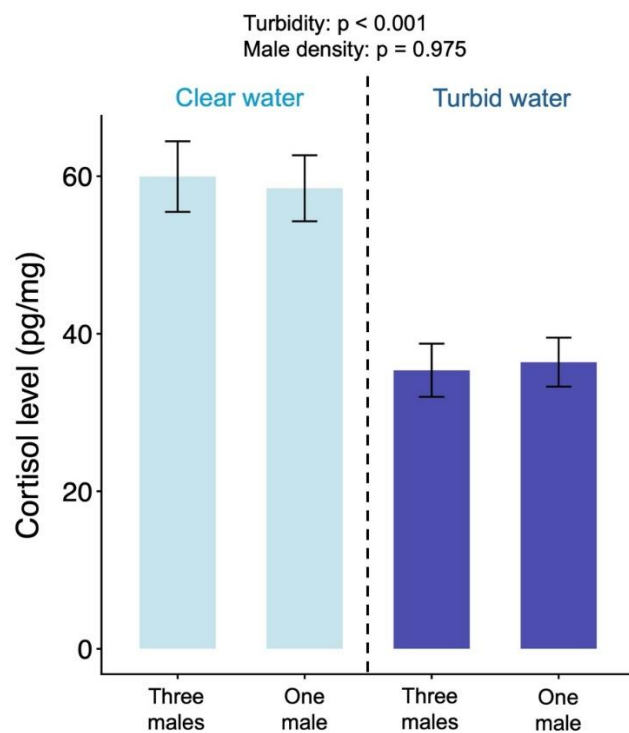


Figure 4: Effect of water turbidity and male density on the cortisol level of females. Data are presented as mean $\pm$ SE. The independent effect of water turbidity was significant ($P < 0.001$), irrespective of male density.

Correlation

Within each of the four treatments there was no significant correlation between cortisol level and either of the two measured behaviours on either day (Table 1).

Table 1: Correlation table between cortisol and measured behaviour of four different treatments at day 1 and 2

Treatment	Mating attempts		Following time	
	Day 1	Day 2	Day 1	Day 2
Clear water and low male density (CL)	-0.167 (n=38, P= 0.315)	0.189 (n=37, P= 0.263)	-0.093 (n= 38, P=0.578)	0.200 (n=37, P= 0.234)
Clear water and high male density (CH)	0.010 (n=41, P=0.948)	-0.032 (n=40, P= 0.846)	0.010 (n=41, P= 0.949)	0.039 (n=40, P=0.812)
Turbid water and low male density (TL)	-0.304 (n=43, P=0.047)	0.048 (n=42, P=0.762)	-0.165 (n=43, P= 0.290)	-0.111 (n=42, P=0.484)
Turbid water and high male density (TH)	0.168 (n=38, P=0.313)	-0.096 (n=40, P= 0.558)	0.224 (n=38, P=0.176)	0.057 (n=40, P=0.725)

Discussion

I tested how environmental change in the form of increased water turbidity affects the level of harassment experienced by females, and whether this ultimately affects their physiological stress response. I exposed female mosquitofish to one of two turbidities (<5 NTU or ~50 NTU) and to either 1 or 3 males for 30 hours. During this time, I measured their reproductive behaviour, namely mating attempts and time followed. After 30 hours, I then measured the level of cortisol (a ‘stress’ hormone) of the females. I found that females in turbid water were followed more often by males and experienced more mating attempts than did females in clear water. Surprisingly, however, despite more harassment, females who were in turbid water had *lower* levels of cortisol than females in clear water.

In addition, within each of the four treatments the level of cortisol did not covary with the level of sexual harassment a female received.

Effect of male density and turbidity on reproductive behaviour

As expected, females housed with three males experienced greater following time and more mating attempts than female housed with one male. This pattern is commonly seen in experimental studies that manipulate male density, where harassment increases with an increased number of males (Jirotkul, 1999; Sih & Krupa, 1995). My results therefore support the prediction that higher male density leads to increased harassment.

Mosquitofish adjusted their mating behaviour in response to the reduced visibility caused by greater turbidity. In turbid water, males followed the female for longer and made more mating attempts than they did when they were in clear water. This may be because females in clear water could see males from further away and were able to maintain a safe distance from males and escape from them more easily. In turbid water, females might have been less able to maintain a safe distance from males and therefore were more closely followed by males and were more often subject to mating attempt. My result is similar to those from other studies where species show behavioural plasticity in their mating behaviour in response to changes in the abiotic environment (Ehlman et al., 2018; Engström-Öst & Candolin, 2007; Heuschele et al., 2009). In turbid water, for example, male guppies (*Poecilia reticulata*) increase all mating behaviour directed towards females (Ehlman et al., 2018). In threespine stickleback (*Gasterosteus aculeatus*), male need to court significantly more in turbid than clear water to receive the same amount of interest from females (Engström-Öst & Candolin, 2007).

Effect of male density and turbidity on female cortisol level

Cortisol, which is a primary ‘stress’ hormone, plays a critical role in female fish reproduction. While short-term elevations can be adaptive, chronic stress and persistently

high cortisol levels can disrupt reproductive processes by suppressing gonadotropin-releasing hormone (GnRH). This can lead to delayed ovulation, reduced egg size, and decreased sexual activity (Campbell et al., 1992). Elevated cortisol also affects brain function, impairing cognitive processes such as learning and memory which are important for mate selection (Kim et al., 2015). For example, higher cortisol level weaken female mating preferences in treefrogs (Davis & Leary, 2015). In addition, several studies have suggested that fish in better condition have lower cortisol levels (Chase et al., 2016; Pasparakis et al., 2022). Poorer body condition (hence higher cortisol levels) is often linked to reduced reproductive output in fish. In zebrafish, a higher level of cortisone (synthetic cortisol) stops oocyte development and decrease attraction to male which ultimately decrease the likelihood of mating, reduces egg number and lowers survival (Ye et al., 2024). In common carp, cortisol affects the brain-pituitary-gonad axis either directly or indirectly and inhibits sexual development (Consten et al., 2001). Any changes in cortisol levels in female mosquitofish therefore has the potential to lower their evolutionary fitness and might also affect population dynamics.

In poeciliids, female harassment by males seems likely to be a chronic stressor which increases their metabolic rates leading to increased energy expenditure (Köhler et al., 2011) that could ultimately reduce female fitness and reproduction (e.g., Gay et al., 2009; Sakurai & Kasuya, 2008). This could be directly due to sexual harassment and/or to the costs of an increased number of matings. In my study, however, there was no difference in the female stress response due to variation in male density which was itself shown to increase the amount of harassment experienced by females. This is the opposite of the pattern expected if the rate of harassment is a major source of physiological stress. Furthermore, there was no correlation between the rate of harassment and cortisol levels within any of the four treatments. In any given environment, females that experienced more harassment did not show higher levels of cortisol. One possibility for these

‘negative’ results is that we tested the cortisol level after 30 hours, so maybe initially female were stressed but cortisol levels dropped back to baseline (control) levels prior to being measured. This might explain the lack of correlation within each treatment or between female house with one or three males, but it cannot explain the significant difference in cortisol levels between females in turbid and clear water. Based on my current results, I therefore conclude that greater male harassment is not stressful for females. This finding aligns with another study which shown that female guppies display plasticity in their physiological traits by spending less energy swimming to reduce the cost of greater harassment (Killen et al., 2016).

My analysis of cortisol levels clearly showed that female mosquitofish in clear water were more stressed than females in turbid water. This is intriguing given that females in turbid water were more often harassed by males. There are at least two plausible reasons for this result. First, females in clear water are more fearful of their surroundings due to a greater risk of predation. Females in the wild tend to prefer to stay near dense vegetation rather than in clear water, especially when predators are present (Pyke, 2005). My results align with a study on Delta smelt (*Hypomesus transpacificus*), which also showed that in turbid water they have lower level of cortisol than clear water (Pasparakis et al., 2023). A second possibility is that turbidity influenced female feeding behaviour. Mosquitofish are planktivorous (Blanco et al., 2004), and our turbid water treatment was made with algae. It is therefore possible that females had more ready access to food in the turbid water treatment if they consumed some of the algae. Some fish studies have shown there are health benefits to consuming algae (Alagawany et al., 2021). Improved body condition might then explain the lower cortisol levels of females in turbid water. We do not, however, have direct evidence that females consumed algae in the current study.

Conclusion

In this study, I found that females in turbid water received more mating attempts and were followed by males more than females in clear water. Even so, females were more physiologically stressed in clear water. This suggests that the level of sexual harassment has weak or no negative effects on female physiological stress. My result also suggests that turbid water is actually beneficial for mosquitofishes, but further studies investigating the effects of long-term turbidity on the physiology of both male and female mosquitofish and reproductive fitness are needed. Studies in other fish are also needed. Crucially, if turbid water generally lowers stress levels in fish this could lead to changes in animal care practices. Currently, most ethics committees expect that water is kept clean and clear. Based on my findings it might, however, actually be more ethical to maintain fish in turbid water to reduce their stress levels.

REFERENCES

- Agrillo, C., Dadda, M., & Bisazza, A. (2006). Sexual harassment influences group choice in female mosquitofish. *Ethology*, 112(6), 592–598.
<https://doi.org/10.1111/j.1439-0310.2006.01188.x>
- Alagawany, M., Taha, A. E., Noreldin, A., El-Tarabily, K. A., & Abd El-Hack, M. E. (2021). Nutritional applications of species of *Spirulina* and *Chlorella* in farmed fish: A review. *Aquaculture*, 542, 736841.
<https://doi.org/10.1016/j.aquaculture.2021.736841>
- Barton, B. A., & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, 1, 3–26. [https://doi.org/10.1016/0959-8030\(91\)90019-G](https://doi.org/10.1016/0959-8030(91)90019-G)
- Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, 2(3), 349–368.
<https://doi.org/10.1038/hdy.1948.21>
- Bisazza, A. (1993). Male competition, female mate choice and sexual size dimorphism in poeciliid fishes. *Marine and Freshwater Behaviour and Physiology*, 23, 257–286. <https://doi.org/10.1080/10236249309378869>
- Blanco, S., Romo, S., & Villena, M. J. (2004). Experimental study on the diet of mosquitofish (*Gambusia holbrooki*) under different ecological conditions in a shallow lake. *International Review of Hydrobiology*, 89(3), 250–262.
<https://doi.org/10.1002/iroh.200310684>
- Brewster, J., & Houde, A. (2003). Are female guppies more likely to flee when approached by two males? *Journal of Fish Biology*, 63(4), 1056–1059.
<https://doi.org/10.1046/j.1095-8649.2003.00222.x>

- Campbell, P. M., Pottinger, T. G., & Sumpter, J. P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biology of Reproduction*, 47(6), 1140–1150. <https://doi.org/10.1095/biolreprod47.6.1140>
- Candolin, U., Salesto, T., & Evers, M. (2007). Changed environmental conditions weaken sexual selection in sticklebacks. *Journal of Evolutionary Biology*, 20(1), 233–239. <https://doi.org/10.1111/j.1420-9101.2006.01207.x>
- Chapman, B. B., Morrell, L. J., & Krause, J. (2009). Plasticity in male courtship behaviour as a function of light intensity in guppies. *Behavioral Ecology and Sociobiology*, 63(12), 1757–1763. <https://doi.org/10.1007/s00265-009-0796-4>
- Chase, D. A., Flynn, E. E., & Todgham, A. E. (2016). Survival, growth and stress response of juvenile tidewater goby, *Eucyclogobius newberryi*, to interspecific competition for food. *Conservation Physiology*, 4(1), cow013. <https://doi.org/10.1093/conphys/cow013>
- Cherry, D. S., Rodgers, J. H., Jr., Cairns, J., Jr., Dickson, K. L., & Guthrie, R. K. (1976). Responses of mosquitofish (*Gambusia affinis*) to ash effluent and thermal stress. *Transactions of the American Fisheries Society*, 105(6), 686–694. [https://dx.doi.org/10.1577/1548-8659\(1976\)105<686:ROMGAT>2.0.CO;2](https://dx.doi.org/10.1577/1548-8659(1976)105<686:ROMGAT>2.0.CO;2)
- Chung, M. H. J., Jennions, M. D., & Fox, R. J. (2019). Novel ablation technique shows no sperm priming response by male eastern mosquitofish to cues of female availability. *Behavioral Ecology and Sociobiology*, 73, 167. <https://doi.org/10.1007/s00265-019-2779-4>
- Clutton-Brock, T. H., & Parker, G. A. (1995). Sexual coercion in animal societies. *Animal Behaviour*, 49(5), 1345–1365. <https://doi.org/10.1006/anbe.1995.0166>

- Condon, C. H. L., & Wilson, R. S. (2006). Effect of thermal acclimation on female resistance to forced matings in the eastern mosquitofish. *Animal Behaviour*, 72(2), 419–426. <https://doi.org/10.1016/j.anbehav.2005.11.016>
- Consten, D., Bogerd, J., Komen, J., Lambert, J. G. D., & Goos, H. J. Th. (2001). Long-term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L. *Biology of Reproduction*, 64(4), 1063–1071. <https://doi.org/10.1095/biolreprod64.4.1063>
- Crino, O. L., Buchanan, K. L., Fanson, B. G., Hurley, L. L., Smiley, K. O., & Griffith, S. C. (2017). Divorce in the socially monogamous zebra finch: Hormonal mechanisms and reproductive consequences. *Hormones and Behavior*, 87, 155–163. <https://doi.org/10.1016/j.yhbeh.2016.11.004>
- Crino, O. L., Wild, K. H., Friesen, C. R., Leibold, D., Laven, N., Peardon, A. Y., Recio, P., Salin, K., & Noble, D. W. A. (2024). From eggs to adulthood: Sustained effects of early developmental temperature and corticosterone exposure on physiology and body size in an Australian lizard. *Journal of Experimental Biology*, 227(24), jeb249234. <https://doi.org/10.1242/jeb.249234>
- Davies-Colley, R. J., & Smith, D. G. (2001). Turbidity, suspended sediment, and water clarity: A review. *JAWRA Journal of the American Water Resources Association*, 37(5), 1085–1101. <https://doi.org/10.1111/j.1752-1688.2001.tb03624.x>
- Davis, A. G., & Leary, C. J. (2015). Elevated stress hormone diminishes the strength of female preferences for acoustic signals in the green treefrog. *Hormones and Behavior*, 69, 119–122. <https://doi.org/10.1016/j.yhbeh.2015.01.005>

- Day, R. L., MacDonald, T., Brown, C., Laland, K. N., & Reader, S. M. (2001). Interactions between shoal size and conformity in guppy social foraging. *Animal Behaviour*, 62(5), 917–925. <https://doi.org/10.1006/anbe.2001.1820>
- Devigili, A., Kelley, J. L., Pilastro, A., & Evans, J. P. (2013). Expression of pre- and postcopulatory traits under different dietary conditions in guppies. *Behavioral Ecology*, 24(3), 740–749. <https://doi.org/10.1093/beheco/ars204>
- Ehlman, S. M., Martinez, D., & Sih, A. (2018). Male guppies compensate for lost time when mating in turbid water. *Behavioral Ecology and Sociobiology*, 72(3), 48.
- Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *The American Naturalist*, 139(S1), S125–S153.
- Engqvist, L. (2005). The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Animal Behaviour*, 70, 967–971. <https://doi.org/10.1016/j.anbehav.2005.01.016>
- Engström-Öst, J., & Candolin, U. (2007). Human-induced water turbidity alters selection on sexual displays in sticklebacks. *Behavioral Ecology*, 18(2), 393–398. <https://doi.org/10.1093/beheco/arl097>
- Evans, J. P., Pilastro, A., & Schlupp, I. (2011). *Ecology and evolution of poeciliid fishes*. University of Chicago Press.
- Gay, L., Eady, P. E., Vasudev, R., Hosken, D. J., & Tregenza, T. (2009). Costly sexual harassment in a beetle. *Physiological Entomology*, 34(1), 86–92. <https://doi.org/10.1111/j.1365-3032.2008.00656.x>
- Guthrie, D. M. (1986). Role of vision in fish behaviour. In T. J. Pitcher (Ed.), *The behaviour of teleost fishes* (pp. 75–113). Springer. https://doi.org/10.1007/978-1-4684-8261-4_4

- Hain, T. J. A., & Neff, B. D. (2007). Multiple paternity and kin recognition mechanisms in a guppy population. *Molecular Ecology*, 16(18), 3938–3946.
<https://doi.org/10.1111/j.1365-294X.2007.03443.x>
- Hess, S., Prescott, L. J., Hoey, A. S., McMahon, S. A., Wenger, A. S., & Rummer, J. L. (2017). Species-specific impacts of suspended sediments on gill structure and function in coral reef fishes. *Proceedings of the Royal Society B: Biological Sciences*, 284(1866), 20171279. <https://doi.org/10.1098/rspb.2017.1279>
- Heuschele, J., Mannerla, M., Gienapp, P., & Candolin, U. (2009). Environment-dependent use of mate choice cues in sticklebacks. *Behavioral Ecology*, 20(6), 1223–1227. <https://doi.org/10.1093/beheco/arp123>
- Hoare, D. J., Krause, J., Peuhkuri, N., & Godin, J.-G. J. (2000). Body size and shoaling in fish. *Journal of Fish Biology*, 57(6), 1351–1366.
<https://doi.org/10.1111/j.1095-8649.2000.tb02217.x>
- Houde, A. E. (1997). *Sex, color and mate choice in guppies*. Princeton University Press.
- Ioannou, C. C., Couzin, I. D., James, R., Croft, D. P., & Krause, J. (2011). Social organisation and information transfer in schooling fish. In C. Brown, K. Laland, & J. Krause (Eds.), *Fish Cognition and Behavior*, 217–239. Wiley-Blackwell.
<https://doi.org/10.1002/9781444342536.ch10>
- Järvenpää, M., & Lindström, K. (2004). Water turbidity by algal blooms causes mating system breakdown in a shallow-water fish, the sand goby *Pomatoschistus minutus*. *Proceedings of the Royal Society B: Biological Sciences*, 271(1555), 2361–2365. <https://doi.org/10.1098/rspb.2004.2870>

- Jirotkul, M. (1999). Operational sex ratio influences female preference and male–male competition in guppies. *Animal Behaviour*, 58(2), 287–294.
<https://doi.org/10.1006/anbe.1999.1149>
- Killen, S. S., Croft, D. P., Salin, K., & Darden, S. K. (2016). Male sexually coercive behaviour drives increased swimming efficiency in female guppies. *Functional Ecology*, 30(4), 576–583. <https://doi.org/10.1111/1365-2435.12527>
- Kim, E. J., Pellman, B., & Kim, J. J. (2015). Stress effects on the hippocampus: A critical review. *Learning & Memory*, 22(9), 411–416.
<https://doi.org/10.1101/lm.037291.114>
- Kimbell, H., & Morrell, L. (2015). Turbidity influences individual and group level responses to predation in guppies, *Poecilia reticulata*. *Animal Behaviour*, 103, 179–188. <https://doi.org/10.1016/j.anbehav.2015.02.027>
- Köhler, A., Hildenbrand, P., Schleucher, E., Riesch, R., Arias-Rodriguez, L., Streit, B., & Plath, M. (2011). Effects of male sexual harassment on female time budgets, feeding behavior, and metabolic rates in a tropical livebearing fish (*Poecilia mexicana*). *Behavioral Ecology and Sociobiology*, 65(8), 1513–1523.
<https://doi.org/10.1007/s00265-011-1161-y>
- Laven, N. E., Pearson, P. R., Wild, K. H., Noble, D. W. A., & Crino, O. L. (2025). Sex steroid profiles align with phenotype in sex-reversed female lizards. *General and Comparative Endocrinology*, 369, 114754.
<https://doi.org/10.1016/j.ygcen.2025.114754>
- Lehtiniemi, M., Engström-Öst, J., & Viitasalo, M. (2005). Turbidity decreases anti-predator behaviour in pike larvae, *Esox lucius*. *Environmental Biology of Fishes*, 73(1), 1–8. <https://doi.org/10.1007/s10641-004-5568-4>

- Meager, J. J., & Batty, R. S. (2007). Effects of turbidity on the spontaneous and prey-searching activity of juvenile Atlantic cod (*Gadus morhua*). *Philosophical Transactions of the Royal Society B: Biological Sciences*, 362(1487), 2123–2130. <https://doi.org/10.1098/rstb.2007.2104>
- Mi, H., Fagherazzi, S., Qiao, G., Hong, Y., & Fichot, C. G. (2019). Climate change leads to a doubling of turbidity in a rapidly expanding Tibetan lake. *Science of the Total Environment*, 688, 952–959. <https://doi.org/10.1016/j.scitotenv.2019.06.339>
- Michelangeli, M., Tuomainen, U., Candolin, U., & Wong, B. B. M. (2015). Habitat alteration influences male signalling effort in the Australian desert goby. *Behavioral Ecology*, 26(4), 1164–1169. <https://doi.org/10.1093/beheco/arv060>
- Mommsen, T. P., Vijayan, M. M., & Moon, T. W. (1999). Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, 9(3), 211–268. <https://doi.org/10.1023/A:1008924418720>
- Nieman, C. L., & Gray, S. M. (2020). Elevated algal and sedimentary turbidity alter prey consumption by emerald shiner (*Notropis atherinoides*). *Ecology of Freshwater Fish*, 29(2), 325–333. <https://doi.org/10.1111/eff.12517>
- Pankhurst, N. W. (2011). The endocrinology of stress in fish: An environmental perspective. *General and Comparative Endocrinology*, 170(2), 265–275. <https://doi.org/10.1016/j.ygcen.2010.07.017>
- Pasparakis, C., Lohroff, T., Biefel, F., Cocherell, D. E., Carson, E. W., Hung, T.-C., Cannon, R. E., Fanguie, N. A., & Todgham, A. E. (2023). Effects of turbidity, temperature and predation cue on the stress response of juvenile delta smelt.

Conservation Physiology, 11(1), coad036.

<https://doi.org/10.1093/conphys/coad036>

Pasparakis, C., Wampler, A. N., Lohroff, T., DeCastro, F., Cocherell, D. E., Carson, E.

W., Hung, T.-C., Connon, R. E., Fangue, N. A., & Todgham, A. E. (2022).

Characterizing the stress response in juvenile delta smelt exposed to multiple stressors. *Comparative Biochemistry and Physiology Part A: Molecular &*

Integrative Physiology, 274, 111303. <https://doi.org/10.1016/j.cbpa.2022.111303>

Perry, J. C., & Rowe, L. (2018). Sexual conflict in its ecological setting. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 373(1757), 20170418.

<https://doi.org/10.1098/rstb.2017.0418>

Pilastro, A., Benetton, S., & Bisazza, A. (2003). Female aggregation and male

competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour*, 65(6), 1161–1167.

<https://doi.org/10.1006/anbe.2003.2118>

Pyke, G. H. (2005). A review of the biology of *Gambusia affinis* and *G. holbrooki*.

Reviews in Fish Biology and Fisheries, 15(4), 339–365.

<https://doi.org/10.1007/s11160-006-6394-x>

Redding, J. M., Schreck, C. B., & Everest, F. H. (1987). Physiological effects on coho

salmon and steelhead of exposure to suspended solids. *Transactions of the American Fisheries Society*, 116(5), 737–744. [https://doi.org/10.1577/1548-](https://doi.org/10.1577/1548-8659(1987)116<737:PEOCSA>2.0.CO;2)

[8659\(1987\)116<737:PEOCSA>2.0.CO;2](https://doi.org/10.1577/1548-8659(1987)116<737:PEOCSA>2.0.CO;2)

Romero, L. M. (2004). Physiological stress in ecology: Lessons from biomedical research. *Trends in Ecology & Evolution*, 19(5), 249–255.

<https://doi.org/10.1016/j.tree.2004.03.008>

Sakurai, G., & Kasuya, E. (2008). The costs of harassment in the adzuki bean beetle.

Animal Behaviour, 75(4), 1367–1373.

<https://doi.org/10.1016/j.anbehav.2007.09.010>

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids

influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, 21(1), 55–89.

<https://doi.org/10.1210/edrv.21.1.0389>

Sarma, R. R., Edwards, R. J., Crino, O. L., Eyck, H. J. F., Waters, P. D., Crossland, M.

R., Shine, R., & Rollins, L. A. (2020). Do epigenetic changes drive corticosterone responses to alarm cues in larvae of an invasive amphibian?

Integrative and Comparative Biology, 60(6), 1481–1494.

<https://doi.org/10.1093/icb/icaa082>

Sih, A., & Krupa, J. J. (1995). Interacting effects of predation risk and male and female density on male/female conflicts and mating dynamics of stream water striders.

Behavioral Ecology, 6(3), 316–325. <https://doi.org/10.1093/beheco/6.3.316>

Sohel, S., & Lindström, K. (2015). Algal turbidity reduces risk assessment ability of the three-spined stickleback. *Ethology*, 121(6), 548–555.

<https://doi.org/10.1111/eth.12370>

Sundin, J., Berglund, A., & Rosenqvist, G. (2010). Turbidity hampers mate choice in a pipefish. *Ethology*, 116(8), 713–721. [https://doi.org/10.1111/j.1439-](https://doi.org/10.1111/j.1439-0310.2010.01787.x)

[0310.2010.01787.x](https://doi.org/10.1111/j.1439-0310.2010.01787.x)

Swanbrow Becker, L. J., & Gabor, C. R. (2012). Effects of turbidity and visual vs. chemical cues on anti-predator response in the endangered fountain darter

(*Etheostoma fonticola*). *Ethology*, 118(10), 994–1000.

<https://doi.org/10.1111/eth.12002>

Utne-Palm, A. C. (2002). Visual feeding of fish in a turbid environment: Physical and behavioural aspects. *Marine and Freshwater Behaviour and Physiology*, 35(1–2), 111–128. <https://doi.org/10.1080/10236240290025644>

Van der Sluijs, I., Gray, S. M., Amorim, M. C. P., Barber, I., Candolin, U., Hendry, A. P., Krahe, R., Maan, M. E., Utne-Palm, A. C., Wagner, H.-J., & Wong, B. B. M. (2011). Communication in troubled waters: Responses of fish communication systems to changing environments. *Evolutionary Ecology*, 25(3), 623–640. <https://doi.org/10.1007/s10682-010-9450-x>

Venkatesan, S., Chung, M.-H. J., Moura-Campos, D., & Head, M. L. (2024). The effect of turbidity on female mate choice in the guppy, *Poecilia reticulata*. *Animal Behaviour*, 213, 107–115. <https://doi.org/10.1016/j.anbehav.2024.05.001>

Wingfield, J. C., & Kitaysky, A. S. (2002). Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? *Integrative and Comparative Biology*, 42(3), 600–609. <https://doi.org/10.1093/icb/42.3.600>

Wishingrad, V., Musgrove, A. B., Chivers, D. P., & Ferrari, M. C. O. (2015). Risk in a changing world: Environmental cues drive anti-predator behaviour in lake sturgeon (*Acipenser fulvescens*) in the absence of predators. *Behaviour*, 152(5), 635–652. <https://doi.org/10.1163/1568539X-00003246>

Wong, B. B. M., Candolin, U., & Lindström, K. (2007). Environmental deterioration compromises socially enforced signals of male quality in three-spined sticklebacks. *The American Naturalist*, 170(2), 184–189. <https://doi.org/10.1086/519398>

Ye, M., Yang, J., Cai, Z., Wu, J., Xiong, W., & Hou, L. (2024). The effect of cortisone on female zebrafish (*Danio rerio*): Reducing reproductive capacity and offspring survival rate. *Aquatic Toxicology*, 277, 107132.

<https://doi.org/10.1016/j.aquatox.2024.107132>

Chapter 5: How do temperature and salinity affect male reproductive traits? - An experimental test in mosquitofish

“If you really think that environment is less important than the economy, try holding your breath while you count your money”- **Guy McPherson**

Introduction

Global climate change appears to be causing a rapid decline in biodiversity, but we still need considerably more research to understand the proximate basis for changes in the reproduction and/or survival of organisms in response to this ongoing environmental shift (IPCC, 2014). Higher temperatures are often listed as the main driver of population decline, but many other anthropogenic disturbances are simultaneously affecting biodiversity. These disturbances include habitat loss, light pollution, and pesticides. Aquatic species that live in freshwater are, for example, being exposing to a growing number of likely stressors (Grizzetti et al., 2017), such as salinization (Cañedo-Argüelles et al., 2013), water acidification (Kroeker et al., 2013; Muniz, 1990) and eutrophication (Smith, 2003; Smith & Schindler, 2009). Understanding how organism will respond to the combined effects of multiple stressors is one of the most important recent challenges in conservation biology and applied ecology (Côté et al., 2016; Schäfer & Piggott, 2018).

Environmental conditions early in life can have long-lasting consequences on an animal's development, which can lower their performance in later life. Many studies have shown that early-life stressors can have significant negative effects on adult morphology, behaviour and physiology, which ultimately lower an individual's reproductive success and survival (Denver et al., 1998; Vallée et al., 1997; Varlinskaya & Spear, 2008). For example, stress during development can negatively affect reproductive physiology (Naguib et al., 2006), reproductive performance (Crino et al., 2017), breeding behaviour (Chin et al., 2009; Spencer & Verhulst, 2007), body size (Pavlovska-Teglia et al., 1995),

and cognitive ability (Farrell et al., 2015; Nowicki et al., 2002). According to the “silver spoon” hypothesis, individuals that face a poor start early in their development can experience metabolic disorders later in life and therefore need to invest more energy in their self-maintenance as adults (Criscuolo et al., 2008). Due to life history trade-offs, an increase in self-maintenance ultimately lowers reproduction investment (Royle et al., 2005). More generally, when animals experience stressful conditions, they increase their energy consumption in the short-term so that they can maintain homeostasis (Chang et al., 2007), which then limits how much they can invest into other life history processes (Sharma, 2019). Ultimately, the pace of life depends on how an organism allocates resources to growth, self-maintenance, gamete production and reproduction (Arnqvist et al., 2022). Early life stress is likely to increase investment in self-maintenance at the cost of reduced investment in gamete production and reproduction.

Temperature is one of the environmental factors that has received the most attention in climate change studies of aquatic species because of its potential effects on key ecological and evolutionary processes. In fish, which are ectothermic, water temperature can significantly impact their reproductive physiology (e.g., gametogenesis, spawning behaviour and embryonic development) because their body temperature and metabolic rate is directly affected by the surrounding water temperature. For example, in Atlantic Salmon (*Salmo salar*), elevated temperatures increase physiological stress and immune responses, and change many of their behaviours (Aprison & Ruvinsky, 2014; Rinehart et al., 2000). The effects of higher temperatures are not, however, limited to affecting the phenotype of adults.

Higher temperatures can strongly influence sperm quality and have been associated with lower sperm count, decrease sperm motility, greater DNA damage and reduced sperm longevity (Cameron & Blackshaw, 1980; Flowers, 2015; Parrish et al., 2017; Peña et al., 2022; Reinhardt et al., 2015). For instance, boar (*Sus scrofa domestica*) kept at high

temperatures produce sperm with greater DNA damage, more morphological abnormalities, and lower motility (Cameron and Blackshaw, 1980; Flowers, 2015; Parrish et al., 2017; Peña et al., 2021). Similar negative effects of temperature on sperm have been observed in other species and are summarised in a recent meta-analysis (Dougherty et al., 2024). These temperature-induced changes in sperm traits can have profound consequences for male fertility and reproductive success (Aprison & Ruvinsky, 2014; Rinehart et al., 2000). For example, in flesh fly (*Sarcophaga crassipalpis*), males that experienced a heat shock become completely infertile, and produce very few sperm (Rinehart et al., 2000).

Alongside rising temperature, higher salinity is another environmental stressor whose effect on aquatic species is increasing because of both climate change and anthropogenic activities. In freshwater ecosystems, natural shifts in salinity arise from variation in evaporation (Forster, 1998) and precipitation, especially droughts, floods (Van De Pol et al., 2010), and storms (Walker, 2001). Global climate change is increasing the rate of occurrence of severe weather events such as droughts and heavy rains that lead to flooding. Anthropogenic activities such as irrigation, mining, power generation, and land clearing can further amplify changes in salinity because these activities affect rates of water run-off, often with severe ecological and economic consequences (Bhuiyan & Dutta, 2012; Karim & Mimura, 2008; Nielsen & Brock, 2009). High salinity significantly harms freshwater fish by disrupting their osmotic balance and forcing them to expend more energy on osmoregulation to survive. This shift in investment can then affect key life history traits by reducing their growth rate, spermatogenesis, testicular homeostasis, sperm quality and reproductive output (Billard, 1978; Chung et al., 2025; Cruz Vieira et al., 2019; Servili et al., 2020; Torres-Rodríguez et al., 2025; Yeganeh et al., 2008). In climate change studies the effect of salinity on the reproductive performance of aquatic species have been less well investigated than the effects of temperature.

Crucially, despite widespread recognition of the importance of both salinity and temperature in shaping reproductive performance, there are even fewer studies that test for the combined effect of these two factors (Hopkins et al., 2017). In addition, surprisingly few studies test how early life salinity affects male reproductive success because of the effects of salinity on sperm production (Servili et al., 2020). This is especially the case for studies testing for the effect of a male's developmental (rather than adult) environment on sperm production. This knowledge gap is highly relevant because increased salinity and higher temperatures are becoming common in many waterways. If we have greater knowledge of how these two factors interact then we can better forecast how environmental changes will influence the reproduction, hence population dynamics, of species in the wild.

The mosquitofish (*Gambusia holbrooki*) is a highly invasive species that occurs in a broad range of habitats (Pyke, 2005, 2008). They can tolerate high salinity and acclimatize to water with up to 25‰ (Nordlie & Mirandi, 1996). However, exposure to high salinity can reduce their performance, including decreased foraging activity, reduced aggression (Alcaraz & García-Berthou, 2007), taking longer to explore a novel environment, and lower sexual activity (Zhou et al., 2022). These behavioural changes have been linked to the energetic costs of osmoregulation (Christensen et al., 2018), which can reduce the capability of *G. holbrooki* to remain in highly saline water. Populations of *Gambusia* are rarely found in permanently high saline habitats (Pyke, 2005; Alcaraz & García-Berthou, 2007), although periodic exposing to high salinity does happen for several reasons, such as agriculture, road salt, urbanization and wastewater (Cañedo-Argüelles, 2020). In a review paper it was argued that if there is a gradual increase in salinity the survival rate of *Gambusia* might even increase due to genetic adaptation (Pyke, 2005).

In this chapter I tested for the combined effect of salinity and temperature on male reproductive traits in a 2 x 2 factor experiment. I assigned newborn babies to develop at either 26°C or 31°C in water that was either 0‰ or 20‰ salinity. Upon male fish reaching sexual maturity I recorded their: time to maturity, size at maturity and gonopodium length at the time of maturity. Then 35-49 days after the males had matured, I measured two of their ejaculate traits, namely sperm count and sperm velocity. These measurements allowed me to quantify the main effects of early life stressor (salinity and temperature) and their possible interaction, on several key life history traits.

The following predictions were made:

1. Male performance will be lower at high temperatures. I define lower performance as: smaller size at maturation (which is usually coupled with a shorter time to reach maturity), a relatively smaller gonopodium, slower adult growth, a lower sperm count and slower swimming sperm.
2. Male performance will be lower (as defined above) at higher salinity.
3. There will be an interaction between temperature and salinity such that the performance of males that develop at a high temperature under high salinity will have a worse performance than predicted by either temperature or salinity acting alone.

Materials and Method

Overview of the study design

Origin of fish: To generate fish stock, pregnant females were collected from Sullivan's Creek, Canberra, Australia in 2025 and transferred to the aquarium facilities at the Australian National University. These females were then transferred to individual 1L tanks to give birth. All pregnant females were kept in freshwater at 26°C (± 1 °C) under a 14 h light:10 h dark cycle and fed brine shrimp twice daily. A net enclosure in the tank

was set up to provide shelter for newborn babies. The tanks were checked twice daily for newborn offspring. Once detected, the offspring from each brood were evenly distributed across four treatment groups: normal temperature and freshwater control (NC), normal temperature and saline water (NS), high temperature and freshwater control (HC), and high temperature and saline water (HS). This design controlled statistically for some of the genetic variation among broods and/or differences in maternal effects that might affect the life history traits of offspring.

Temperature and salinity treatment

Two temperatures (26°C and 31°C) and two salinity levels (0‰ and 20‰) were used to test how temperature and salinity during development affect adult performance. These are all values that mosquitofish encounter and tolerate in nature. Newborn offspring were randomly and evenly assigned from each brood to the four treatments: 26°C and 0‰ (NC), 26°C and 20‰ (NS), 31°C and 0‰ (HC) and 31°C and 20‰ (HS). I used a total of 54 broods comprising 193 offspring (NC= 49, NS= 44, HC= 53 and HS= 47) in this experiment. The desired level of salinity was created by dissolving commercial sea salt into tap water with added aquatic conditioner. Shortly after birth the newborns were directly transferred into 0‰ saline water for the freshwater treatment. For the salinity treatments, to acclimate the newborns to a more saline environment, the babies were first transferred to 2.5‰ saline water for two days, then into 10‰ for the next two days, and then to 20‰ salinity water. Newborns were individually housed in 1L tanks until they matured. A random sample of tanks were checked every two days using a refractometer to test the salinity, and – when necessary - water levels were maintained by topping up tanks with freshwater to maintain the desired salinity. All fish were housed under a 14 h light:10 h dark cycle and fed brine shrimp twice daily.

The tanks were checked twice per week to determine when a male had matured, which was defined as occurring when he had a sharp tip to his gonopodium. To take a photo of the males, they were first anesthetized in AQUI-S solution (20 μ L in 200mL water) for 30s and then photographed alongside a 1 cm scale. Later I measured the standard length (SL, from the snout tip to the end of the vertebral column) and gonopodium length using *ImageJ* software version 1.53t (Abràmoff et al., 2004). Finally, 35-49 days after maturation, the male's sperm traits were measured, and his SL was remeasured. The following traits were measured:

Size at maturity and gonopodia length at maturity

When a male reached maturity, I photographed him to measure his SL and gonopodium length.

Time to maturity

I counted how many days between the date of birth and when the male had a sharp tip to his gonopodium (i.e. was mature).

Adult growth

I measured adult growth as the differences between two size measurements: SL on the day when a male matured (initial size) and again 5-7 weeks later (final size).

Sperm traits

To quantify sperm traits, males were first anesthetized in ice water, and I then stripped them of their sperm. I then waited for five days for a male to produce more sperm, photographed him, stripped him of sperm and then quantified his sperm count and sperm velocity. Velocity was measured at 28.5°C, which is the mean of the two-temperature treatment at which males were housed during their development. This process allowed me to test for differences among the four treatment groups in sperm production under

standardised conditions (Fig 1). Sperm count and velocity were measured following the procedures outlined in Chapter 3 (Akhter et al., 2026) (see also below).

Sperm velocity: Each focal male was anesthetized using ice water and placed on a glass slide coated with a 1% polyvinyl alcohol solution (PVA) under a dissection microscope. I carefully positioned the gonopodium until it was perpendicular to his body. By gently pressing the abdomen (3-5 times per fish), the ejaculates were collected onto the PVA-coated slide. To keep the sperm hydrated, I added 100 μ L of Extender solution (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, and 10 mM Tris (Cl); pH 7.5). From the ejaculate, I collected two sets of five sperm bundles using a micropipette. Each set of five bundles was placed into a separate microtube containing 3 μ L of Extender solution. To measure the sperm velocity, 3 μ L of activator medium was placed on a cell of a 12-cell multi-test slide to activate the sperm. Next, I added 3 μ L of the Extender solution containing the sperm bundles to the activator medium and waited for 30 seconds before placing the cover slip on top. The slide was then placed under the microscope and the temperature set to 28.5°C. I tried to obtain at least 10 motile tracks per sample (mean 29.1; range 1-130). I also recorded the velocity of these motile tracks as the average curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). Here, I only report VCL as all three measures were highly correlated (VSL-VCL: $r = 0.986$; VSL-VAP: $r = 0.998$; and VCL-VAP: $r = 0.992$; all $p < 0.001$). The same procedure was repeated for the second 3 μ L sample to determine the repeatability of the estimate of mean velocity (repeatability: $r \pm SE = 0.473 \pm 0.062$, $p = 0.001$, $N = 169$ males).

Sperm count: After collecting 10 sperm bundles for the velocity measurement, the remaining ejaculate was transferred from the PVA-coated slide to a 1.5 ml Eppendorf tube. Depending on the amount of ejaculate, the sample was diluted with an appropriate amount of extender medium to ensure a workable (i.e. countable) concentration of sperm.

To release the sperm and break up the bundles, the samples were vortexed for about 1 minute and further even distribution of the sperm was then ensured by pipetting the solution up and down 20-30 times. Next, 3 μ L of the sample was placed on a 20-micron capillary slide (Leja). Using the CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100 $\times$ magnification, I took five measurements per male from randomly selected portions of the slide. The number of sperm per sample (standardised to the same volume to correct for differences in dilution) was highly repeatable across males (repeatability: $r \pm SE = 0.920 \pm 0.009$, $p = 0.001$, $N = 177$ males).

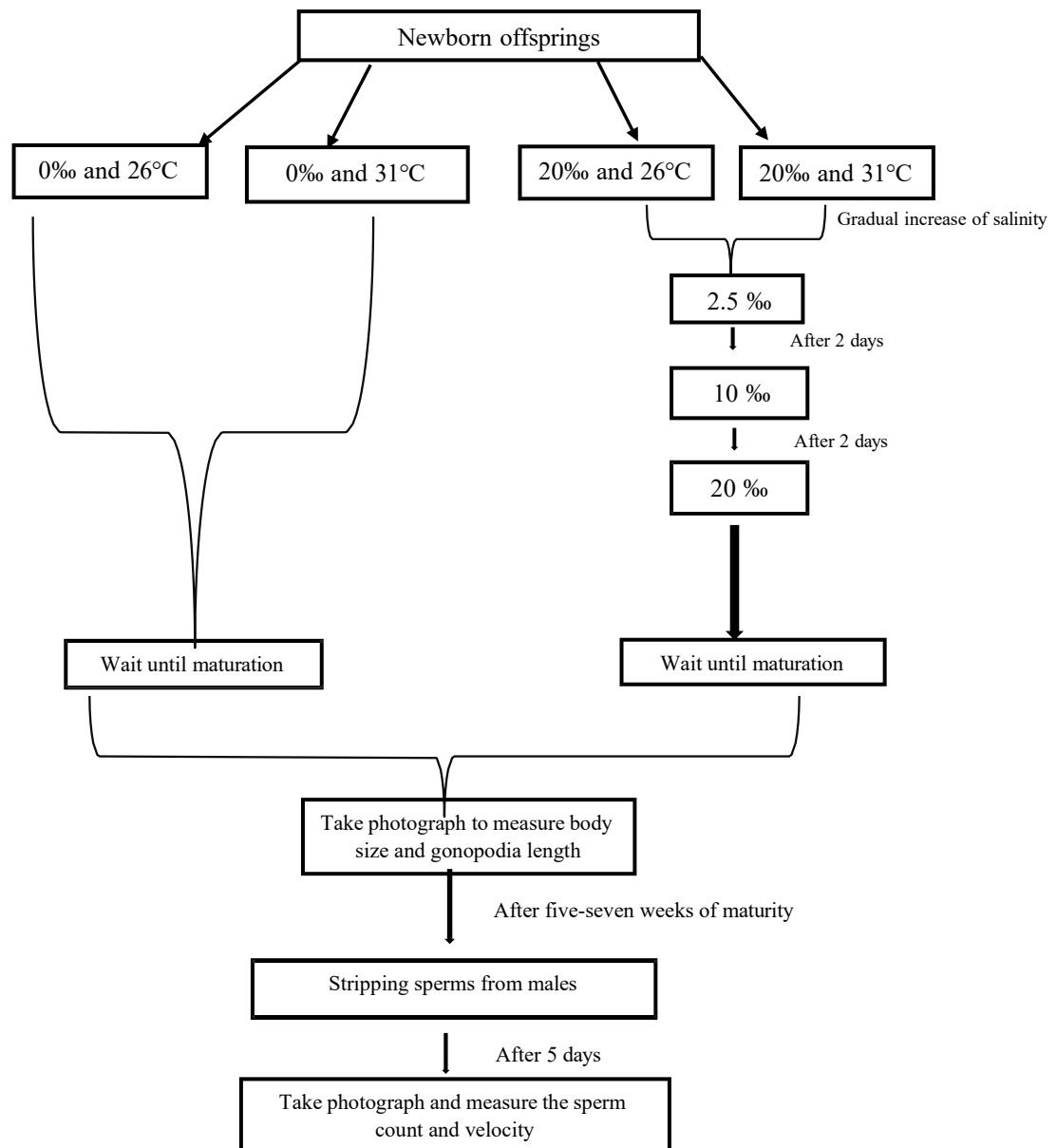


Figure 1: Full experimental design with two temperatures (26°C and 31°C) and two salinity treatments (0‰ and 20‰) to create four treatment groups. I then tested how developmental temperature and salinity stress affect male sperm traits.

Statistical analysis

I preregistered the statistical analysis on the Open Science Framework (OSF, <https://doi.org/10.17605/OSF.IO/GR6MV>). The data were analysed using R (version 4.3.1; R Core Team 2022). For each response variable, a histogram was made to see

whether the data was approximately normally distributed. I then assessed whether transformation of the data was likely to better meet the assumptions of a linear mixed model with Gaussian error. If necessary, an appropriate transformation was applied to the data (details below). After that I checked the appropriateness of the models using the DHARMA package to examine the distribution of residuals, Q-Q plots, and to test for potential overdispersion of residuals.

I ran separate models for each of the six-response variables (time to maturity, size at maturity, relative gonopodium length, adult growth, sperm count, sperm velocity). For all six variables temperature and salinity were treated as fixed factors, and Mother ID as a random factor because there were several offspring per mother. Initially I included the interaction between salinity and temperature in the model. If there was no significant interaction, I removed it from the final model to test for the main effects of temperature and salinity. A log-likelihood ratio test was used to confirm that removal of the interaction did not significantly reduce the model fit. For gonopodium length I also included mature male size as a covariate in the initial model because I was interested in the effect of the treatment on relative gonopodium length. Body size and gonopodium length are highly correlated ($r = 0.722$), so testing for treatment effects on body size is almost equivalent to testing for effects on absolute gonopodium length. For growth, final body size (size after 5-7 weeks of maturity) was used as the dependent variable and size at maturity as a covariate in the model. In the initial model I included all two-way and three-way interactions between initial body size, salinity and temperature.

For sperm count, I initially ran a model without male body size (final body size) as a covariate. I then reran the final model including final body size (but excluding any interactions with other terms). It is well known that larger males produce more sperm (O'Dea et al., 2014). By including male size in the final model, I could test for any

additional effects of salinity or temperature on sperm count beyond any effects directly mediated by male body size.

Summary statistics are presented as mean $\pm$ SE. I set $\alpha = 0.05$ (two-tailed). The outputs of all initial and final models are provided in the Supplementary Material.

Results

To test the effect of salinity and temperature on male reproductive traits, I compared the four treatments for differences in size at maturity, time to mature, relative gonopodium length at maturity, adult growth rate, sperm count and sperm velocity.

Effect of salinity and temperature on size at maturity

Size at maturity was significantly affected by an interaction between salinity and temperature ($\chi^2_1 = 8.54$, $P = 0.003$) (Table 1; see also supplementary material Table S1). This interaction was due to the magnitude of the effect of salinity in reducing male size being greater for fish reared in the hot rather than the normal temperature. Pairwise comparisons showed that fish in the high temperature and freshwater (HC) treatment were significantly larger than those in the high temperature and saline water (HS) treatment (HC vs HS, $P < 0.001$). Likewise, fish in the normal temperature and freshwater (NC) treatment were significantly larger than those in the normal temperature and saline water (NS) (NC vs NS, $P = 0.003$) treatment. The size of fish reared at the normal and hot temperature did not differ in the freshwater (NC vs HC, $P = 0.764$), but fish reared in saline water matured at a significantly smaller size when reared at the hot rather than the normal temperature (NS vs HS, $P = 0.013$) (Fig 2).

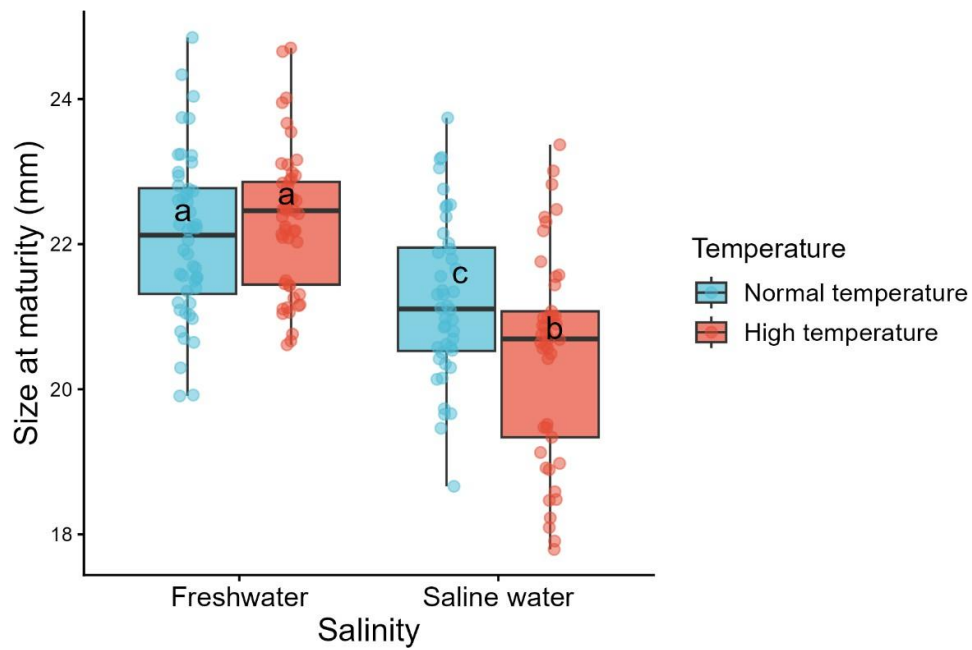


Figure 2: The effect of salinity and temperature on male size at maturity. Data are presented as mean $\pm$ SE.

Effect of salinity and temperature on time to maturity

Time to maturity was significantly affected by salinity ($\chi^2_1 = 4.29$, $P = 0.038$), but not by temperature ($\chi^2_1 = 0.08$, $P = 0.781$) (Table 1). Fish in saline water matured sooner than fish in freshwater (Fig 3). There was no interaction between salinity and temperature (see supplementary material Table S2).

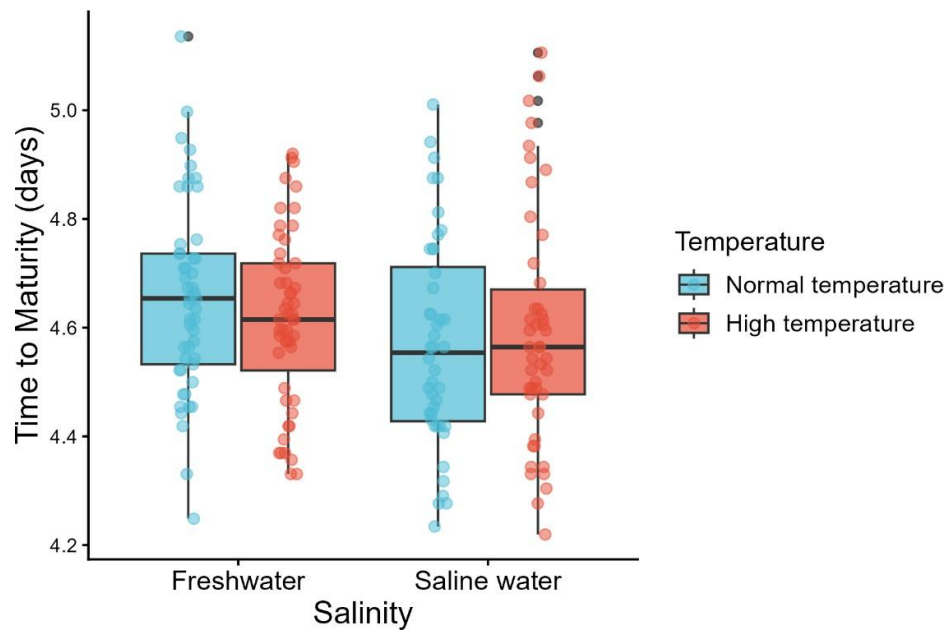


Figure 3: The effect of salinity and temperature on male time to maturity. Data are presented as mean $\pm$ SE.

Effect of salinity and temperature on relative gonopodium length at maturity

After controlling for the effect of body size at maturity ($\chi^2_1 = 117.50$, $P < 0.001$), gonopodium length was significantly affected by salinity ($\chi^2_1 = 11.01$, $P = 0.001$), but not by temperature ($\chi^2_1 = 0.75$, $P = 0.39$) (Table 1) (Figs 4 & 5). Males that developed in saline water had a relatively shorter gonopodium at maturation than males reared in the freshwater. There was no interaction between salinity and temperature (see supplementary material Table S3).

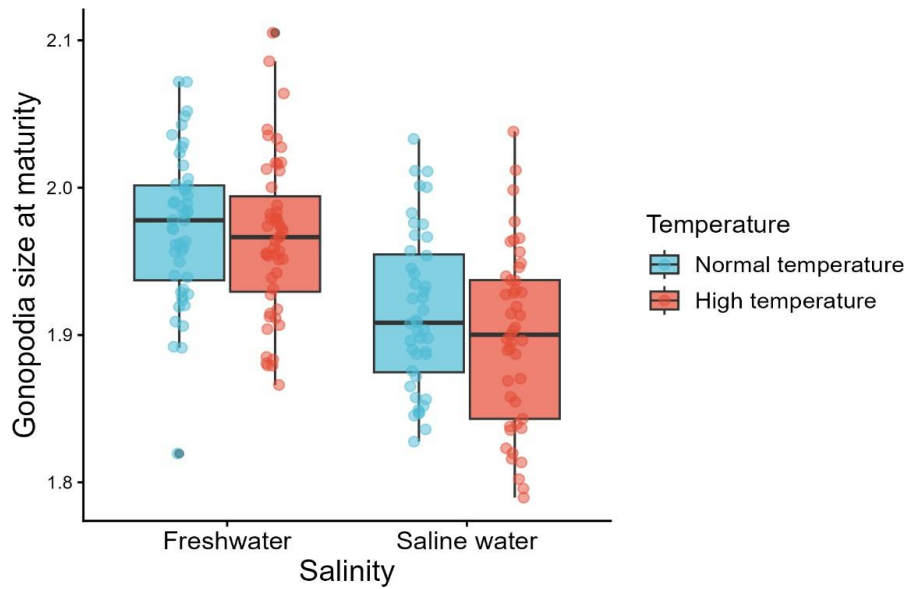


Figure 4: The effect of salinity and temperature on gonopodium length. Data are presented as mean $\pm$ SE.

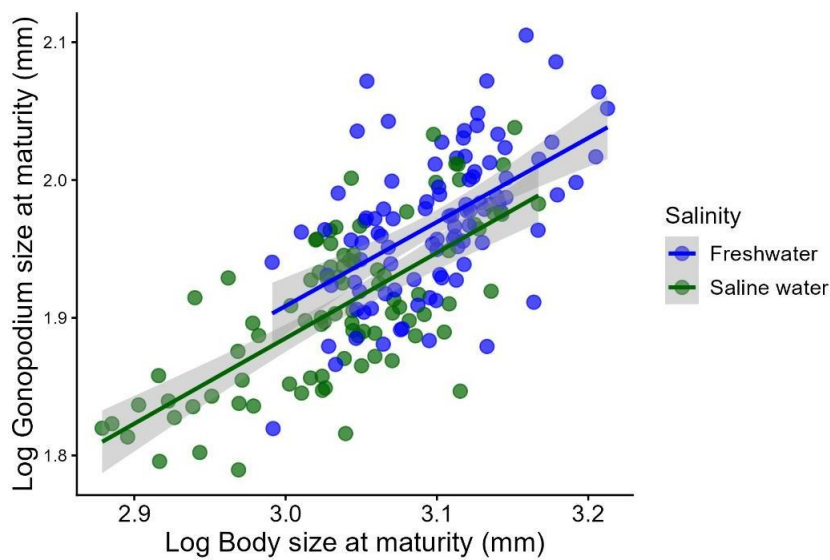


Figure 5: The relationship between body size and gonopodium length in saline and fresh water.

Effect of salinity and temperature on male growth

There was a significant three-way interaction between initial body size, salinity, and temperature ($\chi^2_1 = 4.41$, $P = 0.036$) that affected a male's size after 5-7 weeks. This indicates that growth (i.e. the slope of the relationship between initial and final size) was

affected by an interaction between salinity and temperature (Table 1; see also supplementary material Table S4). In freshwater, males that developed at the normal temperature showed slightly faster growth than males that developed at the high temperature (i.e. steeper slope in Fig 6). But in saline water, males reared at the normal temperature showed slightly slower growth than males reared at the high temperature (Fig 6).

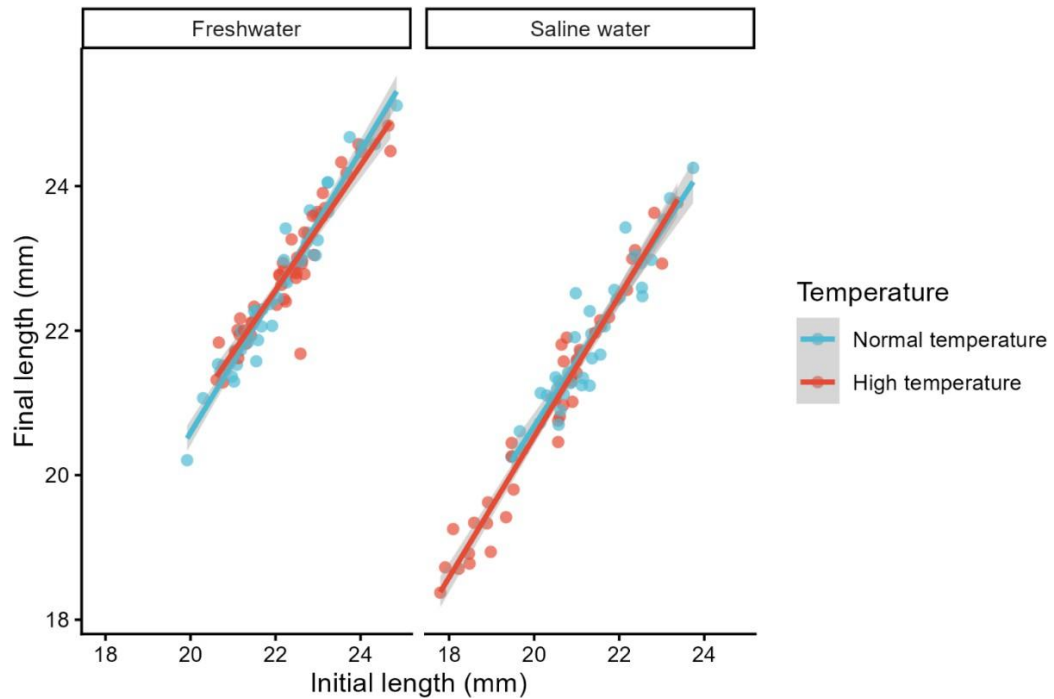


Figure 6: The effect of salinity and temperature on male adult growth.

Effect of salinity and temperature on sperm count

Total sperm count was affected by both salinity ($\chi^2_1 = 11.81$, $P = 0.001$), and temperature ($\chi^2_1 = 6.40$, $P = 0.011$) (Table 1). Males that developed in freshwater produced more sperm than males that developed in saline water; and males that developed at the high temperature produced more sperm than males that developed at the normal temperature (Fig 7). There was no interaction between salinity and temperature (see supplementary material Table S5). Initially we focused on how temperature and salinity affect sperm number, but we recognize that body size can also influence sperm number, but we recognize that body size can also influence sperm number, which is why we also included

body size as covariate. After controlling for body size ($\chi^2_1 = 16.38$, $P < 0.001$), the total sperm count was still significantly influenced by temperature ($\chi^2_1 = 8.69$, $P = 0.003$), but not by salinity ($\chi^2_1 = 1.37$, $P = 0.243$) (Table 1). There was no interaction between salinity and temperature. These results indicate that male body size is the strongest predictor of sperm production, but that temperature still affects sperm count even after controlling for how it affects male size (Fig 8). In contrast, the effect of salinity on sperm count seems to be explicable based on its direct effect on male size.

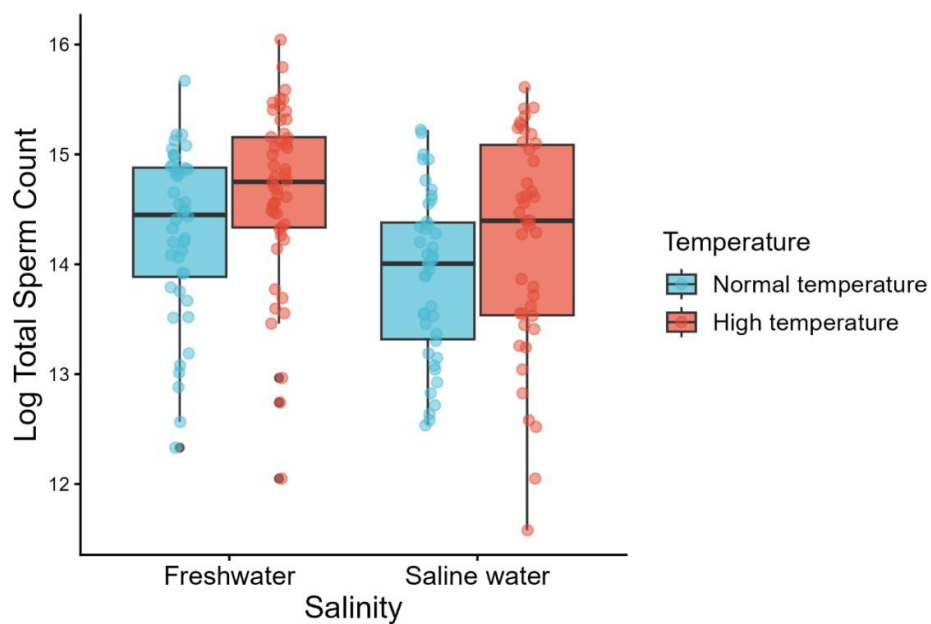


Figure 7: The effect of salinity and temperature on sperm count. Data are presented as mean $\pm$ SE.

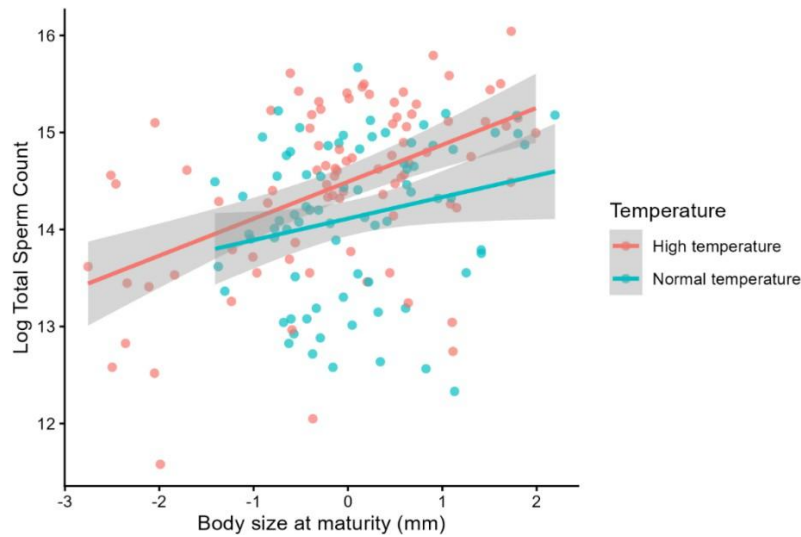


Figure 8: The relationship between body size and sperm number

Effect of salinity and temperature on sperm velocity

Sperm velocity was significantly affected by the salinity a male experienced during development ($\chi^2_1 = 9.77$, $P = 0.002$) but not by temperature ($\chi^2_1 = 0.86$, $P = 0.354$) (Table 1). There was no interaction between salinity and temperature (see supplementary material Table S6). The sperm of males that developed in saline water swam faster than the sperm of males that developed in freshwater (Fig 9).

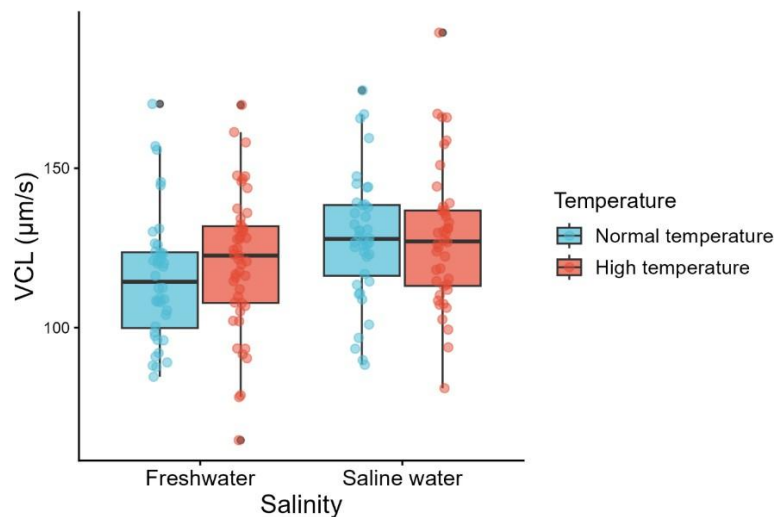


Figure 9: The effect of salinity and temperature on sperm velocity. Data are presented as mean $\pm$ SE.

Table 1: Effect of salinity and temperature on male reproductive traits. Significant estimates are indicated in bold (excluding main effects if there was a two-way interaction, or main effects or two-way effects if there was a significant three-way interaction).

Traits	Predictors	Estimate	SE	χ^2_1	P value
a) Size at maturity	Intercept	22.32	0.16		
	Saline Water	-1.86	0.24	60.83	<0.001
	Normal Temperature	-0.23	0.23	0.96	0.326
	Salinity: Temperature	0.99	0.34	8.54	0.003
b) Time to maturity	Intercept	4.63	0.02		
	Saline Water	-0.06	0.03	4.29	0.038
	Normal Temperature	0.01	0.03	0.08	0.78
c) Gonopodia size at maturity	Intercept	0.01	0.17		
	Saline Water	-0.02	0.01	11.01	0.001
	Normal Temperature	0.01	0.01	0.75	0.385
	Size at maturity	0.60	0.06	117.50	<0.001
d) Growth	Intercept	3.67	0.99		
	Initial body size	0.86	0.04	370.65	<0.001
	Saline Water	-2.49	1.20	4.27	0.039
	Normal Temperature	-2.44	1.37	3.17	0.075
	Initial body size: Saline water	0.11	0.06	3.82	0.051
	Initial body size: Normal temperature	0.11	0.06	3.19	0.074
	Salinity: Temperature	4.00	1.85	4.67	0.031
	Initial body size: Salinity: Temperature	-0.18	0.09	4.41	0.036
e) Sperm count	Intercept	14.63	0.11		
	Saline Water	-0.43	0.13	11.81	0.001
	Normal Temperature	-0.32	0.13	6.40	0.011
f) Sperm count with final body size	Intercept	14.56	0.11		
	Saline Water	-0.17	0.14	1.37	0.243
	Normal Temperature	-0.37	0.12	8.69	0.003
	Final body size	0.29	0.07	16.39	<0.001
g) Sperm velocity	Intercept	119.67	2.76		
	Saline Water	10.02	3.21	9.77	0.002
	Normal Temperature	-3.0	3.23	0.86	0.354

Discussion

Global climate change is creating multiple environmental stressors for wild animals, such as extreme temperatures and large fluctuations in salinity which can significantly influence their reproductive performance. For this reason, measuring the performance of individuals, including the performance of their gametes when they develop under different environmental conditions (such as salinity and temperature) is crucial to understand the reproductive consequences of climate change. For example, higher temperatures and higher salinity have each been shown to accelerate the rate at which fish mature and the production of sperm (Alcaraz & García-Berthou, 2007; Chung et al., 2025; Iglesias-Carrasco et al., 2020). Numerous studies have independently examined the effects of temperature and salinity, but there are very few studies which have investigated the combined effect of the salinity and temperature experienced during development on reproductive performance. Here, I experimentally manipulated the developmental temperature and salinity of male mosquitofish, *Gambusia holbrooki*, to examine the combined effects of these two factors on key life-history and reproductive traits. These traits were: body size at maturity, time to maturity, relative gonopodium length (which has been linked to male attractiveness), adult growth and two ejaculate traits (sperm count and sperm velocity). My study demonstrates that salinity on its own can significantly influence the time to maturation, relative gonopodium length, sperm count and sperm velocity, while temperature on its own can significantly influence a male's sperm count. In addition, temperature and salinity interacted to influence a male's body size at maturity and his rate of adult growth.

Effect of salinity on life history and reproductive traits

Male mosquitofish exposed to higher salinity during development matured earlier, at a smaller body size, and with a relatively shorter gonopodium. These findings are consistent with previous work on *G. holbrooki*, which also found that fish reared in higher salinity grew faster and matured sooner (Alcaraz & García-Berthou, 2007; Chung et al., 2025). Similar effects of salinity have been observed in other fish species. For instance, the congeneric *Gambusia affinis*, another poecilid, the sailfin molly (*Poecilia latipinna*) and the Caspian roach (*Rutilus rutilus caspicus*) all matured sooner in water with higher salinity (Naddafi et al., 2005; Trexler & Travis, 1990; Vondracek et al., 1988); and in black-chinned tilapia (*Sarotherodon melanotheron*) and the bonga shad (*Ethmalosa fimbriata*), fish reared at a high salinity reached sexual maturity at a smaller body size (Panfili, Mbow, et al., 2004; Panfili, Durand, et al., 2004). These patterns suggest that when mosquitofish develop in higher saline water they experience physiological stress because it is predicted that faster development and earlier reproduction will occur in fish as an adaptive response to a poor environment during development (Chung et al., 2025; Vondracek et al., 1988). More generally, life history theory predicts that in unfavourable conditions, given certain background assumptions, organisms develop a fast pace-of-life strategy characterized by earlier maturation and increased and earlier reproductive effort (Stearns, 1992). In environments where adult survival is likely to be lower, or breeding conditions are unstable, organisms do indeed tend to evolve a ‘fast’ strategy characterized by earlier maturation, smaller size at maturity, and increased reproductive effort (i.e. larger clutches) (Alcaraz & García-Berthou, 2007).

Male *G. holbrooki* that experienced higher salinity during development produced fewer sperm, although these sperm were faster swimming. This finding highlights an adaptive reproductive strategy where organisms compensate for lower sperm production by

increasing their sperm velocity which may help them to maintain their fertilization rate, at least in the absence of sperm competition which usually favours producing more sperm (Nissling & Larsson, 2018). Environmental stress such as higher salinity impairs spermatogenesis through germline cell apoptosis, which decrease the number of sperm that can be produced (Cruz Vieira et al., 2019; Edward & Gilburn, 2007). A review by Servili et al. 2020 concludes that males that experience higher salinity (although it is not clear at which life stages) tend to produce fewer sperm, although higher salinity also tends to increase sperm motility. This increased sperm motility might be caused by the release of water from the spermatozoa due to osmosis which increases the internal concentration of certain ions that then activate swimming by the sperm (Servili et al., 2020). It should be noted, however, that when I controlled statistically for the effect of body size, salinity no longer affected a male's sperm count. This suggests that the effect of salinity on sperm count can most parsimoniously be attributed to its direct effect on adult body size: smaller males with smaller testes produce fewer sperm.

In my study, individuals experienced high (or low) salinity from birth. Exposure to high salinity might have imposed high energetic cost due to increased osmoregulation during development. This cost could then affect adult reproductive performance because there would be less energy available for reproduction due to the increased costs of growth and self-maintenance. The increased costs of growth and self-maintenance would also explain why males that developed in high salinity water matured at a smaller size. In addition, higher ongoing somatic maintenance costs because of high salinity might then have shifted subsequent resource allocation away from reproduction towards self-maintenance, thereby lowering sperm production (Haddy & Pankhurst, 2000; Servili et al., 2020). Salinity is thought to be a key factor affecting rates of mosquitofish survival (and reproduction) in wild populations due to the physiological costs of osmoregulation. There are, however, confounding factors. For example, high saline habitats usually undergo

much greater environmental fluctuations than freshwater habitats (Nordlie & Mirandi, 1996), and environmental fluctuations are themselves costly to cope with. In addition, studies on wild populations of *G holbrooki* reveal that there are other factors that tend to covary with salinity, such as temperature, pH and distance from the coast, that could also affect survival and lead to differences among populations (Alcaraz & García-Berthou, 2007). This covariation makes it difficult to quantify the effect of salinity alone on survival or reproduction in *Gambusia*. Similar issues have been highlighted in studies of guppies (*Poecilia reticulata*), where variation in key life-history among populations that is attributed to predation risk may also reflect the effects of environmental factors that covary with predation risk. For example, low predation sites are often in small, shaded streams above waterfalls, while high predation sites are in larger, sunny rivers below waterfalls (Grether et al., 2001; Reznick et al., 2001). This covariation is why controlled experiments, such as the one I have present here, are always necessary to disentangle the causal effects of specific environmental factors on life history traits.

Effect of temperature on life history and reproductive traits

Developmental temperature had fewer effects on male mosquitofish than salinity as it did not significantly influence their time to maturity, relative gonopodium length or sperm velocity. Developing at a higher temperature did, however, significantly increase a male's sperm count, which is consistent with findings in other studies of *G holbrooki* (Meffe, 1991), and in work on other fish species (e.g., Atlantic Cod (*Gadus morhua*), Yoneda & Wright, 2005)). The result for time to maturity was slightly surprising as higher temperatures are known to promote earlier sexual maturation in many fish (e.g., Atlantic Salmon and Grouper (Pino Martinez et al., 2022; Xu et al., 2024)). In teleosts, maturation and reproduction are influenced by the brain-pituitary-gonadal (BPG) axis. When an external stimulus such as a higher temperature stimulates the BPG axis, it releases gonadotropin releasing hormones, which induce the synthesis of gonadotropins and

increase the sperm number (Mateos et al., 2002; Zahangir et al., 2022). The lack of an effect of temperature on the time of maturity in *G. holbrooki* in my study is therefore worthy of further investigation. Developmental temperature did, however, influence a male's body size at maturity and his adult growth, but these effects depended on the salinity level (see the next section).

Combined effect of salinity and temperature on life history and reproductive traits

I found significant interactive effects of salinity and temperature on both body size at maturity and growth in male *G. holbrooki*. Males that developed in saline water matured at smaller size when they developed at the higher temperature, but for males that developed in freshwater there was no effect of temperature on body size. This also meant that there was a negative effect of salinity of body size at maturity, but this was only apparent at the higher temperature (see Fig 2). My results differ from those in a study of another poeciliid, the sailfin molly (*Poecilia latipinna*), where fish tended to mature at a smaller size in freshwater than saltwater at normal temperatures (Trexler & Travis, 1990).

Adult growth was also influenced by an interaction between salinity and temperature during development. In freshwater, growth tends to be higher when males developed at the normal rather than the high temperature. In saline water the opposite trend was observed as higher growth was observed at the higher rather than the normal temperature. In addition, at the normal temperature, males grew slower in saline than freshwater, although the difference was not statistically significant; while at the higher temperature fish grew faster in saline than freshwater, although the difference was again not statistically significant (see Fig 6). A similar pattern was observed in another study of *G. holbrooki* conducted at a normal temperature where high salinity reduced somatic growth (Alcaraz & García-Berthou, 2007). Other studies have shown that fish grow faster in freshwater than saline water regardless of the water temperature (Likongwe et al., 1996;

Xu et al., 2024; Yoneda & Wright, 2005) which is likely to be due to a faster metabolism.

In sum, my results indicate that the effect of temperature on growth is context-dependent and is regulated by the salinity of the water.

Conclusion

My study reveals that *Gambusia holbrooki* males exhibits developmental phenotypic plasticity in response to environmental stressors that act during development. Salinity primarily drove males towards earlier maturation, decreased size at maturation and decreased reproductive investment in the form of a lower sperm count, albeit with faster swimming sperm; while temperature interacted with salinity to influence adult growth and size at maturation. A higher temperature also had the direct effect of elevating a male's sperm count. These results highlight that when predicting life history responses to climate change it is important to consider the potential for multiple environmental factors to interact. In sum, my study suggests that the combined effects of temperature and salinity can have complex and sometimes unexpected effects on life history traits. These outcomes could have major implications for population dynamics, invasion success of introduced species such as *Gambusia*, and ecosystem function. In addition, I note that climate change may not always be detrimental to animal reproduction as I found positive effects of higher temperatures and greater salinity on some of the measured traits. To more fully understand the synergistic effects of temperature and salinity on wild populations it would be useful to now investigate their impact on the life history and reproductive traits of females, and to further test whether there are any transgenerational effects on offspring. The success of a population ultimately depends on the ability of both sexes to reproduce and produce viable, fecund offspring.

REFERENCES

- Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics International*, 11, 36–42.
- Akhter, F., Head, M. L., Jennions, M. D., & Chung, M.H. J. (2026). Disentangling the life-history costs of direct male-male competition and sperm production. *Evolutionary Ecology*, 40(2), 17. <https://doi.org/10.1007/s10682-026-10383-6>
- Alcaraz, C., & García-Berthou, E. (2007). Life history variation of invasive mosquitofish (*Gambusia holbrooki*) along a salinity gradient. *Biological Conservation*, 139(1), 83–92. <https://doi.org/10.1016/j.biocon.2007.06.006>
- Aprison, E. Z., & Ruvinsky, I. (2014). Balanced trade-offs between alternative strategies shape the response of *C. elegans* reproduction to chronic heat stress. *PLOS ONE*, 9(8), e105513. <https://doi.org/10.1371/journal.pone.0105513>
- Arnqvist, G., Rönn, J., Watson, C., Goenaga, J., & Immonen, E. (2022). Concerted evolution of metabolic rate, economics of mating, ecology, and pace of life across seed beetles. *Proceedings of the National Academy of Sciences*, 119(33), e2205564119. <https://doi.org/10.1073/pnas.2205564119>
- Bhuiyan, Md. J. A. N., & Dutta, D. (2012). Assessing impacts of sea level rise on river salinity in the Gorai river network, Bangladesh. *Estuarine, Coastal and Shelf Science*, 96, 219–227. <https://doi.org/10.1016/j.ecss.2011.11.005>
- Billard, R. (1978). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. *Aquaculture*, 14(3), 187–198. [https://doi.org/10.1016/0044-8486\(78\)90094-7](https://doi.org/10.1016/0044-8486(78)90094-7)

- Cameron, R. D., & Blackshaw, A. W. (1980). The effect of elevated ambient temperature on spermatogenesis in the boar. *Journal of Reproduction and Fertility*, 59(1), 173–179. <https://doi.org/10.1530/jrf.0.0590173>
- Cañedo-Argüelles, M. (2020). A review of recent advances and future challenges in freshwater salinization. *Limnetica*, 39(1), 185–211. <https://doi.org/10.23818/limn.39.13>
- Cañedo-Argüelles, M., Kefford, B. J., Piscart, C., Prat, N., Schäfer, R. B., & Schulz, C.-J. (2013). Salinisation of rivers: An urgent ecological issue. *Environmental Pollution*, 173, 157–167. <https://doi.org/10.1016/j.envpol.2012.10.011>
- Chang, J. C. H., Wu, S. M., Tseng, Y. C., Lee, Y. C., Baba, O., & Hwang, P. P. (2007). Regulation of glycogen metabolism in gills and liver of the euryhaline tilapia (*Oreochromis mossambicus*) during acclimation to seawater. *Journal of Experimental Biology*, 210(19), 3494–3504. <https://doi.org/10.1242/jeb.007146>
- Chin, E. H., Love, O. P., Verspoor, J. J., Williams, T. D., Rowley, K., & Burness, G. (2009). Juveniles exposed to embryonic corticosterone have enhanced flight performance. *Proceedings of the Royal Society B: Biological Sciences*, 276(1656), 499–505. <https://doi.org/10.1098/rspb.2008.1294>
- Christensen, E. A. F., Illing, B., Iversen, N. S., Johansen, J. L., Domenici, P., & Steffensen, J. F. (2018). Effects of salinity on swimming performance and oxygen consumption rate of shiner perch *Cymatogaster aggregata*. *Journal of Experimental Marine Biology and Ecology*, 504, 32–37. <https://doi.org/10.1016/j.jembe.2018.04.002>
- Chung, M. H. J., Noble, D. W. A., Fox, R. J., Harrison, L. M., & Jennions, M. D. (2025). Fluctuating salinity during development impacts fish life histories.

Journal of Animal Ecology, 94(9), 1848–1865. <https://doi.org/10.1111/1365-2656.70095>

- Côté, I. M., Darling, E. S., & Brown, C. J. (2016). Interactions among ecosystem stressors and their importance in conservation. *Proceedings of the Royal Society B: Biological Sciences*, 283(1824), 20152592. <https://doi.org/10.1098/rspb.2015.2592>
- Crino, O. L., Klaassen van Oorschot, B., Crandell, K. E., Breuner, C. W., & Tobalske, B. W. (2017). Flight performance in the altricial zebra finch: Developmental effects and reproductive consequences. *Ecology and Evolution*, 7(7), 2316–2326. <https://doi.org/10.1002/ece3.2775>
- Criscuolo, F., Monaghan, P., Nasir, L., & Metcalfe, N. B. (2008). Early nutrition and phenotypic development: 'Catch-up' growth leads to elevated metabolic rate in adulthood. *Proceedings of the Royal Society B: Biological Sciences*, 275(1642), 1565–1570. <https://doi.org/10.1098/rspb.2008.0148>
- Cruz Vieira, A. B., Weber, A. A., Ribeiro, Y. M., Luz, R. K., Bazzoli, N., & Rizzo, E. (2019). Influence of salinity on spermatogenesis in adult Nile tilapia (*Oreochromis niloticus*) testis. *Theriogenology*, 131, 1–8. <https://doi.org/10.1016/j.theriogenology.2019.03.013>
- Denver, R. J., Mirhadi, N., & Phillips, M. (1998). Adaptive plasticity in amphibian metamorphosis: Response of *Scaphiopus hammondi* tadpoles to habitat desiccation. *Ecology*, 79(6), 1859–1872. [https://doi.org/10.1890/0012-9658\(1998\)079\[1859:APIAMR\]2.0.CO;2](https://doi.org/10.1890/0012-9658(1998)079[1859:APIAMR]2.0.CO;2)
- Dougherty, L. R., Frost, F., Maenpaa, M. I., Rowe, M., Cole, B. J., Vasudeva, R., Pottier, P., Schultner, E., Macartney, E. L., Lindenbaum, I., Smith, J. L., Carazo, P.,

Graziano, M., Weaving, H., Canal Domenech, B., Berger, D., Meena, A., Bishop, T. R., Noble, D. W. A., ... Price, T. A. R. (2024). A systematic map of studies testing the relationship between temperature and animal reproduction. *Ecological Solutions and Evidence*, 5(1), e12303. <https://doi.org/10.1002/2688-8319.12303>

Edward, D. A., & Gilburn, A. S. (2007). The effect of habitat composition on sexual conflict in the seaweed flies *Coelopa frigida* and *C. pilipes*. *Animal Behaviour*, 74, 343–348. <https://doi.org/10.1016/j.anbehav.2006.07.023>

Farrell, T. M., Neuert, M. A. C., Cui, A., & MacDougall-Shackleton, S. A. (2015). Developmental stress impairs a female songbird's behavioural and neural response to a sexually selected signal. *Animal Behaviour*, 102, 157–167. <https://doi.org/10.1016/j.anbehav.2015.01.018>

Flowers, W. (2015). Factors affecting the efficient production of boar sperm. *Reproduction in Domestic Animals*, 50(S2), 25–30. <https://doi.org/10.1111/rda.12529>

Forster, S. J. (1998). Osmotic stress tolerance and osmoregulation of intertidal and subtidal nematodes. *Journal of Experimental Marine Biology and Ecology*, 224(1), 109–125. [https://doi.org/10.1016/S0022-0981\(97\)00192-5](https://doi.org/10.1016/S0022-0981(97)00192-5)

Grether, G. F., Millie, D. F., Bryant, M. J., Reznick, D. N., & Mayea, W. (2001). Rain forest canopy cover, resource availability, and life history evolution in guppies. *Ecology*, 82(6), 1546–1559. [https://doi.org/10.1890/0012-9658\(2001\)082\[1546:RFCCRA\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2001)082[1546:RFCCRA]2.0.CO;2)

- Grizzetti, B., Pistocchi, A., Liqueste, C., Udias, A., Bouraoui, F., & van de Bund, W. (2017). Human pressures and ecological status of European rivers. *Scientific Reports*, 7(1), 205. <https://doi.org/10.1038/s41598-017-00324-3>
- Haddy, J. A., & Pankhurst, N. W. (2000). The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri*. *Aquaculture*, 188(1), 115–131. [https://doi.org/10.1016/S0044-8486\(00\)00326-4](https://doi.org/10.1016/S0044-8486(00)00326-4)
- Hopkins, G. R., French, S. S., & Brodie, E. D., Jr. (2017). Interacting stressors and the potential for adaptation in a changing world: Responses of populations and individuals. *Royal Society Open Science*, 4(6), 161057. <https://doi.org/10.1098/rsos.161057>
- Iglesias-Carrasco, M., Harrison, L., Jennions, M. D., & Head, M. L. (2020). Combined effects of rearing and testing temperatures on sperm traits. *Journal of Evolutionary Biology*, 33(12), 1715–1724. <https://doi.org/10.1111/jeb.13710>
- IPCC. (2014). *Climate change 2014: Synthesis report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. IPCC.
- Karim, M. F., & Mimura, N. (2008). Impacts of climate change and sea-level rise on cyclonic storm surge floods in Bangladesh. *Global Environmental Change*, 18(3), 490–500. <https://doi.org/10.1016/j.gloenvcha.2008.05.002>
- Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M., & Gattuso, J. P. (2013). Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Global Change Biology*, 19(6), 1884–1896. <https://doi.org/10.1111/gcb.12179>

- Likongwe, J. S., Stecko, T. D., Stauffer, J. R., & Carline, R. F. (1996). Combined effects of water temperature and salinity on growth and feed utilization of juvenile Nile tilapia *Oreochromis niloticus* (Linnaeus). *Aquaculture*, 146(1), 37–46.
[https://doi.org/10.1016/S0044-8486\(96\)01360-9](https://doi.org/10.1016/S0044-8486(96)01360-9)
- Mateos, J., Mañanos, E., Carrillo, M., & Zanuy, S. (2002). Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 132(1), 75–86.
[https://doi.org/10.1016/S1096-4959\(01\)00535-8](https://doi.org/10.1016/S1096-4959(01)00535-8)
- Meffe, G. K. (1991). Life history changes in eastern mosquitofish (*Gambusia holbrooki*) induced by thermal elevation. *Canadian Journal of Fisheries and Aquatic Sciences*, 48(1), 60–66. <https://doi.org/10.1139/f91-009>
- Muniz, I. P. (1990). Freshwater acidification: Its effects on species and communities of freshwater microbes, plants and animals. *Proceedings of the Royal Society of Edinburgh, Section B: Biological Sciences*, 97, 227–254.
<https://doi.org/10.1017/S0269727000005364>
- Naddafi, R., Abdoli, A., Hassanzadeh Kiabi, B., Mojazi Amiri, B., & Karami, M. (2005). Age, growth and reproduction of the Caspian roach (*Rutilus rutilus caspicus*) in the Anzali and Gomishan wetlands, North Iran. *Journal of Applied Ichthyology*, 21(6), 492–497. <https://doi.org/10.1111/j.1439-0426.2005.00669.x>
- Naguib, M., Nemitz, A., & Gil, D. (2006). Maternal developmental stress reduces reproductive success of female offspring in zebra finches. *Proceedings of the Royal Society B: Biological Sciences*, 273(1596), 1901–1905.
<https://doi.org/10.1098/rspb.2006.3526>

- Nielsen, D. L., & Brock, M. A. (2009). Modified water regime and salinity as a consequence of climate change: Prospects for wetlands of Southern Australia. *Climatic Change*, 95(3), 523–533. <https://doi.org/10.1007/s10584-009-9564-8>
- Nissling, A., & Larsson, R. (2018). Population specific sperm production in European flounder *Platichthys flesus*: Adaptation to salinity at spawning. *Journal of Fish Biology*, 93(1), 47–52. <https://doi.org/10.1111/jfb.13667>
- Nordlie, F. G., & Mirandi, A. (1996). Salinity relationships in a freshwater population of eastern mosquitofish. *Journal of Fish Biology*, 49(6), 1226–1232. <https://doi.org/10.1111/j.1095-8649.1996.tb01791.x>
- Nowicki, S., Searcy, W., & Peters, S. (2002). Brain development, song learning and mate choice in birds: A review and experimental test of the "nutritional stress hypothesis." *Journal of Comparative Physiology A*, 188(11), 1003–1014. <https://doi.org/10.1007/s00359-002-0361-3>
- O'Dea, R. E., Jennions, M. D., & Head, M. L. (2014). Male body size and condition affects sperm number and production rates in mosquitofish, *Gambusia holbrooki*. *Journal of Evolutionary Biology*, 27, 2739–2744. <https://doi.org/10.1111/jeb.12534>
- Panfili, J., Durand, J. D., Mbow, A., Guinand, B., Diop, K., Kantoussan, J., Thior, D., Thiaw, O. T., Albaret, J.-J., & Laë, R. (2004). Influence of salinity on life history traits of the bonga shad *Ethmalosa fimbriata* (Pisces, Clupeidae): Comparison between the Gambia and Saloum estuaries. *Marine Ecology Progress Series*, 270, 241–257. <https://doi.org/10.3354/meps270241>
- Panfili, J., Mbow, A., Durand, J. D., Diop, K., Diouf, K., Thior, D., Ndiaye, P., & Laë, R. (2004). Influence of salinity on the life-history traits of the West African black-chinned tilapia (*Sarotherodon melanotheron*): Comparison between the

Gambia and Saloum estuaries. *Aquatic Living Resources*, 17(1), 65–74.

<https://doi.org/10.1051/alr:2004002>

Parrish, J. J., Willenburg, K. L., Gibbs, K. M., Yagoda, K. B., Krautkramer, M. M.,

Loether, T. M., & Melo, F. C. S. A. (2017). Scrotal insulation and sperm production in the boar. *Molecular Reproduction and Development*, 84(9), 969–978. <https://doi.org/10.1002/mrd.22841>

Pavlovska-Teglia, G., Stodulski, G., Svendsen, L., Dalton, K., & Hau, J. (1995). Effect of oral corticosterone administration on locomotor development of neonatal and juvenile rats. *Experimental Physiology*, 80(3), 469–475.

<https://doi.org/10.1113/expphysiol.1995.sp003861>

Peña, F. J., Ortiz-Rodríguez, J. M., Gaitskell-Phillips, G. L., Gil, M. C., Ortega-

Ferrusola, C., & Martín-Cano, F. E. (2022). An integrated overview on the regulation of sperm metabolism (glycolysis–Krebs cycle–oxidative phosphorylation). *Animal Reproduction Science*, 246, 106805.

<https://doi.org/10.1016/j.anireprosci.2021.106805>

Pino Martinez, E., Braanaas, M. F., Balseiro, P., Kraugerud, M., Pedrosa, C., Imsland,

A. K. D., & Handeland, S. O. (2022). Constant high temperature promotes early changes in testis development associated with sexual maturation in male Atlantic salmon (*Salmo salar* L.) post-smolts. *Fishes*, 7(6), 341.

<https://doi.org/10.3390/fishes7060341>

Pyke, G. H. (2005). A review of the biology of *Gambusia affinis* and *G. holbrooki*.

Reviews in Fish Biology and Fisheries, 15(4), 339–365.

<https://doi.org/10.1007/s11160-006-6394-x>

- Pyke, G. H. (2008). Plague minnow or mosquito fish? A review of the biology and impacts of introduced *Gambusia* species. *Annual Review of Ecology, Evolution, and Systematics*, 39, 171–191.
<https://doi.org/10.1146/annurev.ecolsys.39.110707.173451>
- Reinhardt, K., Dobler, R., & Abbott, J. (2015). An ecology of sperm: Sperm diversification by natural selection. *Annual Review of Ecology, Evolution, and Systematics*, 46(1), 435–459. <https://doi.org/10.1146/annurev-ecolsys-120213-091611>
- Reznick, D., Butler, M. J., IV, & Rodd, H. (2001). Life-history evolution in guppies. VII. The comparative ecology of high- and low-predation environments. *The American Naturalist*, 157(2), 126–140. <https://doi.org/10.1086/318627>
- Rinehart, J. P., Yocum, G. D., & Denlinger, D. L. (2000). Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology*, 25(4), 330–336. <https://doi.org/10.1111/j.1365-3032.2000.00201.x>
- Royle, N. J., Lindström, J., & Metcalfe, N. B. (2005). A poor start in life negatively affects dominance status in adulthood independent of body size in green swordtails *Xiphophorus helleri*. *Proceedings of the Royal Society B: Biological Sciences*, 272(1575), 1917–1922. <https://doi.org/10.1098/rspb.2005.3190>
- Schäfer, R. B., & Piggott, J. J. (2018). Advancing understanding and prediction in multiple stressor research through a mechanistic basis for null models. *Global Change Biology*, 24(5), 1817–1826. <https://doi.org/10.1111/gcb.14073>

- Servili, A., Canario, A. V. M., Mouchel, O., & Muñoz-Cueto, J. A. (2020). Climate change impacts on fish reproduction are mediated at multiple levels of the brain-pituitary-gonad axis. *General and Comparative Endocrinology*, 291, 113439. <https://doi.org/10.1016/j.ygcen.2020.113439>
- Sharma, M. (2019). Behavioural responses in effect to chemical stress in fish: A review. *International Journal of Fisheries and Aquatic Studies*, 7(1), 1–5.
- Smith, V. H. (2003). Eutrophication of freshwater and coastal marine ecosystems: A global problem. *Environmental Science and Pollution Research*, 10(2), 126–139. <https://doi.org/10.1065/espr2002.12.142>
- Smith, V. H., & Schindler, D. W. (2009). Eutrophication science: Where do we go from here? *Trends in Ecology & Evolution*, 24(4), 201–207. <https://doi.org/10.1016/j.tree.2008.11.009>
- Spencer, K. A., & Verhulst, S. (2007). Delayed behavioral effects of postnatal exposure to corticosterone in the zebra finch (*Taeniopygia guttata*). *Hormones and Behavior*, 51(2), 273–280. <https://doi.org/10.1016/j.yhbeh.2006.11.001>
- Stearns, S. C. (1992). *The evolution of life histories*. Oxford University Press.
- Torres-Rodríguez, M., Martínez-Rodríguez, G., Rodríguez-Viera, L., Mancera, J. M., & Martos-Sitcha, J. A. (2025). Physiological effects of water salinity on metabolism and fatty acid biosynthesis in the model fish *Fundulus heteroclitus*. *Animals*, 15(17), 2549. <https://doi.org/10.3390/ani15172549>
- Trexler, J. C., & Travis, J. (1990). Phenotypic plasticity in the sailfin molly, *Poecilia latipinna* (Pisces: Poeciliidae). I. Field experiments. *Evolution*, 44(1), 143–156. <https://doi.org/10.1111/j.1558-5646.1990.tb04285.x>

Vallée, M., Mayo, W., Dellu, F., Le Moal, M., Simon, H., & Maccari, S. (1997).

Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: Correlation with stress-induced corticosterone secretion.

Journal of Neuroscience, 17(7), 2626–2636.

<https://doi.org/10.1523/JNEUROSCI.17-07-02626.1997>

Van de Pol, M., Ens, B. J., Heg, D., Brouwer, L., Krol, J., Maier, M., Exo, K.-M.,

Oosterbeek, K., Lok, T., Eising, C. M., & Koffijberg, K. (2010). Do changes in the frequency, magnitude and timing of extreme climatic events threaten the population viability of coastal birds? *Journal of Applied Ecology*, 47(4), 720–

730. <https://doi.org/10.1111/j.1365-2664.2010.01842.x>

Varlinskaya, E. I., & Spear, L. P. (2008). Social interactions in adolescent and adult

Sprague–Dawley rats: Impact of social deprivation and test context familiarity.

Behavioural Brain Research, 188(2), 398–405.

<https://doi.org/10.1016/j.bbr.2007.11.024>

Vondracek, B., Wurtsbaugh, W. A., & Cech, J. J. (1988). Growth and reproduction of

the mosquitofish, *Gambusia affinis*, in relation to temperature and ration level: Consequences for life history. *Environmental Biology of Fishes*, 21(1), 45–57.

<https://doi.org/10.1007/BF02984442>

Walker, N. D. (2001). Tropical storm and hurricane wind effects on water level, salinity, and sediment transport in the river-influenced Atchafalaya-Vermilion Bay

system, Louisiana, USA. *Estuaries*, 24(4), 498–508.

<https://doi.org/10.2307/1353252>

Xu, W., Soyano, K., & Manabe, S. (2024). High water temperature triggers early sexual maturation in the juvenile red spotted grouper *Epinephelus akaara* via regulation of reproduction-related hormones in the brain-pituitary-gonadal axis. *Animal*

Reproduction Science, 268, 107546.

<https://doi.org/10.1016/j.anireprosci.2024.107546>

Yeganeh, S., Amiri, B. M., & Alavi, S. M. H. (2008). Motility of *Mugil cephalus* L. spermatozoa in coelomic fluid, seminal fluid and saline media. *Journal of Applied Ichthyology*, 24(4), 517–518. <https://doi.org/10.1111/j.1439-0426.2008.01143.x>

Yoneda, M., & Wright, P. J. (2005). Effect of temperature and food availability on reproductive investment of first-time spawning male Atlantic cod, *Gadus morhua*. *ICES Journal of Marine Science*, 62(7), 1387–1393. <https://doi.org/10.1016/j.icesjms.2005.04.018>

Zahangir, M. M., Rahman, M. L., & Ando, H. (2022). Anomalous temperature interdicts the reproductive activity in fish: Neuroendocrine mechanisms of reproductive function in response to water temperature. *Frontiers in Physiology*, 13, 902257. <https://doi.org/10.3389/fphys.2022.902257>

Zhou, L., Liu, K., Zhao, Y., Cui, L., Dong, C., & Wang, Z. (2022). Increasing salinization of freshwater limits invasiveness of a live-bearing fish: Insights from behavioral and life-history traits. *Environmental Pollution*, 308, 119658. <https://doi.org/10.1016/j.envpol.2022.119658>

Chapter 6: General Conclusion

“Nothing in life is to be feared; it is only to be understood”- **Marie Curie**

This thesis was designed to investigate costs related to sexual conflict and how environmental variation affects the reproductive strategies of the Eastern mosquitofish (*Gambusia holbrooki*). This species is known for its intense level of sexual conflict and highly adaptable nature. To investigate these costs and environmental effects, I formulated four data chapters. In chapters 2 and 3, I looked at the effects of the social environment, while in chapters 4 and 5, I looked at the effects of the physical environment. In chapter 2, I looked at sexual conflict from a female's perspective by disentangling the cost of sexual harassment by males and the possible benefits (or costs) of multiple mating. While in chapter 3, I looked at sexual conflict from a male's perspective and quantified the cost of fighting with rivals and the cost of sperm production. In chapter 4, I determined the effect of turbidity on the level of male sexual harassment and how the direct effects of turbidity and/or any indirect effects mediated by changes in the level of sexual harassment affect the physiological stress of females. Finally, in chapter 5, I examined how two environmental stressors – temperature and salinity - individually or together shape the expression of male reproductive traits. Together all these findings reveal that sexual conflict in mosquitofish creates measurable costs for both sexes, and that sexual conflict is context dependent.

In a polyandrous situation, females face constant sexual harassment as multiple males try to mate. In that scenario, it is hard to measure the cost of harassment independent of the effects of mating multiply. In my chapter 2, I successfully differentiated the cost of sexual harassment from the costs/benefits of multiple mating by ablating (physically cutting) the gonopodia of males. Through this experimental approach I successfully managed to control the rate of mating while keeping the harassment level same. This chapter shows

that persistent male harassment is bad for the female as it reduces their growth rate and makes them produce fewer eggs, but when that harassment is accompanied by multiple mating, the adverse effect of harassment can be partly mitigated. This finding provides a reasonable explanation why female mosquitofish or other species evolve resistance strategies to avoid male harassment.

To maximize reproductive success a male needs to invest energy in both fighting with his rivals and producing sperm. Both fighting and sperm production are likely to be energetically costly for a male. In chapter 3, I differentiated the cost of male-male fighting from the cost of continuous sperm production. My results showed that fighting with rivals is less expensive than continuous sperm production. If an organism repeatedly ejaculates and produces sperm, then its reproductive capability may eventually decline as the resources and machinery to produce sperm are limited, which creates a trade-off between continuous sperm production and maintaining long term fertility.

Aquatic animals who rely on vision for most of their activities are very sensitive to the clarity of water. In chapter 4, I showed that turbid water, which significantly reduces visibility, probably makes females more visible to males and females were therefore less likely to avoid costly male encounters compared to the rate of encounter in clear water. My findings also indicated that an environmental factor, specifically water clarity, significantly influences female physiological stress, while there was no detectable effect of male harassment on female physiological stress. This result challenges our conventional assumption that sexual harassment imposes high physiological costs on females.

Temperature and salinity are two major environmental factors that influence the reproductive performance of male fish. In chapter 5, I showed that developmental stress due to increase temperature and salinity negatively affects the performance of adult

males and that when there are multiple stressors then the effect is exacerbated. My results also show that to maintain fertilization success organisms seem to show some adaptive strategies. For example, when there is higher salinity the males produced fewer sperm, but they also increased the swimming speed of the sperm, which should ensure a higher rate of fertilization success. This chapter also shows that male mosquitofish can tolerate a wide range of temperatures as there were only a few significant differences in life history traits observed between the two experimental temperatures. Altogether, the findings from this chapter indicate that salinity is more stressful than temperature for mosquitofish. However, when mosquitofish face both a high temperature and higher salinity together then the situation becomes far worse. Although I did not explicitly look at how temperature and salinity combine to affect sexual conflict, it seems likely the observed changes in male resource allocation will affect how males interact with females.

Taken together my findings convey a broader message that sexual conflict is context-dependent which means that it needs to be studied in different ecological settings. My thesis highlights the methodological challenges involved in distinguishing the cost of sexual harassment from those of multiple mating; as well as the challenge of separating the cost of male-male fighting from the cost of continuous sperm production. The approach I used to successfully disentangle these costs provides a useful template for future researchers who want to investigate the cost of these activities in other species. My thesis shows that the level of sexual conflict could be altered through habitat modification. By looking at the effect of temperature, salinity and turbidity, it is clear that environmental factors can strongly influence the intensity of sexual conflict. Mosquitofish are an invasive species in Australia, so understanding how they will react under different environmental conditions may contribute to improved management strategies.

Finally, my thesis opens several promising directions for future research. Given that sexual conflict is highly context dependent, future studies can investigate how sexual conflict might be influenced by diet or food availability and photoperiod (i.e. low light conditions are equivalent to greater water turbidity). Finding no negative effect of turbidity on female stress level also opens another research direction, which is to check the stress level in male as well. Future work could also be done to test whether turbidity and varying level of harassment influence the physiological stress of males. Negative effect of salinity and high temperature on male mosquitofish suggest that checking the responses of female mosquitofish under salinity and high temperature would also be worthwhile so that we can test for sex differences in the effect of these factors on fitness and, by extension, the likely effects on sexual conflict. Bringing these research questions will provide a more comprehensive understanding about the evolutionary consequences of sexual conflict in mosquitofish under different environmental conditions.

Appendix A

Reduced water turbidity rather than male harassment elevates female physiological stress in a fish with intense sexual conflict (Chapter 4)

Fatema Akhter, Ondi L Crino, Michael D Jennions, Megan L Head, Meng-Han Joseph Chung

Submitted to Proceeding of the Royal Society B (under review)

Abstract

There is sexual conflict over mating because additional matings typically increase the reproductive success of males, but less so that of females. Consequently, males often aggressively pursue females and attempt to mate, while females evade these attempts. Male harassment is assumed to be stressful for females. The level of stress likely depends on the harassment rate and how females respond; and both factors might vary across environments. In mosquitofish (*Gambusia holbrooki*), males persistently attempt forced matings. We tested how increased sexual harassment (three vs one male) affected female cortisol ('stress hormone') levels in either clear or turbid water. We predicted that turbidity would affect cortisol levels because mosquitofish rely heavily on vision: males to locate females, and females to evade males. Females in turbid water experienced more sexual harassment from males compared to females in clear water. However, more sexual harassment did not increase female cortisol levels. Instead, cortisol levels were *lower* in turbid water, regardless of the number of males. We conclude that sexual harassment does not increase physiological stress in females despite intense sexual conflict in this species. Instead, females had higher cortisol levels in clear than turbid water, perhaps because clear water increases their susceptibility to predators.

Keywords: Cortisol, glucocorticoids, reproductive costs, sexual coercion, sex ratio, sexual selection

Introduction

There is often sexual conflict over mating. Male reproductive success tends to increase with the number of matings or mates because this increases opportunities to sire offspring (reviewed in [1]). In contrast, one or two matings often provide sufficient sperm to fertilise a female's eggs, such that females in many species gain few or no benefits from additional mates or matings (reviewed in [2]; but see [3, 4]). The optimal number of matings is therefore lower for females than males, generating conflict over whether to mate. This sexual conflict has led to the evolution of male sexual harassment, which includes behaviours such as forced copulations, sexual coercion, and punishment of non-receptive females (reviews: [5, 6]).

Male harassment generates costs for females that can reduce their reproductive success and survival. For example, sexual harassment can increase physiological stress, reduce foraging efficiency, and increase predation risk due to the conspicuousness of harassing behaviours, all of which can reduce female fitness [6-8]. In polyandrous species where females tend to mate with multiple males, selection also favours male traits that lower the probability that a female remates, such as seminal toxic compounds in insects [9] and penile spines in mammals [10]. These sexually selected traits can injure females and reduce their lifelong reproductive success [11-14]. In response, there has been selection for female traits that reduce male-imposed costs, such as evasive manoeuvres [15], forming female-only groups to dilute harassment [16], enhanced immunity to repair body damage [17], and aggression toward males [18].

The degree of sexual conflict in a population or species varies based on the relative costs for males to pursue matings and females to evade harassment. In this way, environmental

conditions are likely to alter the degree of sexual conflict [19, 20]. First, environmental conditions can affect selection on survival that then moderates male-female interactions. For example, in environments with high predation risk, female guppies (*Poecilia reticulata*) have reduced defences against male harassment, whereas males exhibit more coercive behaviours, increasing the level of sexual conflict [21-23]. Second, environmental conditions can alter mate encounter rates. For example, increased spatial complexity or reduced visibility often lowers mate encounter rates [24], reducing the likelihood of females of being harassed [25]. Third, the environment can influence a female's physiological condition. For example, females in environments with low food availability may have fewer energetic resources to repair damage from unwanted matings [26], increasing the costs associated with coercive mating. As such, the environment may not only affect the rate of male harassment but also affect the capacity of females to cope with harassment.

For aquatic organisms, one environmental condition that might affect the cost of sexual conflict is water turbidity. Turbidity is caused by suspended particles, which scatter light and reduce visibility [27-30]. Changes in turbidity can influence foraging efficiency and predation pressure, which then influence physiological stress and body condition [31-34]. For fish that heavily rely on visual information to mate, greater turbidity can also alter mating interactions, such as courtship [35], sneak copulations [36], and mate choice [37, 38]. As a result, water turbidity may affect the cost of sexual conflict by altering the level of male sexual harassment and/or female physiological condition and tolerance of harassment. Currently, turbidity is increasing in waterways around the world because of deforestation, agricultural runoff, urbanization, and extreme weather events [39-41]. However, little is known about how water turbidity affects sexual conflict. Given that sexual conflict results in costs to females (such as reduced fecundity), understanding how

environmental factors (such as turbidity) affect sexual conflict is critical to understand the population resilience of animals in human-altered environments.

Here we tested the effects of water turbidity on the level of sexual harassment and physiological stress experienced by female mosquitofish, *Gambusia holbrooki*. Sexual conflict over mating is intense in *G. holbrooki*. Males constantly chase females and attempt to mate, while females either attack or evade males [16, 18]. Sexual conflict often imposes costs to female mosquitofish and closely related species, such as reduced foraging efficiency, lower reproductive output, and increased physiological stress (i.e., cortisol) [42, 43]. Although sexual conflict has been widely studied, evidence for environment-dependent sexual conflict primarily comes from observational studies that compare behavioural differences in male-female interactions among populations or species [44, 45]. Therefore, it remains unclear if the observed links between environmental conditions and the degree of sexual conflict are causal or correlational, and how environment-dependent harassment levels affect traits linked to female fitness.

We conducted a 2×2 experiment that altered levels of sexual harassment and water turbidity. We aimed to (1) test the effects of turbidity on the rate of male sexual harassment, (2) evaluate the independent effects of male sexual harassment and water turbidity on female cortisol levels, and (3) test for a potential interaction between male sexual harassment and water turbidity that affects female cortisol levels. Cortisol is a glucocorticoid hormone that regulates behaviours and energy availability to enable vertebrates to cope with stressors and disturbances [46-48]. However, cortisol can also inhibit reproduction by suppressing the production of sex steroids and reduce body condition and immune function [49]. For these reasons, water turbidity could impose costs to females through changes in cortisol resulting directly from environmental conditions, indirectly through changes in male harassment, or from an interaction between these direct and indirect effects.

We tested three predictions. First, sexual harassment decreases with greater water turbidity because lower visibility reduces the ability of males to approach females and allows females to evade males more easily. Second, females exposed to higher levels of sexual harassment have elevated physiological stress (cortisol levels). Third, the number of males and water turbidity interact to affect sexual harassment, hence the physiological stress of females; that is, harassment levels are disproportionately high in clear water when three males are present, and females in this environment have disproportionately high cortisol levels. It is well documented that environmental changes shape physiology and mating interaction in many vertebrate [50-52]. However, experiments that test for environment-driven differences in sexual conflict are rare which limit our understanding of mating costs in vertebrates. These costs may generate variation in fecundity and net female productivity [53, 54], ultimately affecting population viability in changing environments.

Materials and Method

Origin and maintenance of animals

We captured juvenile mosquitofish from a stream (35°180'27" S, 149°070'27.9" E) in Canberra, Australia. These juveniles were kept in 90-L aquaria (max 50 fish/aquarium) and checked twice weekly to determine their sex: males were identified by an elongated anal fin (called 'gonopodium'), and females by eggs being visible through the body wall. Immature fish were then transferred to single sex 90L aquaria (max 50 fish/aquarium) to ensure their virginity. All fish were housed at 26°C (±1 °C) on a 14:10 h light: dark cycle and fed commercial fish flakes and *Artemia* brine shrimp twice daily.

Experimental design

We conducted a 2×2 factorial experiment by manipulating water turbidity and the number of harassing males. There were four females per experimental block (N = 43

blocks). Female were randomly allocated to each of the four treatments: one male/clear water; one male/turbid water; three males/clear water; three males/turbid water. To generate turbid water (~50 NTU, measured using a turbidity tube), we added 70 mg of algal (*Spirulina*) paste (algal dry weight = 23.8mg) to 1L of conditioned (Seachem, Madison, USA) tap water. For the clear water treatment, we used conditioned tap water (turbidity < 5NTU). The water was briefly stirred every 5-6 hours during the light cycle to keep the algae in suspension.

Ablation surgery

Female responsiveness to mating and hormone regulation are often affected by pregnancy [55, 56]. We therefore quantified cortisol levels in virgin females. To ensure virginity, we ablated males to prevent them from inseminating females. We anaesthetised each male in an Aquí-S solution (1µl/10mL water) for 30 seconds and then carefully removed the tip of his gonopodium using a scalpel. These ‘ablated’ males show normal harassment behaviour but cannot inseminate females [57, 58]. Males were returned to their stock tanks for at least three days before the start of the experiment to ensure they had fully recovery from the minor surgery [59, 60].

Manipulating levels of sexual harassment

On the first day of the experimental treatment, we placed a female and males (one or three) in a 37×26×12 cm tank with a water depth of 7.5 cm (Fig S1). The female was placed in a plastic cylinder in the centre of the tank, and the males were placed outside the cylinder. After 10 minutes, we removed the cylinder and thereafter the fish could interact freely. We recorded the fishes’ behaviour with a digital camera (Model: NCDOTIR21-SCAT500MA) positioned above the tank. We made a 15-minute recording on Day 1 shortly after the fish began interacting, and another recording on Day 2 one hour after the start of the light cycle. We measured: (a) mating attempts: the number of times

that males directed a gonopodial thrust at the female; and (b) time followed: the total time the female was followed by a male (<2 body lengths behind the female). Male following behaviour was readily visible, even in turbid water, because the water was shallow and fish mostly swam near the surface. In contrast, horizontal visibility (the distance over which fish could see each other) was low. We used the number of mating attempts received by a female and the time spent being followed as measures of sexual harassment.

Measuring whole body cortisol levels in females

After a female had been housed with males for 30 hours, she was moved to a smaller tank, euthanised with Aquí-S (0.05% v/v), and immediately stored at -80 °C for later analysis. All females were euthanised within four minutes of initial disturbance (mean= 202 seconds $\pm$ 37 SD). The time to euthanise females did not affect the cortisol levels once we accounted for the effects of water turbidity and male number (χ^2 = 0.043; P = 0.836; Supplementary Materials Table S1). We measured whole body cortisol levels using Arbor Assay Enzyme Immunoassay (EIA) kits (Cat. No. K003) with methods modified from Sarma et al [61]. Fish were first thawed on ice, patted dry using Kimwipes, and weighed ($\pm$ 0.1mg). Fish were then homogenized with an electric tissue homogenizer (LK Lab, HGD-10) in 2.2–4.4 mL of chilled assay buffer to obtain a tissue homogenate concentration of ~20mg/mL. We extracted cortisol from 1 mL aliquots of homogenate using a double wash of dichloromethane. For each wash, homogenates were combined with 4 mL of dichloromethane, vortexed briefly, and mixed on a test tube shaker for 20 minutes. We centrifuged homogenates (3000 rpm at 4°C for 3 minutes) to isolate the organic layer. We combined the organic layers from each wash and dried the samples under nitrogen gas at 35°C. The dehydrated samples were then stored at -20°C.

Extracted samples were reconstituted in 1 mL of assay buffer prior to conducting EIA assays. We assayed all samples and standards in duplicate across five microplates that

were read on a FLUOstar Omega microplate reader at 450nm. We calculated cortisol levels from a four parameter, seven-point curve ranging from 50–3,200 pg/mL. An external standard of 400 pg/mL was assayed on every plate. Intra- and inter-plate variation were 4.64% and 2.94%, respectively. We used mass-corrected cortisol levels (pg/mg) in our statistical analysis.

To determine the extraction efficiency, we stripped cortisol from samples of pooled tissue homogenate using a 1% Dextran coated charcoal solution (Sigma, Cat. No. C6241). We spiked aliquots of stripped homogenate with cortisol (320 pg/mL) and extracted cortisol from spiked and control aliquots (methods as above). We assayed a spiked and control sample on each EIA plate and compared the percentage of cortisol recovered from the spiked sample relative to the control sample to determine extraction efficiency [62, 63]. The average extraction efficiency was $91 \% \pm 11$ SD. We tested assay parallelism by serially diluting a pooled extracted sample to six dilutions from 1:4 to 1:128. The slopes of antibody binding relative to the cortisol concentrate of the serially diluted samples to the standard curve did not differ ($F = 0.236$, $P = 0.639$; Fig S2, Table S2).

Statistical analysis

We analysed the number of mating attempts, time followed and cortisol levels using separate models. For behavioural traits (mating attempts, time followed), we used generalized linear mixed models (GLMMs) with a quasi-Poisson error distribution. We treated turbidity (2 levels: clear or turbid), male density (2 levels: high or low) and day of testing (2 levels: day 1 or 2) as fixed factors. We treated Block ID (a set of four tanks) and Female ID as random factors to account for potential temporal effects related to when tanks were set up and repeated behavioural observations of each female (on days 1 and 2). We tested all possible two-way and three-way interactions among turbidity, male density and day of testing in initial models. Non-significant interactions were removed to

quantify the main effects of turbidity, male density and day of testing in the final models [64].

We ran a linear mixed model (LMM) for the analysis of cortisol level, as Q-Q plots indicated that model residuals fulfilled the model assumptions (normality, homoscedascity, and linearity). In the initial model, we treated turbidity, male density and their two-way interaction as fixed factors, and Block ID and microplate ID as random factors. The two-way interaction was not significant and was removed from the final model to allow interpretation of the main effects of turbidity and male density.

Exclusion of non-significant interactions did not significantly reduce the model fit in any of the analyses (see Supplementary Material). We used Wald chi-square tests to calculate *P* values (*Anova* function in the *car* package), with type III and type II sum of squares for models with and without interaction terms, respectively. We set alpha at 0.05 (two-tailed). Final sample sizes per treatment ranged from 38-43 due to missing video recordings for five of the 172 tanks.

Finally, to test if sexual harassment levels are directly linked to female stress response, we calculated Pearson correlations between female cortisol levels and harassment behaviors (i.e., mating attempts received, and time spent being followed) for each of the four treatment combinations on both Day 1 and 2.

Results

The amount of sexual harassment

A higher density of males increased the amount of sexual harassment experienced by females (Fig 1), which is consistent with past studies [65, 66]. Specifically, females experienced more mating attempts when there were three males rather than one male ($\chi^2 = 45.83$; $P < 0.001$). Additionally, females were followed by males for longer periods of

time when housed with three rather than one male ($\chi^2 = 66.38$; $P < 0.001$). Contrary to our first prediction, however, females experienced more mating attempts ($\chi^2 = 10.96$; $P < 0.001$) and spent more time being followed ($\chi^2 = 10.48$; $P < 0.001$) in turbid than clear water (Fig 1). There was also an effect of day of testing with females experiencing less harassment on Day 2 (number of mating attempts: $\chi^2 = 52.32$; $P < 0.001$; time followed: $\chi^2 = 64.85$; $P < 0.001$).

There were no significant three-way or two-way interactions between water turbidity, male density and the day of testing that affected the time a female spent being followed, or how many mating attempts she received (all $\chi^2 < 2.29$; $P > 0.130$; Tables S3, S4).

Female cortisol levels

Surprisingly, females in clear water had higher cortisol levels than those in turbid water ($\chi^2 = 39.55$; $P < 0.001$; Fig 2), even though they experienced less male harassment. In contrast, male density did not affect female cortisol levels ($\chi^2 = 0.001$; $P = 0.975$; Table S5), despite the increased male harassment when there were three males present (see above). Contrary to our third prediction, water turbidity and male density did not interact to affect female cortisol levels ($\chi^2 = 0.102$; $P = 0.750$).

Correlation between cortisol levels and the amount of sexual harassment

Our finding that cortisol levels did not align with experimentally induced changes in the level of harassment, suggests that greater male harassment does not increase physiological stress in females. To further test this conclusion, we analysed the correlation between cortisol levels and the level of harassment that females received in each of 8 treatment combinations (= 2 levels of turbidity $\times$ 2 levels of male density $\times$ 2 days of testing). In 15 of 16 tests, there was no correlation between the amount of harassment and the cortisol levels (all $|r| < 0.165$, all $P > 0.175$; Table S6). The only exception was for females in turbid water with one male on Day 1, where females that received more mating

attempts had lower cortisol levels ($r = -0.304$, $P = 0.047$). Overall, there is therefore no robust evidence that cortisol levels were affected by the level of sexual harassment.

Discussion

Sexual conflict is common because males tend to benefit from additional matings whereas females do not. Environmental conditions can affect the physiological costs of sexual harassment for females, but few studies have empirically evaluated this hypothesis. To test how water turbidity affects male-female mating interactions, and how any resultant changes in sexual harassment influence female physiological stress, we exposed female *G. holbrooki* to either one or three males in clear or turbid water. Females in turbid water were more often followed by males and subject to more mating attempts. Surprisingly, however, despite greater sexual harassment, females in turbid water had *lower* cortisol levels than those in clear water. In addition, within each treatment, female cortisol levels were not related to the rate of male harassment. Our findings show that an environmental factor (i.e., clear rather than turbid water) directly affects cortisol levels, whereas increased male harassment does not. Our result is extremely clearcut because the effect of water turbidity was almost identical in environments with either one male or three males (see Fig 2). Below we offer some potential reasons for our unexpected findings and discuss the implications.

Levels of sexual harassment increase with male density and water turbidity

Females housed with three males spent more time being followed by males and experienced more mating attempts than females housed with one male. This outcome is expected (and was part of the experimental rationale) but it was not inevitable: increased male-male competition might cause each male to spend less time harassing females if he spends more time fighting rivals [67-69]. This could decrease the total number of mating attempts received by females, even if there are more males present. That said, our findings

of greater harassment in the presence of more males align well with the general pattern that females experience more sexual harassment when the sex ratio is more male biased [65, 66].

Interestingly, there was no evidence that water turbidity interacted with male density to affect the amount of sexual harassment. This contrasts with our prediction and with results from studies in other taxa, in which manipulation of temperature, water availability, or habitat complexity interacted with male density to affect the amount of sexual harassment [70-74]. In our study, water turbidity alone altered the amount of sexual harassment, irrespective of male density. Males followed females for longer and made more mating attempts in turbid than clear water. In mosquitofish, males approach females from behind to get close enough to thrust their gonopodia into females' gonopores prior to detection (so-called 'sneaky mating'; [75]). Females often respond to the presence of a male behind them with a burst of speed, swimming rapidly forward [18]. We expected that females would more easily escape or avoid males in turbid water because the reduced visibility would impede the ability of males to locate and approach females. However, females may have been hesitant to swim forward rapidly to evade males in turbid water. This might occur if decreased visibility increases the perceived risk of swimming towards a predator. For example, female guppies (*P. reticulata*) were more likely to freeze in turbid water when detecting a predator [76]. Another explanation is that males increase their mating effort in turbid water to compensate for a reduced mate encounter rate and fewer mating opportunities. For example, male guppies spent less time with females in turbid water, so they made more sneak mating attempts when encountering a female [36]. Likewise, male three-spined stickleback (*Gasterosteus aculeatus*) courted more in turbid than clear water to attract female attention [77].

Female cortisol levels decline with greater water turbidity, irrespective of male density or the level of sexual harassment

Cortisol, which is often described as a primary hormone indicating stress, plays a critical role in reproduction in female fish [78]. Chronic stress and persistently high cortisol levels can suppress gonadotropin-releasing hormone which delays ovulation, reduces egg size, and decreases sexual activity [79, 80]. In addition to the direct impact of a high cortisol level on reproductive traits, it can also reduce body condition [81] and thereby lower fecundity [82]. Elevated cortisol levels in clear water are therefore likely to lower the reproductive success of females [83]. Our results indicate that sexual harassment is lower in clearer water, but this does not lower female cortisol levels. Instead, we found the opposite: turbid water decreased female cortisol levels, despite turbidity increasing the amount of male sexual harassment. The challenge is therefore to explain why greater turbidity decreases physiological stress, while sexual harassment has no effect.

In poeciliid fish, male harassment increases female vigilance to evade unwanted mating [84], reduces feeding time [8], and forces females to use sites with greater predation risk [85]. Additionally, females that experience male harassment respond more strongly to acute stressors (i.e., higher elevation of cortisol levels) than females that are not exposed to harassment [43]. Given this, we expected male harassment to elevate female cortisol levels. Why then was the stress response of female mosquitofish unaffected by how much harassment they received? One explanation is that we tested the cortisol level after 30 hours, so maybe initially female were stressed but cortisol levels dropped back to baseline (control) levels prior to being measured. A second explanation is that adaptive plasticity in cortisol production in response to varying levels of sexual harassment has not evolved in female mosquitofish. That is, females may not adjust their cortisol levels because it is rare for them not to be pursued by males [86]. If females can always anticipate (in the evolutionary sense) the presence of males attempting sneak copulation, there may be no

advantage to adjusting stress responses when the amount of harassment temporarily changes [87].

Why were cortisol levels lower in more turbid water? It is possible that females might perceive a greater risk of predation in clear water which increased cortisol secretion[47]. Indeed, female mosquitofish in the wild prefer sites with dense vegetation and lower visibility rather than open water, especially when predators are present [88]. If so, the cortisol response to water turbidity is likely to vary depending upon a species ecological niche (e.g., habitat use, trophic position). Consistent with this idea, studies on other species have revealed contrasting cortisol responses to greater water turbidity – a decrease in cortisol in *Hypomesus transpacificus* [89] but an increase in *Oncorhynchus kisutch* and *Salmo gairdneri* [90]. Another explanation might be that, although mosquitofish primarily consume small insects, they also consume detritus [91]. It is possible that females in the turbid water treatment consumed some of the algae that we used to increase turbidity. If algal consumption improved female body condition, this might have lowered the secretion of cortisol (e.g. [92]), as feeling satiated might make them feel less stressed.

Conclusion

It is widely assumed that male sexual harassment imposes stress on females that reduces female fitness. However, we found no evidence that male harassment increases cortisol levels in female mosquitofish. While this finding at first seems anomalous relative to that in other studies on sexual conflict [93], we note that the failure of more harassment to induce a stronger stress response does not preclude male harassment lowering female fitness by other means. Males could still impose costs when harassing females such as physical injury and increased energy expenditure. There might be no adaptive value for females to elevate cortisol if male sexual harassment is consistently high. Finally, we note that our study has practical application for animal husbandry. If clear water is more

stressful for some species, then general welfare guidelines to maintain fish in highly filtered 'clean' water might be counterproductive.

Supplementary material: Supplementary material has been uploaded as a separate file.

Data accessibility: Raw data and R code can be downloaded from Zenodo:

<https://doi.org/10.5281/zenodo.19211782>

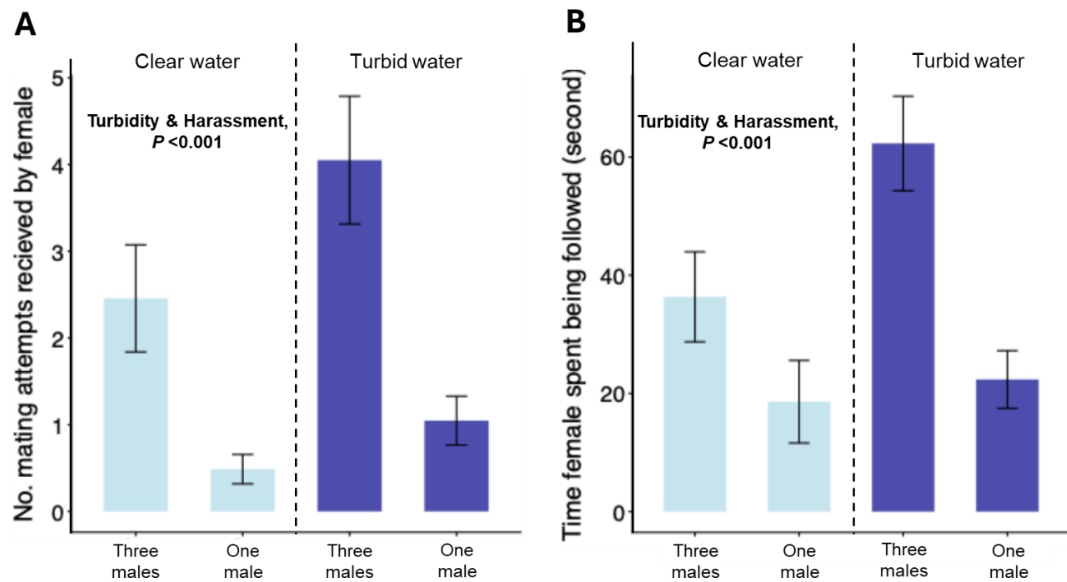


Figure 1. Effect of water turbidity and male density on the amount of sexual harassment received by females: (A) number of mating attempts and (B) time spent being followed. Data are presented as mean $\pm$ SE. The independent effect of water turbidity and the level of sexual harassment were both significant ($P < 0.001$).

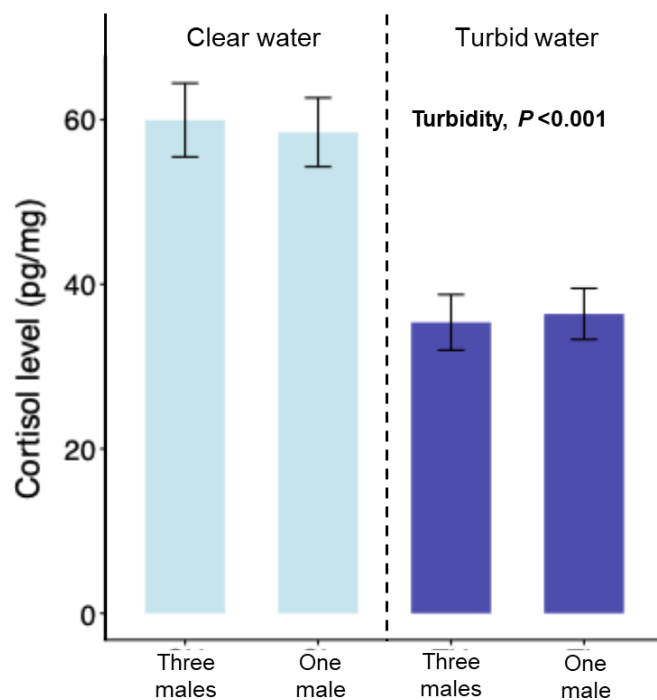


Figure 2. Effect of water turbidity and male density on the cortisol level of females. Data are presented as mean $\pm$ SE. The independent effect of water turbidity was significant ($P < 0.001$), irrespective of male density.

References

1. Janicke T., Häderer I.K., Lajeunesse M.J., Anthes N. 2016 Darwinian sex roles confirmed across the animal kingdom. *Sci Adv* **2**, e1500983.
2. Slatyer R.A., Mautz B.S., Backwell P.R.Y., Jennions M.D. 2012 Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. *Biol Rev* **87**, 1–33.
3. Fromonteil S., Marie-Orleach L., Winkler L., Janicke T. 2023 Sexual selection in females and the evolution of polyandry. *PLoS Biol* **21**, e3001916.
4. Kokko H., Jennions M.D. 2023 Is more always better when it comes to mating? *PLoS Biol* **21**.
5. Cluttonbrock T.H., Parker G.A. 1995 Sexual coercion in animal societies. *Anim Behav* **49**, 1345–1365.
6. Arnqvist G., Rowe L. 2005 *Sexual conflict*, Princeton University Press.
7. Colpitts J., Jarvis W.M.C., Agrawal A.F., Rundle H.D. 2022 Quantifying male harm and its divergence. *Evolution* **76**, 829–836.
8. Köhler A., Hildenbrand P., Schleucher E., Riesch R., Arias-Rodriguez L., Streit B., Plath M. 2011 Effects of male sexual harassment on female time budgets, feeding behavior, and metabolic rates in a tropical livebearing fish (*Poecilia mexicana*). *Behav Ecol Sociobiol* **65**, 1513–1523.
9. Lung O., Tram U., Finnerty C.M., Eipper-Mains M.A., Kalb J.M., Wolfner M.F. 2002 The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics* **160**, 211–224.
10. Stockley P. 2002 Sperm competition risk and male genital anatomy: comparative evidence for reduced duration of female sexual receptivity in primates with penile spines. *Evol Ecol* **16**, 123–137.

11. Johnstone R.A., Keller L. 2000 How males can gain by harming their mates: Sexual conflict, seminal toxins, and the cost of mating. *Am Nat* **156**, 368–377.
12. Smit N. 2025 Strategies, costs and counter-strategies to sexual coercion. *Biol Rev* **100**, 1557–1577.
13. Hotzy C., Arnqvist G. 2009 Sperm competition favors harmful males in seed beetles. *Curr Biol* **19**, 404–407.
14. Morrow E.H., Arnqvist G., Pitnick S. 2003 Adaptation versus pleiotropy: why do males harm their mates? *Behav Ecol* **14**, 802–806.
15. Brewster J., Houde A. 2003 Are female guppies more likely to flee when approached by two males? *J Fish Biol* **63**, 1056—1059.
16. Agrillo C., Dadda M., Bisazza A. 2006 Sexual harassment influences group choice in female mosquitofish. *Ethology* **112**, 592–598.
17. Dougherty L.R., van Lieshout E., McNamara K.B., Moschilla J.A., Arnqvist G., Simmons L.W. 2017 Sexual conflict and correlated evolution between male persistence and female resistance traits in the seed beetle. *Proc R Soc B* **284**, 20170132.
18. Condon C.H.L., Wilson R.S. 2006 Effect of thermal acclimation on female resistance to forced matings in the eastern mosquitofish. *Anim Behav* **72**, 585–593.
19. Perry J.C., Rowe L. 2018 Sexual conflict in its ecological setting. *Philos Trans R Soc B* **373**, 20170418.
20. Plesnar-Bielak A., Lukasiewicz A. 2021 Sexual conflict in a changing environment. *Biol Rev* **96**, 1854–1867.
21. Adler M.I., Bonduriansky R. 2014 Sexual conflict, life span, and aging. *Cold Spring Harb Perspect Biol* **6**, a017566.
22. Magurran A.E., Seghers B.H. 1994 Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proc R Soc B* **255**, 31–36.

23. Magurran A.E., Seghers B.H. 1994 A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proc R Soc B* **258**, 89–92.
24. Heuschele J., Salminen T., Candolin U. 2012 Habitat change influences mate search behaviour in three-spined sticklebacks. *Anim Behav* **83**, 1505–1510.
25. Osijo K., Agrawal A.F., Rundle H.D. 2026 On the expression of male harm in *Drosophila melanogaster*: impacts of density and structural complexity of the mating environment. *Evolution* **80**, 496–503.
26. Rostant W.G., Mason J.S., de Coriolis J.C., Chapman T. 2020 Resource-dependent evolution of female resistance responses to sexual conflict. *Evol Lett* **4**, 54–64.
27. Davies-Colley R.J., Smith D.G. 2001 Turbidity suspended sediment, and water clarity: a review. *JAWRA* **37**, 1085–1101.
28. Utne-Palm A.C. 2002 Visual feeding of fish in a turbid environment: Physical and behavioural aspects. *Mar and Freshw Behav Physiol* **35**, 111–128.
29. Zanghi C., Ioannou C.C. 2025 The impact of increasing turbidity on the predator-prey interactions of freshwater fishes. *Freshw Biol* **70**, e14354.
30. Sohel S., Lindström K. 2015 Algal turbidity reduces risk assessment ability of the three-spined stickleback. *Ethology* **121**, 548–555.
31. Clinchy M., Zanette L., Boonstra R., Wingfield J.C., Smith J.N. 2004 Balancing food and predator pressure induces chronic stress in songbirds. *Proc R Soc B* **271**, 2473–2479.
32. Monclús R., Palomares F., Tablado Z., Martínez-Fontúrbel A., Palme R. 2009 Testing the threat-sensitive predator avoidance hypothesis: physiological responses and predator pressure in wild rabbits. *Oecologia* **158**, 615–623.
33. Killen S.S., Marras S., Metcalfe N.B., McKenzie D.J., Domenici P. 2013 Environmental stressors alter relationships between physiology and behaviour. *Trends Ecol Evol* **28**, 651–658.

34. Clinchy M., Sheriff M.J., Zanette L.Y. 2013 Predator-induced stress and the ecology of fear. *Funct Ecol* **27**, 56–65.
35. Michelangeli M., Tuomainen U., Candolin U., Wong B.B.M. 2015 Habitat alteration influences male signalling effort in the Australian desert goby. *Behav Ecol* **26**, 1164–1169.
36. Ehlman S.M., Martinez D., Sih A. 2018 Male guppies compensate for lost time when mating in turbid water. *Behav Ecol Sociobiol* **72**, 1–9.
37. Venkatesan S., Chung M.H.J., Moura-Campos D., Head M.L. 2024 The effect of turbidity on female mate choice in the guppy, *Poecilia reticulata*. *Anim Behav* **213**, 107–115.
38. Sundin J., Berglund A., Rosenqvist G. 2010 Turbidity hampers mate choice in a pipefish. *Ethology* **116**, 713–721.
39. Mi H., Fagherazzi S., Qiao G., Hong Y., Fichot C.G. 2019 Climate change leads to a doubling of turbidity in a rapidly expanding Tibetan lake. *Sci Total Environ* **688**, 952–959.
40. Clermont M., Kinnard C., Dubé-Richard D., Campeau S., Bordeleau P.A., de Grandpre A., Ziyad J., Roy A. 2023 Using remote sensing to assess how intensive agriculture impacts the turbidity of a fluvial lake floodplain. *J Great Lakes Res* **49**, 102240.
41. Zhou Q., Wang J., Tian L., Feng L., Li J., Xing Q. 2021 Remotely sensed water turbidity dynamics and its potential driving factors in Wuhan, an urbanizing city of China. *J Hydrol* **593**, 125893.
42. Pilastro A., Benetton S., Bisazza A. 2003 Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Anim Behav* **65**, 1161–1167.

43. Thakur S., Adams E., Prah C., Vuong A., Ramsey M., Cummings M.E. 2025 Different social experiences drive the development of divergent stress coping styles in female swordtails (*Xiphophorus nigrensis*). *Horm Behav* **168**, 105675.
44. Gosden T.P., Svensson E.I. 2009 Density-dependent male mating harassment, female resistance, and male mimicry. *Am Nat* **173**, 709–721.
45. De Lisle S.P., Goedert D., Reedy A.M., Svensson E.I. 2018 Climatic factors and species range position predict sexually antagonistic selection across taxa. *Philos Trans R Soc B* **373**, 1757.
46. Mommsen T.P., Vijayan M.M., Moon T.W. 1999 Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fisher* **9**, 211–268.
47. Sapolsky R.M., Romero L.M., Munck A.U. 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* **21**, 55–89.
48. Wingfield J.C., Kitaysky A.S. 2002 Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? *Integr Comp Biol* **42**, 600–609.
49. Crino O.L., Jensen S.M., Buchanan K.L., Griffith S.C. 2018 Evidence for condition mediated trade-offs between the HPA-and HPG-axes in the wild zebra finch. *Gen Comp Endocr* **259**, 189–198.
50. Rahman T., Candolin U. 2022 Linking animal behavior to ecosystem change in disturbed environments. *Front Ecol Evol* **10**, 893453.
51. Lemonnier C., Bize P., Boonstra R., Dobson F.S., Criscuolo F., Viblanc V.A. 2022 Effects of the social environment on vertebrate fitness and health in nature: Moving beyond the stress axis. *Horm Behav* **145**, 105232.
52. Sydeman W.J., Poloczanska E., Reed T.E., Thompson S.A. 2015 Climate change and marine vertebrates. *Science* **350**, 772–777.

53. Arnqvist G., Tuda M. 2010 Sexual conflict and the gender load: correlated evolution between population fitness and sexual dimorphism in seed beetles. *Proc R Soc B* **277**, 1345–1352.
54. Berger D., Martinossi-Allibert I., Grieshop K., Lind M.I., Maklakov A.A., Arnqvist G. 2016 Intralocus sexual conflict and the tragedy of the commons in seed beetles. *Am Nat* **188**, 98–112.
55. Butler J.M., Whitlow S.M., Rogers L.S., Putland R.L., Mensinger A.F., Maruska K.P. 2019 Reproductive state-dependent plasticity in the visual system of an African cichlid fish. *Horm Behav* **114**, 104539.
56. Tanner J.C., Garbe L.M., Zuk M. 2019 When virginity matters: age and mating status affect female responsiveness in crickets. *Anim Behav* **147**, 83-90.
57. Chung M.H.J., Fox R.J., Jennions M.D. 2020 Fine-scale genital morphology affects male ejaculation success: an experimental test. *Biol Letters* **16**, 20200251.
58. Kwan L., Cheng Y.Y., Rodd F.H., Rowe L. 2013 Sexual conflict and the function of genitalic claws in guppies (*Poecilia reticulata*). *Biol Lett* **9**, 20130267.
59. Chung M.H.J., Jennions M.D., Fox R.J. 2021 Quantifying the costs of pre- and postcopulatory traits for males: evidence that costs of ejaculation are minor relative to mating effort. *Evol Lett* **5**, 315–327.
60. Chung M.H.J., Jennions M.D., Fox R.J. 2019 Novel ablation technique shows no sperm priming response by male eastern mosquitofish to cues of female availability. *Behav Ecol Sociobiol* **73**, 167.
61. Sarma R.R., Edwards R.J., Crino O.L., Eyck H.J.F., Waters P.D., Crossland M.R., Shine R., Rollins L.A. 2020 Do epigenetic changes drive corticosterone responses to alarm cues in larvae of an invasive amphibian? *Integr Comp Biol* **60**, 1481–1494.
62. Crino O.L., Wild K.H., Friesen C.R., Leibold D., Laven N., Peardon A.Y., Recio P., Salin K., Noble D.W.A. 2024 From eggs to adulthood: sustained effects of early

developmental temperature and corticosterone exposure on physiology and body size in an Australian lizard. *J Exp Biol* **227**, jeb249234.

63. Laven N.E., Pearson P.R., Wild K.H., Noble D.W.A., Crino O.L. 2025 Sex steroid profiles align with phenotype in sex-reversed female lizards. *Gen Comp Endocr* **369**, 114754.

64. Engqvist L. 2005 The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim Behav* **70**, 967–971.

65. Reale D., Bousses P., Chapuis J.L. 1996 Female-biased mortality induced by male sexual harassment in a feral sheep population. *Can J Zool* **74**, 1812–1818.

66. Løvlie H., Pizzari T. 2007 Sex in the morning or in the evening? Females adjust daily mating patterns to the intensity of sexual harassment. *Am Nat* **170**, 1–13.

67. Jirotkul M. 1999 Operational sex ratio influences female preference and male-male competition in guppies. *Anim Behav* **58**, 287–294.

68. Karatgi R., Fuller R.C. 2025 When rivals are absent: male aggression towards females in bluefin killifish. *Ecol Evol* **15**, e71048.

69. Smith C.C. 2007 Independent effects of male and female density on sexual harassment, female fitness, and male competition for mates in the western mosquitofish. *Behav Ecol Sociobiol* **61**, 1349–1358.

70. García-Roa R., Chirinos V., Carazo P. 2019 The ecology of sexual conflict: Temperature variation in the social environment can drastically modulate male harm to females. *Funct Ecol* **33**, 681–692.

71. Londoño-Nieto C., García-Roa R., Garcia-Co C., González P., Carazo P. 2023 Thermal phenotypic plasticity of pre-and post-copulatory male harm buffers sexual conflict in wild *Drosophila melanogaster*. *eLife* **12**, e84759.

72. Byrne P.G., Rice G.R., Rice W.R. 2008 Effect of a refuge from persistent male courtship in the *Drosophila* laboratory environment. *Am Zool* **48**, e1.

73. Vincent A., Head M.L., Iglesias-Carrasco M. 2020 Sexual conflict and the environment: teasing apart effects arising via males and females. *Anim Behav* **162**, 57–66.
74. García-Roa R., Garcia-Gonzalez F., Maroto V., Chirinos V., Márquez-Rosado A., Iglesias-Carrasco M., Carazo P. 2025 Temperature can reverse sexual conflict, facilitating population growth. *Evol Lett* **9**, 558–566.
75. Langerhans R.B. 2011 Genital evolution. In *Ecology and evolution of poeciliid fishes* (eds. Evans J.P., Pilastro A., Schlupp I.), pp. 228–240. Chicago, IL, University of Chicago Press.
76. Kimbell H.S., Morrell L.J. 2015 Turbidity influences individual and group level responses to predation in guppies, *Poecilia reticulata*. *Anim Behav* **103**, 179–185.
77. Engström-Öst J., Candolin U. 2007 Human-induced water turbidity alters selection on sexual displays in sticklebacks. *Behav Ecol* **18**, 393–398.
78. Faught E., Vijayan M.M. 2018 Maternal stress and fish reproduction: The role of cortisol revisited. *Fish Fish* **19**, 1016–1030.
79. Pankhurst N.W. 2016 Reproduction and development. In *Fish Physiology* (pp. 295–331), Academic Press.
80. Murugananthkumar R., Sudhakumari C.C. 2022 Understanding the impact of stress on teleostean reproduction. *Aqua Fish* **7**, 553–561.
81. Jerez-Cepa I., Gorissen M., Mancera J.M., Ruiz-Jarabo I. 2019 What can we learn from glucocorticoid administration in fish? Effects of cortisol and dexamethasone on intermediary metabolism of gilthead seabream (*Sparus aurata* L.). *Comp Biochem and Physiol A* **231**, 1–10.
82. Barneche D.R., Robertson D.R., White C.R., Marshall D.J. 2018 Fish reproductive-energy output increases disproportionately with body size. *Science* **360**, 642–644.

83. Lambert Y. 2008 Why should we closely monitor fecundity in marine fish populations? **41**, 93–106.
84. Killen S.S., Croft D.P., Salin K., Darden S.K. 2016 Male sexually coercive behaviour drives increased swimming efficiency in female guppies. *Funct Ecol* **30**, 576–583.
85. Darden S.K., Croft D.P. 2008 Male harassment drives females to alter habitat use and leads to segregation of the sexes. *Biol Lett* **4**, 449–451.
86. Wilson R.S. 2005 Temperature influences the coercive mating and swimming performance of male eastern mosquitofish. *Anim Behav* **70**, 1387–1394.
87. DeWitt T.J., Sih A., Wilson D.S. 1998 Costs and limits of phenotypic plasticity. *Trends Ecol Evol* **13**, 77–81.
88. Pyke G.H. 2005 A Review of the biology of *Gambusia affinis* and *G holbrooki*. *Rev Fish Biol Fisher* **15**, 339–365.
89. Pasparakis C., Lohroff T., Biefel F., Cocherell D.E., Carson E.W., Hung T.-C., Cannon R.E., Fangue N.A., Todgham A.E. 2023 Effects of turbidity, temperature and predation cue on the stress response of juvenile delta smelt. *Conserv Physiol* **11**, coad036.
90. Redding J.M., Schreck C.B., Everest F.H. 1987 Physiological effects on coho salmon and steelhead of exposure to suspended solids. *Trans Am Fish Soc* **116**, 737–744.
91. Blanco S., Romo S., Villena M.J. 2004 Experimental study on the diet of mosquitofish (*Gambusia holbrooki*) under different ecological conditions in a shallow lake. *Int Rev Hydrobiol* **89**, 250–262.
92. Klaren P.H., van Dalen S.C., Atsma W., Spanings F.T., Hendriks J., Flik G. 2013 Voluntary timing of food intake increases weight gain and reduces basal plasma cortisol levels in common carp (*Cyprinus carpio* L.). *Physiol Behav* **122**, 120–128.

93. Aspbury A.S., Grayson K.L., Fantaye S., Nichols I., Myers-Burton M., Ortiz-Mangual X., Gabor C.R. 2017 The association between male-biased sex ratio and indicators of stress in red-spotted newts. *Physiol Behav* **173**, 156–162.

Appendix B

Chapter 2: Supplementary material

Statistical outputs from models

Table S1. Rapid approaches to male

(a) Initial model including the interaction between treatment and treatment duration;
and treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	8.891 (1)	0.003
Week	0.028 (2)	0.986
Treatment	1.614 (2)	0.446
Initial body size	5.714 (1)	0.017
Treatment: Week	12.261 (4)	0.016
Treatment: Initial body size	5.847 (2)	0.054

Random effect	Variance	<i>SD</i>	Number of groups
Female ID	0.121	0.35	129
Block ID	0.135	0.37	45

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	8.689 (1)	0.003
Week	0.025 (2)	0.987
Treatment	1.357 (2)	0.507
Initial body size	4.584 (1)	0.032
Treatment: Week	12.390 (4)	0.015

Random effect	Variance	<i>SD</i>	Number of groups
Female ID	0.194	0.44	129
Block ID	0.141	0.38	45

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	15	1357.8	1415.8	-663.92	1327.8	5.587	0.061
Final model (b)	13	1359.4	1409.7	-666.71	1333.4		

Table S2. Number of times female rejected the males

(a) Initial model including the interaction between treatment and treatment duration; and treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	653.001 (1)	<0.001
Week	0.263 (2)	0.877
Treatment	55.943 (2)	<0.001
Initial body size	0.126 (1)	0.723
Treatment: Week	22.191 (4)	<0.001
Treatment: Initial body size	1.346 (2)	0.510

Random effect	Variance	SD	Number of groups
Female ID	0.030	0.17	129
Block ID	0.043	0.21	45

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	651.742 (1)	<0.001
Week	0.254 (2)	0.881
Treatment	55.3 (2)	<0.001
Initial body size	0.268 (1)	0.604
Treatment: Week	22.222 (4)	<0.001

Random effect	Variance	SD	Number of groups
Female ID	0.032	0.18	129
Block ID	0.044	0.21	45

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	15	2602.5	2660.4	-1286.2	2572.5	1.336	0.513
Final model (b)	13	2599.8	2650.1	-1286.9	2573.8		

Table S3. Number of mating attempts/harassment received from males

(a) Initial model including the interaction between treatment and treatment duration; and treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	767.073 (1)	<0.001
Week	0.326 (2)	0.850
Treatment	37.337 (2)	<0.001
Initial body size	1.173 (1)	0.279
Treatment: Week	19.389 (4)	0.001
Treatment: Initial body size	0.380 (2)	0.827

Random effect	Variance	<i>SD</i>	Number of groups
Female ID	<0.001	0.00	129
Block ID	0.080	0.28	45

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	776.746 (1)	<0.001
Week	0.328 (2)	0.849
Treatment	37.074 (2)	<0.001
Initial body size	0.853 (1)	0.356
Treatment: Week	19.242 (4)	0.001

Random effect	Variance	<i>SD</i>	Number of groups
Female ID	<0.001	0.00	129
Block ID	0.078	0.28	45

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	df	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model							
(a)	15	2665.0	2723.0	-1317.5	2635.0	0.380	0.827
Final model							
(b)	13	2661.4	2711.6	-1317.7	2635.4		

Table S4. Probability of giving birth

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	3.919 (1)	0.048
Treatment	4.943 (2)	0.084
Initial body size	0.852 (1)	0.356
Treatment: Initial body size	4.719 (2)	0.094

Random effect	Variance	SD	Number of groups
Block ID	0.58	0.76	45

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	3.795 (1)	0.051
Treatment	5.067 (2)	0.079
Initial body size	0.243 (1)	0.622

Random effect	Variance	SD	Number of groups
Block ID	0.05	0.71	45

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model							
(a)	7	179.86	199.88	-82.93	165.86	5.196	0.074
Final model							
(b)	5	181.06	195.36	-85.53	171.06		

Table S5. Gestation period

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	296.845 (1)	<0.001
Treatment	0.909 (2)	0.635
Initial body size	1.276 (1)	0.259
Treatment: Initial body size	6.520 (2)	0.038

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	54.8	7.40	36

Table S6. Total number of offspring

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	61.655 (1)	<0.001
Treatment	1.531 (2)	0.465
Initial body size	2.451 (1)	0.117
Treatment: Initial body size	3.149 (2)	0.207

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.20	0.45	36

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	55.440 (1)	<0.001
Treatment	2.030 (2)	0.362
Initial body size	0.099 (1)	0.753

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.22	0.48	36

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	8	144.39	161.01	-64.19	128.39	3.16	0.206
Final model (b)	6	143.55	156.01	-65.77	131.55		

Table S7. Offspring size

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	10981.427 (1)	<0.001
Treatment	0.113 (2)	0.945
Initial body size	0.029 (1)	0.865
Treatment: Initial body size	0.910 (2)	0.634

Random effect	Variance	<i>SD</i>	Number of groups
Female ID	0.046	0.214	57
Block ID	<0.001	<0.001	35

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	10997.337 (1)	<0.001
Treatment	0.147 (2)	0.929
Initial body size	1.081 (1)	0.299

Random effect	Variance	<i>SD</i>	Number of groups
Female ID	0.046	0.22	57
Block ID	<0.001	<0.001	35

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	9	288.21	322.80	-135.10	270.21	0.907	0.635
Final model (b)	7	285.12	312.02	-135.56	271.12		

Table S8. Relative reproductive investment

(a) Initial model including the interaction between treatment and soma mass

	χ^2 (df)	<i>P</i>
Intercept	0.218 (1)	0.641
Treatment	3.494 (2)	0.174
Soma mass	8.101 (1)	0.004
Treatment: soma mass	3.347 (2)	0.188

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.01	0.09	44

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	3.300 (1)	0.069
Treatment	7.203 (2)	0.027
Soma mass	27.984 (1)	<0.001

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.01	0.09	44

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2_2	<i>P</i>
Initial model (a)	8	-79.30	-58.30	47.65	-95.30	3.31	0.191
Final model (b)	6	-79.99	-64.24	46.0	-91.99		

Table S9. Total egg number (excluded two females who gave birth twice)

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	1583.089 (1)	<0.001
Treatment	7.590 (2)	0.022
Initial body size	4.016 (1)	0.045
Treatment: Initial body size	6.281 (2)	0.043

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.02	0.13	44

Table S10. Growth

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	23308.928 (1)	<0.001
Treatment	21.605 (2)	<0.001
Initial body size	62.357 (1)	<0.001
Treatment: Initial body size	3.380 (2)	0.185

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.267	0.52	44

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	23465.518 (1)	<0.001
Treatment	21.549 (2)	<0.001
Initial body size	121.895 (1)	<0.001

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.256	0.51	44

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2	<i>P</i>
Initial model							
(a)	8	325.72	347.03	-154.86	309.72	3.51	0.173
Final model							
(b)	6	325.23	341.21	-156.62	313.23		

Table S11. Immunity

(a) Initial model including the interaction between treatment and final body size

	χ^2 (df)	<i>P</i>
Intercept	5.320 (1)	0.021
Treatment	5.018 (2)	0.081
Final body size	0.044 (1)	0.834
Treatment: Final body size	3.343 (2)	0.188

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.014	0.11	44

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	4.351 (1)	0.037
Treatment	5.891 (2)	0.053
Final body size	1.186 (1)	0.276

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.011	0.11	44

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2	<i>P</i>
Initial model							
(a)	8	20.01	41.17	-2.01	4.01	3.47	0.177
Final model							
(b)	6	19.48	35.35	-3.74	7.48		

Table S12. Body condition index

(a) Initial model including the interaction between treatment and final body size

	χ^2 (df)	<i>P</i>
Intercept	11.078 (1)	<0.001
Treatment	4.607 (2)	0.100
Final body size	96.705 (1)	<0.001
Treatment: Final body size	4.700 (2)	0.095

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.003	0.06	44

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	45.182 (1)	<0.001
Treatment	3.701 (2)	0.157
Final body size	273.095 (1)	<0.001

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.003	0.054	44

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	8	-180.15	-158.92	98.08	-196.15	4.56	0.103
Final model (b)	6	-179.59	-163.67	95.80	-191.59		

Table S13. Egg mass (excluding two females who gave birth twice)

(a) Initial model including the interaction between treatment and initial body size

	χ^2 (df)	<i>P</i>
Intercept	3949.164 (1)	<0.001
Treatment	4.995 (2)	0.082
Initial body size	0.438 (1)	0.508
Treatment: Initial body size	9.180 (2)	0.010

Random effect	Variance	<i>SD</i>	Number of groups
Block ID	0.04	0.20	44

Appendix C

Chapter 3: Supplementary material

Statistical outputs from models

Table S1. Effect of competitive environment on time spent following females

(a) Initial model including possible two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	2026.173 (1)	<0.001
Week	37.656 (2)	<0.001
Environment	27.580 (2)	<0.001
Initial body size	2.949 (1)	0.086
Environment: Week	1.408 (4)	0.843
Environment: Initial body size	0.842 (2)	0.657

Random effect	Variance	<i>SD</i>
Male ID	0.130	0.361
Block ID	0.032	0.179

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Intercept	2430.163 (1)	<0.001
Week	60.526 (2)	<0.001
Environment	39.210 (2)	<0.001
Initial body size	8.716 (1)	0.003

Random effect	Variance	<i>SD</i>
Male ID	0.124	0.353
Block ID	0.037	0.192

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	15	3713.5	3771.4	-1841.7	3683.5	2.382	0.882
Final model (b)	9	3703.8	3738.6	-1842.9	3685.8		

Table S2. Effect of competitive environment on number of mating attempts

(a) Initial model including possible two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	233.982 (1)	<0.001
Week	21.650 (2)	<0.001
Environment	3.263 (2)	0.196
Initial body size	0.801 (1)	0.371
Environment: Week	5.522 (4)	0.238
Environment: Initial body size	2.090 (2)	0.352

Random effect	Variance	<i>SD</i>
Male ID	0.211	0.459
Block ID	<0.001	<0.001

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Intercept	237.338 (1)	<0.001
Week	23.70 (2)	<0.001
Environment	0.083 (2)	0.959
Initial body size	11.186 (1)	<0.001

Random effect	Variance	<i>SD</i>
Male ID	0.195	0.442
Block ID	<0.001	<0.001

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	15	2056.8	2114.7	-1013.4	2026.8	7.896	0.246
Final model (b)	9	2052.6	2087.4	-1017.3	2034.6		

Table S3. Effect of competitive environment (b vs c only) on number of aggressions showed

(a) Final model including significant two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	129.995 (1)	<0.001
Week	3.421 (2)	0.181
Environment	0.191 (1)	0.662
Initial body size	1.218 (1)	0.270
Environment: Week	8.488 (2)	0.014
Environment: Initial body size	5.580 (1)	0.018

Random effect	Variance	<i>SD</i>
Male ID	0.046	0.215
Block ID	0.023	0.152

Table S4. Effect of competitive environment (b vs c only) on number of aggressions received

(a) Initial model including possible two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	135.853 (1)	<0.001
Week	0.191 (2)	0.909
Environment	5.149 (1)	0.023
Initial body size	0.641 (1)	0.423
Environment: Week	4.960 (2)	0.084
Environment: Initial body size	5.198 (1)	0.023

Random effect	Variance	<i>SD</i>
Male ID	<0.001	<0.001
Block ID	0.128	0.358

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	165.653 (1)	<0.001
Week	6.668 (2)	0.036
Environment	0.610 (1)	0.403
Initial body size	0.575 (1)	0.448
Environment: Initial body size	4.108 (1)	0.043

Random effect	Variance	SD
Male ID	<0.001	<0.001
Block ID	0.105	0.324

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	11	1559.1	1597	-768.53	1537.1	4.890	0.087
Final model (b)	9	1560.0	1591	-770.98	1542.0		

Table S5. Effect of competitive environment on growth

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	221780 (1)	< 0.001
Environment	0.974 (2)	0.615
Initial body size	2927.4 (1)	< 0.001
Environment: Initial body size	0.564 (2)	0.754

Random effect	Variance	SD
Block ID	<0.001	<0.001

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Environment	0.967 (2)	0.617
Initial body size	8808.632 (1)	< 0.001

Random effect	Variance	SD
Block ID	<0.001	<0.001

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	8	36.931	58.314	-10.466	20.931	0.596	0.742
Final model (b)	6	33.527	49.564	-10.763	21.527		

Table S6. Effect of competitive environment on immunity

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Environment	0.148 (2)	0.929
Week 8 body size	4.460 (1)	0.035
Environment: Week 8 body size	1.377 (2)	0.502

Random effect	Variance	<i>SD</i>
Block ID	0.020	0.141

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Environment	0.145 (2)	0.930
Week 8 body size	4.511 (1)	0.034

Random effect	Variance	<i>SD</i>
Block ID	0.019	0.137

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log- likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	8	-3.827	16.172	9.914	-19.827	1.451	0.482
Final model (b)	6	-6.377	8.622	9.188	-18.377		

Table S7. Effect of competitive environment on sperm count

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	9642.056 (1)	<0.001
Environment	0.760 (2)	0.684
Week 8 body size	10.842 (1)	0.001
Environment: Week 8 body size	2.404 (2)	0.301

Random effect	Variance	<i>SD</i>
Block ID	0.216	0.464

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	9814.401 (1)	<0.001
Environment	0.414 (2)	0.813
Week 8 body size	31.806 (1)	<0.001

Random effect	Variance	<i>SD</i>
Block ID	0.212	0.461

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	8	241.84	262.43	-112.92	225.84	2.526	0.283
Final model (b)	6	240.36	255.81	-114.18	228.36		

Table S8. Effect of competitive environment on sperm velocity

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	705.111 (1)	<0.001
Environment	0.734 (2)	0.693
Week 8 body size	1.305 (1)	0.253
Environment: Week 8 body size	1.077 (2)	0.584

Random effect	Variance	<i>SD</i>
Block ID	84.74	9.205

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	724.359 (1)	<0.001
Environment	1.019 (2)	0.601
Week 8 body size	1.700 (1)	0.192

Random effect	Variance	<i>SD</i>
Block ID	93.51	9.67

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	8	886.74	907.33	-435.37	870.74	1.129	0.569
Final model (b)	6	883.87	899.31	-435.93	871.87		

Table S9. Effect of past sperm investment (ablated or not) on time spent following females.

(a) Initial model including two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	1013.315 (1)	<0.001
Week	14.315 (2)	<0.001
Past sperm investment	6.386 (1)	0.012
Initial body size	6.078 (1)	0.014
Past sperm investment: Week	0.633 (2)	0.729
Past sperm investment: Initial body size	3.002 (1)	0.083

Random effect	Variance	<i>SD</i>
Male ID	0.123	0.351
Tank ID	<0.001	<0.001

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Intercept	1151.520 (1)	<0.001
Week	18.358 (2)	<0.001
Past sperm investment	8.4124 (1)	0.004
Initial body size	3.104 (1)	0.078

Random effect	Variance	<i>SD</i>
Male ID	0.139	0.373
Tank ID	<0.001	<0.001

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_2	<i>P</i>
Initial model (a)	11	2141.2	2178.6	-1059.6	2119.2	3.335	0.343
Final model (b)	8	2138.6	2165.7	-1061.3	2122.6		

Table S10. Effect of past sperm investment (ablated or not) on number of mating attempts

(a) Initial model including two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	157.141 (1)	<0.001
Week	4.130 (2)	0.127
Past sperm investment	2.164 (1)	0.141
Initial body size	5.855 (1)	0.016
Past sperm investment: Week	0.630 (2)	0.730
Past sperm investment: Initial body size	3.084 (1)	0.079

Random effect	Variance	<i>SD</i>
Male ID	<0.001	<0.001
Tank ID	0.103	0.321

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Intercept	160.867 (1)	<0.001
Week	4.056 (2)	0.132
Past sperm investment	2.941 (1)	0.086
Initial body size	2.702 (1)	0.100

Random effect	Variance	<i>SD</i>
Male ID	0.021	0.145
Tank ID	0.109	0.331

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	11	1257.6	1294.9	-617.78	1235.6	3.460	0.326
Final model (b)	8	1255.0	1282.2	-619.51	1239.0		

Table S11. Effect of past sperm investment (ablated or not) on number of aggressions showed

(a) Initial model including two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	100.257 (1)	<0.001
Week	11.298 (2)	0.004
Past sperm investment	1.206 (1)	0.272
Initial body size	4.125 (1)	0.042
Past sperm investment: Week	11.296 (2)	0.004
Past sperm investment: Initial body size	0.187 (1)	0.665

Random effect	Variance	<i>SD</i>
Male ID	0.072	0.268
Tank ID	0.066	0.256

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	100.719 (1)	<0.001
Week	11.635 (2)	0.003
Past sperm investment	1.159 (1)	0.282
Initial body size	5.853 (1)	0.016
Past sperm investment: Week	11.801 (2)	0.003

Random effect	Variance	<i>SD</i>
Male ID	0.074	0.272
Tank ID	0.063	0.251

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	11	1454.5	1491.9	-716.27	1432.5	0.187	0.665
Final model (b)	10	1452.5	1486.7	-716.36	1432.7		

Table S12. Effect of past sperm investment (ablated or not) on number of aggressions received

(a) Initial model including two-way interactions

	χ^2 (df)	<i>P</i>
Intercept	69.270 (1)	<0.001
Week	13.745 (2)	0.001
Past sperm investment	0.932 (1)	0.334
Initial body size	2.996 (1)	0.083
Past sperm investment: Week	0.870 (2)	0.647
Past sperm investment: Initial body size	0.549 (1)	0.459

Random effect	Variance	<i>SD</i>
Male ID	0.066	0.258
Tank ID	0.081	0.285

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Intercept	92.119 (1)	<0.001
Week	19.199 (2)	<0.001
Past sperm investment	0.447 (1)	0.504
Initial body size	3.067 (1)	0.080

Random effect	Variance	<i>SD</i>
Male ID	0.078	0.279
Tank ID	0.092	0.304

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	11	1478.8	1516.2	-728.41	1456.8	1.268	0.737
Final model (b)	8	1474.1	1501.2	-729.05	1458.1		

Table S13. Effect of past sperm investment (ablated or not) on growth

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	132020 (1)	< 0.001
Past sperm investment	0.562 (1)	0.453
Initial body size	1329.2 (1)	< 0.001
Past sperm investment: Initial body size	0.012 (1)	0.914

Random effect	Variance	<i>SD</i>
Tank ID	<0.001	<0.001

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Past sperm investment	0.561 (1)	0.454
Initial body size	3030.506 (1)	< 0.001

Random effect	Variance	<i>SD</i>
Tank ID	<0.001	<0.001

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_2	<i>P</i>
Initial model (a)	6	40.228	52.991	-14.114	28.228	0.012	0.911
Final model (b)	5	38.241	48.876	-14.120	28.241		

Table S14. Effect of past sperm investment (ablated or not) on immunity

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	4.583(1)	< 0.032
Past sperm investment	0.034 (1)	0.853
Week 8 body size	0.905 (1)	0.341
Past sperm investment: Week 8 body size	0.224 (1)	0.636

Random effect	Variance	<i>SD</i>
Tank ID	0.003	0.057

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Past sperm investment	0.023 (1)	0.879
Week 8 body size	0.826 (1)	0.363

Random effect	Variance	<i>SD</i>
Tank ID	0.005	0.069

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_2	<i>P</i>
Initial model (a)	6	-5.147	6.897	8.574	-17.147	0.234	0.629
Final model (b)	5	-6.914	3.123	-8.457	-16.914		

Table S15. Effect of past sperm investment (ablated or not) on sperm count

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	10735.489 (1)	<0.001
Past sperm investment	33.089 (1)	<0.001
Week 8 body size	4.617 (1)	0.032
Past sperm investment: Week 8 body size	0.909 (1)	0.340

Random effect	Variance	<i>SD</i>
Tank ID	0.104	0.322

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	10711.840 (1)	<0.001
Past sperm investment	33.865 (1)	<0.001
Week 8 body size	4.195 (1)	0.041

Random effect	Variance	<i>SD</i>
Tank ID	0.116	0.340

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	6	132.15	144.51	-60.075	120.15	0.9647	0.326
Final model (b)	5	131.12	141.42	-60.558	121.12		

Table S16. Effect of past sperm investment (ablated or not) on sperm velocity

(a) Initial model including the two-way interaction

	χ^2 (df)	<i>P</i>
Intercept	525.140 (1)	<0.001
Past sperm investment	0.010 (1)	0.921
Week 8 body size	1.440 (1)	0.230
Past sperm investment: Week 8 body size	0.386 (1)	0.534

Random effect	Variance	<i>SD</i>
Tank ID	368.5	19.2

(b) Final model excluding the non-significant interaction

	χ^2 (df)	<i>P</i>
Intercept	529.075 (1)	<0.001
Past sperm investment	0.010 (1)	0.920
Week 8 body size	3.578 (1)	0.059

Random effect	Variance	<i>SD</i>
Block ID	368.9	19.21

(c) Exclusion of the non-significant interaction did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	6	539.44	551.80	-263.72	527.44	0.415	0.520
Final model (b)	5	537.85	548.15	-263.72	527.85		

Appendix D

Chapter 4: Supplementary material

Statistical outputs from models

Table S1: Test if the samples show the same response as the standard curve through Cortisol assay parallelism

	F value	<i>P</i>
Cortisol concentration (log)	1287.060	<0.001
Sample Type (standard or experimental)	0.492 (1, 9)	0.501
Cortisol concentration (log): Sample Type	0.236 (1, 9)	0.639

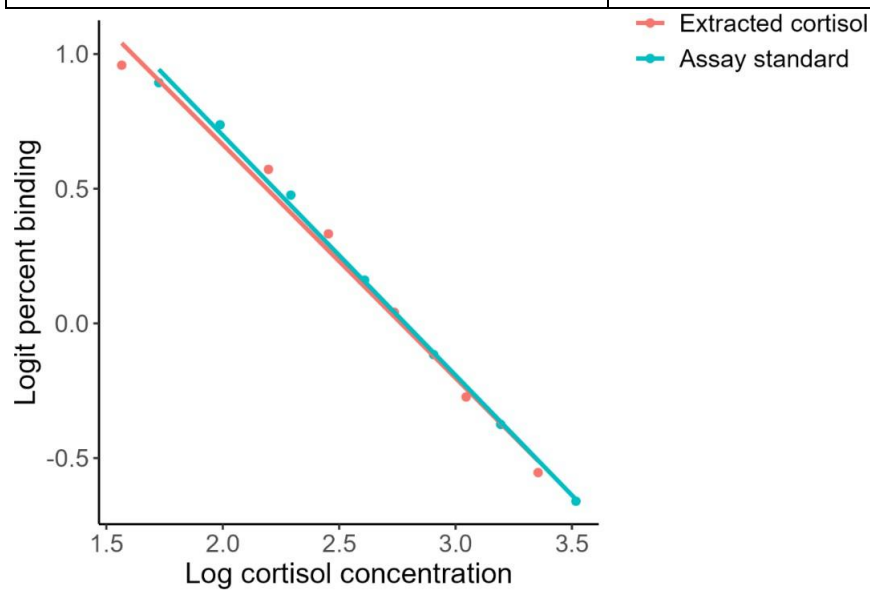


Fig 1: Cortisol assay parallelism

Table S2: Effect of a Changed environment on the number of gonopodial thrusts received by females (mating attempts)

(a) Initial full model including possible three-way interactions

	χ^2 (df)	<i>P</i>
Intercept	38.37 (1)	<0.001
Turbidity	3.50 (1)	0.06
Testing day	22.00 (1)	<0.001
Male density	16.78 (1)	<0.001
Turbidity: Testing day	3.48 (1)	0.06
Turbidity: Male density	0.04 (1)	0.85
Male density: Testing day	1.38 (1)	0.24
Turbidity: Testing day: Male density	1.56 (1)	0.21

Random effect	Variance	SD
Female ID	0.18	0.42
Block ID	0.07	0.27

(b) Model excluding the three-way interaction

	χ^2 (df)	<i>P</i>
Intercept	36.58 (1)	<0.001
Turbidity	4.75 (1)	0.029
Testing day	24.76 (1)	<0.001
Male density	16.15 (1)	<0.001
Turbidity: Testing day	2.28 (1)	0.131
Turbidity: Male density	0.13 (1)	0.721
Male density: Testing day	0.05 (1)	0.836

Random effect	Variance	SD
Female ID	0.15	0.38
Block ID	0.08	0.28

(c) Final model excluding all the non-significant interactions:

	χ^2 (df)	<i>P</i>
Intercept	37.92 (1)	<0.001
Turbidity	10.96 (1)	<0.001
Testing day	52.32 (1)	<0.001
Male density	45.83 (1)	<0.001

Random effect	Variance	SD
Female ID	0.12	0.35
Block ID	0.08	0.29

(d) Exclusion of non-significant interactions did not significantly reduce model fit between the consecutively reduced models

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	11	930.21	971.871	-454.11	908.21	1.531	0.216
Initial model (b)	10	929.74	967.61	-454.87	909.74	2.627	0.453
Final model (c)	7	926.37	952.88	-454.87	912.37		

Table S3: Effect of a Changed environment on the following time

(a) Initial full model including possible three-way interactions

	χ^2 (df)	<i>P</i>
Intercept	453.01 (1)	<0.001
Turbidity	5.40 (1)	0.020
Testing day	20.31 (1)	<0.001
Male density	14.74 (1)	<0.001
Turbidity: Testing day	0.58 (1)	0.45
Turbidity: Male density	0.37 (2)	0.55
Male density: Testing day	0.40 (1)	0.53
Turbidity: Testing day: Male density	0.26 (1)	0.61

Random effect	Variance	<i>SD</i>
Female ID	0.23	0.48
Block ID	0.15	0.38

(b) Model excluding the three-way interaction

	χ^2 (df)	<i>P</i>
Intercept	456.98 (1)	<0.001
Turbidity	6.60 (1)	0.010
Testing day	22.45 (1)	<0.001
Male density	15.60 (1)	<0.001
Turbidity: Testing day	0.33 (1)	0.563
Turbidity: Male density	0.96 (1)	0.326
Male density: Testing day	2.26 (1)	0.133

Random effect	Variance	<i>SD</i>
Female ID	0.23	0.48
Block ID	0.15	0.39

(c) Final model excluding non-significant interactions

	χ^2 (df)	<i>P</i>
Intercept	656.51 (1)	<0.001
Turbidity	10.48 (1)	<0.001
Testing day	64.85 (1)	<0.001
Male density	66.38 (1)	<0.001

Random effect	Variance	SD
Female ID	0.20	0.45
Block ID	0.14	0.37

(d) Exclusion of non-significant interactions did not significantly reduce model fit

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	11	2405.2	2446.9	-1191.6	2383.2	0.258	0.612
Initial model (b)	10	2403.5	2441.3	-1191.7	2383.5	3.749	0.290
Final model (c)	7	2401.2	2427.7	-1193.6	2387.2		

Table S4: Effect of water turbidity and male harassment on female cortisol level

(a) Initial full model including interactions

	F value	<i>P</i>
Turbidity	39.252 (1)	<0.001
Male density	0.0016 (1)	0.968
Turbidity: Male density	0.1019 (1)	0.750

Random effect	Variance	SD
Block ID	0.00	0.00
Plate ID	27.63	5.26

(b) Final model excluding the non-significant interaction

	F value	<i>P</i>
Turbidity	39.547 (1)	<0.001
Male density	0.001 (1)	0.975

Random effect	Variance	SD
Block ID	0.00	0.00
Plate ID	27.78	5.27

- (c) Exclusion of the non-significant interaction did not significantly reduce the model fit

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2	<i>P</i>
Initial model (a)	7	1521.6	1543.3	-753.80	1507.6	0.106	0.745
Final model (b)	6	1519.7	1538.3	-753.85	1507.7		

Table S5: Effect of killing time on cortisol level

a. Full model

	F value	<i>P</i>
Turbidity	39.023 (1)	<0.001
Male density	0.0004 (1)	0.984
Killing Time	0.0431 (1)	0.836

Random effect	Variance	<i>SD</i>
Block ID	<0.001	<0.001
Plate ID	29.31	5.41

Appendix E

Chapter 5: Supplementary material

Statistical outputs from models

Table S1. Effect of salinity and temperature on size at maturity

(e) Initial model including interactions

	χ^2 (df)	<i>P</i>
Intercept	18566.694 (1)	<0.001
Salinity	60.831 (1)	<0.001
Temperature	0.964 (1)	0.326
Salinity: Temperature	8.544 (1)	0.003

Random effect	Variance	<i>SD</i>
Mother ID	0.011	0.104

Table S2. Effect of salinity and temperature on time to mature

(a) Initial model including interactions

	χ^2 (df)	<i>P</i>
Salinity	4.289 (1)	0.039
Temperature	0.078 (1)	0.780
Salinity: Temperature	1.317 (1)	0.251

Random effect	Variance	<i>SD</i>
Mother ID	0.001	0.034

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Salinity	4.287 (1)	0.038
Temperature	0.077 (1)	0.781

Random effect	Variance	<i>SD</i>
Mother ID	0.001	0.035

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_1	<i>P</i>
Initial model (a)	6	-88.8	-69.3	50.4	-100.8	1.341	0.247
Final model (b)	5	-89.5	-73.2	49.7	-99.5		

Table S3. Effect of salinity and temperature on gonopodia size

(a) Initial model including interactions

	χ^2 (df)	<i>P</i>
Salinity	10.995 (1)	0.001
Temperature	0.738 (1)	0.390
Log Size at maturity	119.635 (1)	<0.001
Salinity: Temperature	2.210 (1)	0.137

Random effect	Variance	<i>SD</i>
Mother ID	<0.001	0.011

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Salinity	11.015 (1)	0.001
Temperature	0.754 (1)	0.385
Log Size at maturity	117.498 (1)	<0.001

Random effect	Variance	<i>SD</i>
Mother ID	<0.001	0.012

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_1	<i>P</i>
Initial model (a)	7	-646.0	-623.4	330.0	-660.02	2.251	0.134
Final model (b)	6	-645.8	-626.4	328.9	-657.8		

Table S4. Effect of salinity and temperature on growth

(a) Initial model including interactions

	χ^2 (df)	<i>P</i>
Intercept	13.603 (1)	<0.001
Pre_size	370.650 (1)	<0.001
Salinity	4.266 (1)	0.039
Temperature	3.175 (1)	0.075
Pre_size : Salinity	3.818 (1)	0.051
Pre_size : Temperature	3.186 (1)	0.074
Salinity: Temperature	4.665 (1)	0.031
Pre_size: Salinity: Temperature	4.407 (1)	0.036

Random effect	Variance	<i>SD</i>
Mother ID	0.009	0.093

Table S5. Effect of salinity and temperature on sperm count

(a) Initial model including interactions

	χ^2 (df)	<i>P</i>
Salinity	11.746 (1)	0.001
Temperature	6.372 (1)	0.012
Salinity: Temperature	0.057 (1)	0.811

Random effect	Variance	<i>SD</i>
Mother ID	0.007	0.082

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Salinity	11.806 (1)	0.001
Temperature	6.395 (1)	0.011

Random effect	Variance	<i>SD</i>
Mother ID	0.007	0.084

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_1	<i>P</i>
Initial model (a)	6	446.2	465.2	-217.1	434.2	0.058	0.809
Final model (b)	5	444.2	460.1	-217.1	434.2		

Table S6. Effect of salinity and temperature on sperm velocity

(a) Initial model including interactions

	χ^2 (df)	<i>P</i>
Salinity	9.691 (1)	0.002
Temperature	0.842 (1)	0.359
Salinity: Temperature	0.590 (1)	0.442

Random effect	Variance	<i>SD</i>
Mother ID	14.97	3.869

(b) Final model excluding the non-significant interactions

	χ^2 (df)	<i>P</i>
Salinity	9.767 (1)	0.002
Temperature	0.858 (1)	0.354

Random effect	Variance	<i>SD</i>
Mother ID	17.19	4.146

(c) Exclusion of the non-significant interactions did not significantly reduce model fit in the final model

	No. parameter	AIC	BIC	Log-likelihood	Deviance	χ^2_1	<i>P</i>
Initial model (a)	6	1516.3	1535.1	-752.1	1504.3	0.610	0.435
Final model (b)	5	1514.9	1530.5	-752.45	1504.9		