Title

Causes and consequences of intraspecific variation in sexually selected traits in western mosquitofish (Gambusia affinis)

Author

Weiler, Doriane Elaine

Publication Date

2023

UNIVERSITY OF CALIFORNIA
SANTA CRUZ

CAUSES AND CONSEQUENCES OF INTRASPECIFIC VARIATION IN SEXUALLY SELECTED TRAITS IN WESTERN MOSQUITOFISH (*GAMBUSIA AFFINIS*)

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ECOLOGY AND EVOLUTIONARY BIOLOGY

by

Doriane E. Weiler

June 2023

The Dissertation of Doriane E. Weiler
is approved:

Professor Suzanne Alonzo, Chair

Professor Eric Palkovacs

Professor Molly Cummings

Peter Biehl
Vice Provost and Dean of Graduate Studies

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ABSTRACT

Causes and Consequences of Intraspecific Variation in Sexually Selected Traits in Western Mosquitofish (*Gambusia affinis*)

by

Doriane E. Weiler

The field of eco-evolutionary dynamics has highlighted the myriad ways that ecological and evolutionary processes are intertwined. One key revelation emerging from this framework is the importance of intraspecific variation, which can have ecological effects comparable to or greater than the effects of species incidence and abundance. Despite the rapid expansion of research highlighting the consequences of intraspecific variation, most work in this area has focused on traits shaped primarily by natural selection, excluding sexual selection, a potent driver of phenotypic diversity. In this dissertation, I studied the evolutionary drivers and ecological consequences of intraspecific variation in sexually selected traits, using Western Mosquitofish (*Gambusia affinis*) as a focal organism. Mosquitofish mating behavior is characterized by male sexual harassment, in which males actively attempt copulations with unreceptive females. Although ambient factors (e.g., temperature) that drive plastic variation in this behavior have been described previously, few have examined the evolutionary drivers of variation in mating behavior, especially with respect to abiotic factors. Temperature is among the most influential abiotic factors because it governs the rates of all biological processes, influencing the physiology, morphology, and behavior of organisms. In **Chapter 1**, I used common rearing and behavioral assays to ask whether divergent average temperatures generated systematic differences in the thermal plasticity of mating behavior among six populations of mosquitofish. While harassment was sensitive to ambient temperature, peaking at intermediate temperatures and declining at thermal extremes, I found no evidence that divergent source temperatures could explain evolved behavioral differences among populations, highlighting the inherent challenges in identifying the drivers of intraspecific diversity. In **Chapter 2**, I used a free-swimming paired choice behavioral assay to determine whether differences in male harassment could explain population divergence in female gravid spots, which likely serve as a sexual signal due to the established correlation between gravid spot size and female fertility. Male preferences for gravid spots differed among populations but were inversely correlated with female gravid spot sizes within populations, an outcome in direct opposition to theoretical predictions regarding the coevolution of mate preferences and traits. In **Chapter 3**, I tested how intraspecific variation in male sexual harassment behavior influences aquatic ecosystems. Sexual harassment intensity modulated the effects of mosquitofish introduction for zooplankton communities, demonstrating the ecological importance of sexually selected traits. In summary, my dissertation shows how sexual selection, a ubiquitous evolutionary force, is fertile ground for exploring the interplay of ecological and evolutionary processes and warrants further attention in the framework of eco-evolutionary dynamics.

ACKNOWLEDGEMENTS

Completing a dissertation of this magnitude would not have been possible without the encouragement and support of my community in Santa Cruz and beyond. I feel deep and overwhelming gratitude for the mentors, colleagues, family, friends, and organizations that inspired me to persevere and contributed to my work in diverse forms throughout my PhD.

First, I would like to express my deepest appreciation to my advisor **Dr. Suzanne Alonzo** for her brilliant research advice, empathetic mentorship, and steadfast support throughout my journey as a graduate student. She was a fearless guide when my confidence waivered and I felt lost in the confusing cloud of uncertainty that often characterizes the process of doing research. I aspire to handle difficult moments with such grace. Completing a PhD is among the most challenging things I have done, and I could not have asked for a better advisor over the past seven years– the positive impact of her mentorship will persist long past my defense.

I would also like to extend my deepest gratitude to my co-advisor **Dr. Eric Palkovacs**, who graciously adopted me into his lab when I shifted my research focus to the eco-evolutionary dynamics of mosquitofish early in my graduate career. I am especially thankful for the times he responded to my panicked texts at 5 am when I was sampling mesocosms, and for the moments he volunteered to feed my mosquitofish when I was sick or desperately needed a break. I appreciate that Eric

constantly went above and beyond to ensure my success, both in my research and in my professional goals– he even facilitated professional connections that helped me discover my post-PhD career path!

I would like to offer my sincere appreciation for my qualifying committee members, dissertation reading committee members, and co-authors for their valuable intellectual contributions. I thank **Dr. Molly Cummings,** who provided insightful feedback on my dissertation and was always willing to share her expertise in Poecillid behavior. I thank **Dr. Stephan Munch** for his advice on statistical analyses and patience in responding to my quantitative questions. I also thank **Dr. James Estes**, whose work on trophic cascades inspired my interest in ecology before I even knew who he was! Jim's enthusiasm for my research provided the confidence boost I desperately needed as a new graduate student. I am especially grateful to **Dr. David Fryxell**, the original Palkovacs Lab mosquitofish wrangler who laid the groundwork for my research by identifying the study sites used in my first and second chapters. I am thankful for his impressive patience while sharing his expertise in mosquitofish and have a newfound appreciation for the time he drove eight hours immediately after his defense to help me collect fish in Bishop, CA!

I would like to acknowledge and thank my heroic army of research assistants (also known as the *Gambusia* Gang) for the 3450+ hours they collectively spent caring for mosquitofish, analyzing videos, measuring images, and helping with hands-on experiments that were often extremely physically demanding. These students taught me the importance of collaboration in scientific research and helped maintain my enthusiasm for mosquitofish behavior. First, I would like to thank **Samantha Wong** for her impressive resilience in assisting with behavioral assays for Chapters 1 & 2, and *especially* for her assistance with the mesocosm experiment in Chapter 3. Sam sampled tanks, measured behavior, and caught fish, all while coping with a global pandemic, fires that turned the sky orange, RBG dying, and her mentor (me) almost passing out because I sliced my hand open on a broken glass aquarium. Truly, this chapter would not have been possible without her 450+ hours of assistance. My work also would not have been possible without the contributions of undergraduate researchers A. Laing, H. Thacker, D. Alvarado, B. McCormick, N. Rosen, A. Smith, C. Eagan, M. Reed, M. Glenn, N. Theunissen, D. Muttineni, J. Leung, A. Marsolais, L. Siouffey, J. Flores, A. Nguyen, F. Chan, V. Panelli, E. Hennings, G. Chien, B. Munoz, S. Noguera, C. Dong, E. Wahl, E. Palma, S. Siegman, N. Zanger, and E. Yang. I also thank high school SIP interns S. Park, T. Jojan, C. Mackenzie, M. Wayne, C. McGinnis, A. Guerrero, and C. Xu for spending their summers assisting with research.

I thank all members of the Alonzo and Palkovacs Labs for the community they provided over the past seven years. Thank you for reading my awkward first drafts, sharing your creativity, and offering the type of camaraderie and connection that can only come from labmates. I am especially thankful to **Regina Spranger**, my friend and cohort-mate who is one of the few to understand the struggles of working with live animals long-term, the stress of late-night calls that power is out at the Coastal Campus, and the patience that can only be learned from managing enormous teams of undergrads. I am also thankful to **Matt Kustra** for answering every random text about coding and/or weird animal behavior, and to **Sabrina Beyer** for sharing the role of Suzanne's first UCSC grad students and for teaching me so much about GLMMs.

I am grateful to the people who intervened with honest advice and encouraged me to persist with my PhD when I was at my lowest, who believed I could finish even when I lost faith. I thank **Katie Kobayashi** for bringing me orange-colored food and staying with me at the Coastal Campus late into the first night of my Comprehensive Exam to make sure I didn't send the email declaring my resignation from grad school. I thank **Karen Tanner** for her wisdom as an experienced graduate student– our conversation outside the Seymour Center my first year was more impactful than she knows. I thank **Judy Straub** for walking with me around the Coastal Campus trails and reminding me that I could write my papers. Her relentless support of graduate students is unmatched, and I am so grateful she is the EEB Grad Program Coordinator.

I thank my UCSC IRAPS colleagues for helping me discover my passion for assessment research and for offering the light at the end of the tunnel that I needed to stay motivated in the last year of my PhD. I am grateful to **Anna Sher** for hiring an enthusiastic animal behavior researcher with little social science experience as GSR, and ensuring I had the skills to become a successful Assessment Analyst. I thank **Priscilla Sung** for her thoughtful mentorship, patience, and impressive organizational abilities, which made it so much easier to learn new skills.

I thank **Corey Stoesser** for teaching me to prioritize self-care, and for imbuing my grad school experience with joyful memories. I am so grateful for the help he offered throughout my PhD, from carrying sand bags around hardware stores to shoveling buckets of sediment. He was there when I needed support, and I'll never forget that.

Graduate school would not have been nearly as fun without the people who made the world feel bigger than academia. **Liz Babalola**, thank you for showing me what it's like to have the confidence of a 30-something year old. **Mia Garrard,** thank you for taking me on my first backpacking trip– it truly expanded my world, and I look forward to future adventures together! **Rémi Bourgeois**, our serendipitous meeting was so lucky. During the last three months before my defense, which could have easily been among the worst in my life, you renewed my spirit and offered safe harbor from the storm. I am endlessly grateful to you.

I am thankful to my oldest friend, **Alex Phillips**, who bravely started graduate school before me and was always willing to share her wisdom. Watching you finish your PhD was an inspiration, and I couldn't have made it to the finish line without your pep talks and creative ideas for staying organized and motivated. We have come so far the days of reviewing bio flashcards with your mom at your dining room table, and I can't wait to see where we go next– wherever it is, I'll be cheering you on!

I am thankful to my forever friend and literal lifesaver **Sara Karabashlieva**, who reliably called me several times a week to catch up over the past seven years. Being your friend has made me a better person, and I am so grateful we have been able to grow together. I feel overwhelmingly lucky to have someone in my life who has witnessed my journey firsthand, recognizes my growth, accepts where I am now, and believes in the potential of my future. You kept me grounded through tumultuous times, and I love that we can stay as close as we are despite the distance between us. Thank you for being there for me, no matter what– and know that I'll always be there for you too.

It is no exaggeration to say I could not have made it through grad school without the friendship and support of my dear friend **Stephanie Rich**. I feel overwhelming gratitude that we found each other. It is such a gift to come home to you at the end of the day and decompress with a cup of tea and laughter. Thank you for being my rock through this tumultuous journey of grad school, a friend who celebrated my highest highs and offered comfort during the lowest lows. I wish I could list every specific thank you from everything we have experienced the past few years, but it would likely double the length of my dissertation. So instead I'll say this– I know that we have learned that “different can be good,” but I hope I never have to experience a life where “different” means you aren’t around.

I thank my two dogs **Pepper** and **Rupert** for their emotional support and encouragement via tail wags. And while I’m thanking animals, I’d like to add my gratitude to all the mosquitofish who sacrificed their lives for my research.

I thank my sisters, **Amber Andersen** and **Kristal Salcido**, for being role models throughout grad school and my entire life. I feel so lucky to be part of a family so filled with love. Kristal, I am so glad that I’ve accidentally followed you from place to place as we’ve moved through our lives. You have been a steadfast and grounded supporter through both personal and professional challenges, and I am grateful that you are in Santa Cruz with me. Amber, my fellow animal lover, I am inspired by your capacity to build the life you want, and by your relentless creativity when you want to make a change.

Lastly, I am deeply thankful to my parents for their unconditional love and support through my PhD journey, and always. I am grateful to my mom, **Nancy Weiler**, for being my role model of resilience. Thank you for teaching me that when the going gets tough, the tough get going, but also understanding that sometimes to persevere, we need to be surrounded by love and light. Thank you for coming to Berkeley when I needed you most. You helped me understand that I can persevere, and I am strong now because of it. I am also thankful for the inspiration of my dad, **Clifford Weiler**. Without his influence, I may not have become an ecologist. He taught me that there is always time to admire nature's beauty, and he always caught me when I was too distracted looking at tidepools as a kid to notice the encroaching waves. I appreciate that you remind me to have a sense of humor (but please don't let the raccoons in the house). Mom and Dad, thank you for always finding ways to support my budding love of ecology and fostering my creativity, from sending me to reptile camp and letting me catch bugs in the backyard to ensuring I always had enough art supplies to capture my inspiration. I hope you know how grateful I am for everything you've done. I love you.

Dedication

I dedicate this work to all future PhD students, the brave pioneers who make countless sacrifices to produce new knowledge for all of society to share. Know that you aren't in this alone. You stand on the towering shoulders of academic giants, but if you tumble– and if you're asking hard enough questions, you undoubtably will– remember that there are people around you who will help pick you up again.

Funding sources

This work was supported the National Science Foundation's Graduate Research Fellowship Program, American Fisheries Society, UCSC's Ecology and Evolutionary Biology Department, and UCSC's Graduate Student Association. I appreciate the financial support of these organizations during my time as a graduate student.

INTRODUCTION

The natural world is characterized by an impressive array of diverse individuals, each with unique phenotypic traits. Identifying and understanding the processes that shape and maintain phenotypic diversity is a fundamental goal in the field of evolutionary biology. Researchers have long established that divergent ecological contexts can select for distinct phenotypes within species, as selection shapes traits to enhance fitness in predominant environmental conditions. While this causal relationship between ecology and evolutionary outcomes has been well-established, there is growing recognition that evolved phenotypes can also feedback on ecosystems (Palkovacs & Hendry, 2010; Post & Palkovacs, 2009). Indeed, intraspecific phenotypic diversity can have ecological consequences comparable to the effects of species presence or absence (Des Roches et al., 2018). While the importance of intraspecific diversity has been replicated in diverse systems, most work has focused on traits shaped primarily by natural selection, such as feeding morphology (Palkovacs & Post, 2009), overlooking another important evolutionary driver of intraspecific trait diversity: sexual selection (Alpedrinha et al., 2019; Giery & Layman, 2019; Svensson, 2019). In this dissertation, I explore the causes and consequences of intraspecific variation in sexually selected traits.

Sexual selection is a potent driver of phenotypic diversity that underlies many of the most remarkable morphologies and behaviors in the natural world. Darwin's original conception of sexual selection included two mechanisms: competition within sexes for access to mates (intrasexual selection) and mate choice (intersexual selection) (Darwin, 1871). More recently, there is growing recognition that opposing evolutionary interests among sexes arising from asymmetric parental investment can generate other mating strategies, such as sexual coercion (Chapman et al., 2003). Sexual coercion, or forced copulation, occurs when optimal mating rates diverge among the sexes. While male fitness typically increases with the number of mates they obtain, female fitness plateaus or declines at high mating rates, depending on the cost of excess mating (Bateman, 1948; Daly, 1978). Sexual harassment, in which males actively attempt copulations with unreceptive females, is a common outcome of selection favoring divergent optimal mating rates among sexes. Coercive mating can have profound implications for speciation rates (Gavrilets, 2000, 2014), population dynamics (Le Galliard et al., 2005; Wearmouth et al., 2012), and extinction risk (Kokko & Brooks, 2003; Le Galliard et al., 2005), yet much remains unknown regarding the causes and consequences of variation in sexual harassment behavior.

Ecological context can generate and maintain intraspecific diversity in mating behavior, including sexual harassment. Among populations inhabiting unique ecological settings, selection can favor divergent phenotypes that optimize fitness in prevailing environmental conditions, generating intraspecific diversity (Perry & Rowe, 2018). For example, differences in predation risk are associated with parallel changes in sexual harassment rates in water striders (Rowe et al., 1994). In populations where predators are present, male coercion is reduced because harassment is conspicuous, and mating individuals are at higher risk of predation (Rowe, 1994; Sih et al., 1990). Much research has been devoted to determining how biotic factors such as predation rates, sex ratios, and population density affect sexual conflict, yet our understanding of how abiotic factors affect sexual conflict remains relatively limited (Arnqvist & Rowe, 2005; García-Roa et al., 2020). In my first chapter, I explore how temperature, one of the most important abiotic factors for organismal physiology, affects the evolution and plasticity of sexual harassment behavior.

Understanding the drivers of variation in sexual harassment is important because this variation can have extensive evolutionary consequences, especially for female traits. Male harassment can be extremely costly for female fitness. Evidence across diverse taxa demonstrates how harassment reduces female feeding efficiency (Magurran & Seghers, 1994; Stone, 1995), increases vulnerability to predators (Arnqvist, 1989; Magnhagen, 1991), elevates infection risk (Daly, 1978), and causes physical injury or death (Clutton-Brock & Parker, 1995; Mckinney et al., 1983; Morrow & Arnqvist, 2003). These fitness costs can be a powerful selective force favoring the evolution of female traits that minimize the negative consequences of harassment (Hosken et al., 2016). In some species, sexual signaling may modulate the costs of sexual harassment (Rooker & Gavrilets, 2018). For example, in species with cyclic fertility, selection may favor female traits that signal only during periods of physiological receptivity, thereby isolating male attention to periods when copulations are required for fertilization (Rooker & Gavrilets, 2018). Male preferences for these signals would be evolutionarily advantageous for both sexes, as males would optimize mating success by allocating more energy toward the most fertile females, and females would only experience costs of harassment temporarily. However, the evolutionary dynamics of male mate choice are poorly understood relative to that of female choice. While models of female choice and male signaling predict that coevolutionary dynamics between sexes could lead to parallel variation in traits and preferences among populations (Edwards, 2000; Houde, 1993; Jennions & Petrie, 1997), whether these patterns occur with male choice and female signaling is uncertain. Comparing male harassment rates and female signals among populations, the objective of my second chapter, is a powerful first step in understanding coevolutionary outcomes between male behavior and female traits.

Intraspecific variation in male harassment phenotypes may not only have evolutionary consequences for female traits, but also ecological consequences. The field of eco-evolutionary dynamics has spawned numerous studies establishing foundational insights on the ecological consequences of intraspecific phenotypic diversity (Bolnick et al., 2011; Des Roches et al., 2018). However, these studies have largely focused on traits shaped primarily by natural selection, with little attention toward the ecological consequences of variation in sexually selected traits, despite the well-established potential of sexual selection to generate impressive intraspecific diversity (Giery & Layman, 2019; Svensson, 2019). Changes in predator-prey interactions caused by differences in sexual harassment are a potential mechanism through which sexually selected traits could modify communities and ecosystems. Sexual harassment has been previously linked to changes in feeding behavior (Arrington et al., 2009; Pilastro et al., 2003). In strong ecosystem interactors, subtle differences in consumption rates can have strong direct and indirect ecosystem effects through top-down trophic control, with cascading consequences for species abundance and composition across trophic levels (Attayde & Hansson, 2001; Ripple et al., 2016; Schmitz et al., 2004). Although several researchers have reviewed the potential ecological consequences of intraspecific variation in sexually selected traits, few studies have empirically tested how these traits affect ecosystem structure and function (Giery & Layman, 2019; Svensson, 2019). In my third chapter, I address this deficit by measuring the ecological consequences of intraspecific variation in sexual harassment behavior.

In this dissertation, I use western mosquitofish (*Gambusia affinis*) as a model system to consider the causes and consequences of intraspecific diversity in mating behavior. Mosquitofish are a widespread species of freshwater fish with a coercive mating system dominated by intense sexual harassment (Deaton, 2008; Pyke, 2005, 2008). Female mosquitofish are rarely receptive to male mating attempts because they require few copulations to successfully reproduce, and excess mating can have negative consequences, such as reduced body condition (Makowicz & Schlupp, 2013), fecundity (Makowicz & Schlupp, 2013), and overall fitness (Gasparini et al., 2012; Magurran, 2011; Makowicz & Schlupp, 2013). Males circumvent female choice using gonopodial thrusts, in which males approach females from behind and force copulation using their gonopodium, a modified anal fin that functions as an intromittent organ (Pilastro et al., 1997; Pyke, 2005). Although characterizations of intraspecific diversity in mosquitofish mating behavior are rare, evolved differences in behavior are likely, since mosquitofish inhabit diverse aquatic habitats and may adapt to local conditions. Mosquitofish have been deliberately introduced across aquatic ecosystems to control the spread of mosquito-borne diseases due to their efficacy at consuming mosquito larvae and their impressive physiological tolerance of broad environmental conditions. Among the diverse environments mosquitofish inhabit, optimal male harassment rates may differ, and selection could favor behavior that maximizes fitness in each unique population, generating intraspecific diversity in harassment intensity. Consequently, mosquitofish are an ideal focal species to explore the evolutionary drivers and ecological implications of sexual harassment behavior.

Collectively, my dissertation chapters explore the causes and consequences of variation in mosquitofish sexual harassment behavior and other sexually selected traits. In **Chapter 1**, I use behavioral assays to detect evolved differences in male mating behavior among six populations of mosquitofish and ask whether divergent average source temperatures affect sexual harassment rates. In **Chapter 2**, I explore the implications of divergent male mating behavior for female traits. Specifically, I ask whether differences in male mate preferences among populations can explain differences in female gravid spots, a trait that remains an evolutionary mystery but may serve as a sexual signal to module male attention. Lastly, in **Chapter 3**, I explore the ecological consequences of intraspecific variation in male mosquitofish sexual harassment behavior using a mesocosm experiment. Collectively, this body of work reveals that the drivers of intraspecific diversity in mating behavior may deviate from expected predictions based on evolutionary theory, and that variation in sexually selected traits may have more important ecological consequences than previously considered.

CHAPTER 1: Evolved differences in thermal plasticity of mosquitofish (*Gambusia* affinis) mating behavior are unrelated to source temperature

INTRODUCTION

Temperature governs the rates of all biological processes, influencing the physiology, morphology, and behavior of organisms (Angilletta, 2009). Consequently, changing thermal regimes caused by anthropogenic climate change represent an ongoing threat to species on a global scale (Deutsch et al., 2008; Maclean & Wilson, 2011; Midgley & Hannah, 2019). When dispersal to suitable thermal habitats is constrained, organisms must rely on phenotypic plasticity or evolutionary change (Gienapp et al., 2008). However, since the rate of evolution may, for many species, lag behind the rapid pace of climate change, phenotypic plasticity is likely vital for persistence under changing thermal regimes (Huey et al., 2012; Skelly et al., 2007). Predictions for how plasticity allows organisms to cope with climate change often assume that responses will be uniform within species, but intraspecific variation in phenotypic plasticity is common (Alonzo, 2015; Foster, 1999, 2013). Assuming plastic responses are homogenous can produce inaccurate predictions about the consequences of climate change (Pearman et al., 2010; Peterson et al., 2019; Tüzün & Stoks, 2018; Valladares et al., 2014). Thus, to accurately assess the potential for plasticity to mediate climate change effects, we must measure and identify causes of individual variation and population-level differences in thermal plasticity.

Theoretical predictions assume that differences in plasticity among populations will evolve in response to variation in historical thermal regimes, since contrasting thermal environments impose unique selective pressures (Huey & Kingsolver, 1989; Kingsolver, 2009; Sinclair et al., 2012). These assumptions are rooted in the thermodynamics of chemical reactions, which scale up to influence the physiological responses of organisms (Angilletta et al., 2002; Huey & Kingsolver, 1989). Differences in average temperatures among populations can be a powerful driver of evolutionary divergence in phenotypic plasticity, causing plastic responses to shift in response to local conditions (Angilletta et al., 2002; Angilletta, 2009; Angilletta et al., 2010; Huey & Kingsolver, 1989) (Fig 1.1). This prediction has been empirically tested by comparing plasticity among populations spanning natural thermal gradients, such as those generated by spatial differences in latitude (Fragata et al., 2016; Pereira et al., 2017), altitude (Caldwell et al., 2017), urbanization (Tüzün et al., 2017), or geothermal environments (D. C. Fryxell et al., 2020; O'Gorman et al., 2014). Studies identifying population differences in plasticity caused by divergent temperatures have largely focused on traits related to whole-organism performance, including heat tolerance (Carbonell & Stoks, 2020; Mesas et al., 2021; Pereira et al., 2017), growth rates (Carbonell & Stoks, 2020; Tüzün et al., 2017), and locomotion (Mesas et al., 2021; Richter-Boix et al., 2015). These thermally-sensitive whole-organism traits have indirect implications that can impact other traits such as behavior (Abram et al., 2017; Ord & Stamps, 2017), although comparisons of behavioral plasticity among populations spanning a thermal gradient are relatively rare. Given the importance of behavioral plasticity for mediating the effects climate change (Beever et al., 2017; Riddell et al., 2018; Wolff et al., 2020), this represents a major gap in our understanding of thermal adaptation.

Population differences in behavioral plasticity across a thermal gradient can be conceptualized using thermal performance curves, a type of reaction norm that illustrates how phenotypes change as a function of ambient environmental temperature (Kingsolver, 2009). The general shape of thermal performance curves is typically concave, increasing with temperature to a maximum performance level before declining rapidly (Huey & Kingsolver, 1989; Izem & Kingsolver, 2005) (Fig 1.1A). Population differences in plasticity across a thermal gradient can be characterized by shifts in the location of two key landmarks of thermal performance curves: the peak of the curve (maximum performance), and the temperature at which the peak occurs (thermal optimum).These landmark values are predicted to shift across thermal gradients of source temperature, with three potential outcomes (Fig 1.1): (i) To maintain maximum performance at local temperatures, thermal performance curves may shift horizontally such that the thermal optimum within a population increases with average source temperature (Izem & Kingsolver, 2005) (Fig 1.1B). (ii) If evolutionary and environmental forces are opposed, counter-gradient selection may occur, leading to a vertical shift in which maximum performance decreases with temperature (Conover & Schultz, 1995; Hodgson & Schwanz, 2019) (Fig 1.1C). (iii) A hotter-is-better shift may occur, leading to a correlated increase in both the thermal optimum and maximum performance, since warm-adapted populations may be subject to fewer physiological constraints on maximum performance than populations in colder environments (Angilletta et al., 2010; Huey & Kingsolver, 1989; Kingsolver, 2009) (Fig 1.1D). Although the evolutionary theory underlying these predictions has been rigorously developed (Angilletta, 2009), empirical tests identifying these possible outcomes in natural systems are rare, especially for plastic behavioral traits (Arnold et al., 2019).

Precopulatory mating behaviors are promising candidates for testing how historical differences in average temperature across a thermal gradient affect behavioral plasticity (García-Roa et al., 2020). Plastic responses to temperature have been documented in the mating behavior of several taxa, including fish (Condon & Wilson, 2006; Wilson, 2005), songbirds (Coomes & Derryberry, 2021), anurans (Oseen & Wassersug, 2002), and insects (Leith et al., 2021; Macchiano et al., 2019). Since mating behavior precedes and thus constrains all other aspects of successful reproduction, these behaviors are closely linked to fitness and may be subject to strong selection. Additionally, selection may act indirectly on behavioral plasticity through the thermal dependence of related traits. For example, evolved differences in the plasticity of locomotory ability may affect an individual's ability to find, pursue, or court potential mates (Bennett, 1990), and differences in the plasticity of metabolic rates across a thermal gradient may impact the energetic costs of engaging in mating behavior (Cummings & Gelineau-Kattner, 2009; Moffett et al., 2018). Thus, if populations differ in the plasticity of these related traits across a thermal gradient, we might expect parallel changes in the plasticity of mating behavior. Since thermal limits to reproduction can be more consequential for species persistence than other survival-related traits in the context of climate change (Parratt et al., 2021), understanding how plasticity in mating behavior varies across a thermal gradient is crucial.

Western mosquitofish (*Gambusia affinis*) are a powerful focal organism for examining how historical exposure to divergent average temperatures affects the plasticity of mating behavior. Mosquitofish are well-known for their impressive thermal tolerance and plastic responses to temperature, a trait that has enhanced their ability to invade ecosystems on a global scale and made them an ideal model organism in evolutionary biology (D. C. Fryxell et al., 2022; Pyke, 2005, 2008). Additionally, mosquitofish mating behavior is a common focus of studies examining sexual selection, mating behavior, and sexual conflict (Bisazza & Marin, 1995; Cummings, 2018; Kim et al., 2021; Wang et al., 2015). Mosquitofish mating behavior includes both courtship displays, characterized by males twisting their body into a sigmoid shape near potential mates, and coercive behavior, in which males obtain copulations by persistently chasing and attempting copulations with unreceptive females (Bisazza, 1993; Pilastro et al., 1997). Fertilization occurs internally, and successful copulation requires insertion of the male gonopodium, an extended anal fin used for sperm transfer, into the female gonoduct (Pyke, 2005). Mating behavior of mosquitofish is plastic in response to ambient temperature (Laudien & Schlieker, 1981; Wilson, 2005), although reproductive traits can be highly variable depending on local conditions, suggesting that behavior may also vary depending on historical thermal environments (Pyke, 2005).

In this study, we used common-reared western mosquitofish from geothermal springs spanning a broad thermal gradient to ask how historical differences in average temperature impact the plasticity of mating behavior. Selected focal springs maintained large differences in mean temperatures with minimal thermal variation due to the influx of geothermal spring water, an ideal setting for testing the evolutionary consequences of divergent average temperatures on thermal plasticity. We specifically asked 1) if male mosquitofish mating behavior (courtship displays, copulation attempts, copulations, and mating efficiency) was plastic in response to ambient environmental temperature; 2) if plastic responses in male mosquitofish mating behavior differed among populations with average temperatures spanning a broad thermal gradient; and 3) if estimated landmarks in the thermal performance curve of mating behavior, including maximum performance and thermal optima, depended on source temperature, following predicted patterns of landmark shifts described above and in Figure 1.1. In answering these three questions, we aim to determine whether divergent thermal regimes can generate temperature-dependent predictable patterns of variation in plasticity across populations that correspond to theoretical expectations.

METHODS

Study system

Western mosquitofish are a small, livebearing species of freshwater fish in the poeciliid family. They are native to the southeastern United States and have been introduced globally to consume and control larval mosquitos (Pyke, 2005, 2008). Mosquitofish from Texas were introduced to California in 1922 and have since been distributed throughout the state, although detailed records of their spread are rare (Dill & Cordone, 1997). Populations of mosquitofish used in this study originate from seven geothermal ponds in Inyo and Mono counties, CA (Fig 1.2A). Thermal environments in these geothermal ponds are governed by an influx of constant-temperature spring water, with each pond maintaining a relatively constant average temperature throughout the year despite seasonal changes in air temperature (Fig 1.2B). Among the seven ponds, average source temperatures span a thermal gradient ranging from 18.8 to 33.3 °C (Fig 1.2B, Table A1.1). Mosquitofish within these springs are constantly exposed to water within a narrow temperature range, and physical isolation among ponds limits gene flow. Although the exact date of mosquitofish introduction to these ponds is unknown, mosquitofish generation times are approximately 3 months long (Pyke, 2005), offering sufficient time for evolutionary change within most California populations to occur. Indeed, previous work in this system has found evidence of local adaptation to source temperature in several traits, including metabolic rates, juvenile growth rates, and reproductive effort (D. C. Fryxell et al., 2020; Moffett et al., 2018).

Mosquitofish collection and rearing

Mosquitofish from each geothermal pond were obtained and reared following protocols described below (for further information see Fryxell et al. 2020). Wild-caught fish (F0s) were collected from each pond in February 2018 and were transported to the University of California, Santa Cruz, where F0, F1, and F2 fish were reared and housed in an environmentally-controlled greenhouse. Throughout the rearing process, all tanks were maintained at 26 ^{o}C, the average of the highest and lowest source pond temperatures in the natural thermal gradient. Adult fish were fed Tetramin (Tetra Holding, Blacksburg, VA, USA) flake food *ad libitum* twice per day, and juveniles were fed additional Frystartr (Skretting Inc, Stavanger, Norway) once per day to encourage growth. Mosquitofish reproductive activity is sensitive to photoperiod (Pyke, 2005), so to encourage reproduction throughout the rearing process, the greenhouse photoperiod was set to 14:10 h daylight: dark using overhead full-spectrum lighting. To maintain water quality and remove waste, half of the water in each tank was siphoned and replaced twice weekly.

After collection, adult wild-caught fish from each population were randomly assigned to 568 L tanks. Collection and rearing of the first generation of lab-reared fish (F1s) began one month after adult collection to ensure that offspring were not exposed to their parent’s thermal environment at their source ponds during development. Fry were collected from a fry retention device within each tank (Appendices, Fig. A1.1) and were transferred to fry baskets hung in 57 L tanks to protect maturing fish from adult filial cannibalism. When at least 90 F1 fish were collected from each population, approximately one month after collection commenced, F0 fish were euthanized, and their tanks were drained and reset with clean water. F1 fish were introduced to these tanks, and in November 2018, collection of second-generation laboratory reared offspring (F2s) began, following the fry collection process described above. Up to 10 newborn fish per population were collected each day to ensure representative genetic diversity in F2s. When at least 150 individuals were collected per population, F2 fish were transferred to 132 L tanks to provide additional room for growth, and were subsequently introduced to larger 568 L tanks as they matured.

To provide easier access to fish during behavioral assays, mature F2 fish were transferred from greenhouse tanks to 24 L aquaria in a controlled environment room (TriMark R. W. Smith, San Diego, CA, USA) in June 2019. 14 fish were introduced to each aquarium in a 1:1 male: female sex ratio, with between 4 and 7 replicate tanks per population. During transfer, each male was injected with a single visible implant elastomer tag adjacent to their dorsal fin for individual identification during behavioral assays (Northwest Marine technology, Inc., Anacortes, WA, USA). To reduce animal stress and minimize disease risk, tanks were maintained with 1.25 g of aquarium salt (API Aquarium Salt, Chalfont, PA, USA) per liter and included artificial plants. Controlled environment rooms were set to an air temperature of 26 ± 0.5^{o} C with a photoperiod of 14:10 h daylight: dark supplied by overhead lighting and lights above each aquarium. To maintain water quality and homogenize tank temperatures, each tank was equipped with an aquarium filter (Marina Power Filter, Hagen Inc., Mansfield, MA, USA). Waste was siphoned from tanks during one-third water changes once per week.

Standardized female fish were used in behavioral assays to minimize differences in male behavior that could be caused be differences in female traits. Female fish were obtained from a pond separate from the focal source populations (TMo Fishery, Sacramento, CA USA). These fish were housed in 568 L tanks within an environment-controlled greenhouse on the UC Santa Cruz campus. Tanks were maintained using identical protocols described for F1 fish above.

Behavioral Assays

The mating behavior of each male was observed at 5 ecologically relevant ambient temperatures (15, 20, 26, 32, and 37 ºC) that encompassed the thermal gradient of source populations. Based on previous studies of mosquitofish reproductive behavior, these temperatures were also broad enough to encompass the declining performance at extreme temperatures characteristic of thermal performance curves (Haynes, 1993; Wilson, 2005). The order of observation temperatures was randomized for individual males, although all males were first observed at intermediate temperatures (20, 26, 32) because we anticipated possible mortality at extreme temperatures due to thermal stress (Otto, 1973; Wilson, 2005).

Behavioral assays were performed within a controlled environment room that enabled precise manipulation of air temperature (TriMark R. W. Smith, San Diego, CA, USA). Mosquitofish acclimation to each ambient temperature began at least 16 hours prior to behavioral observations. During acclimation, male fish were isolated in individual fry baskets within temporary holding aquaria to control for behavioral variation among males caused by recent exposure to females. Female fish acclimated within mixed-sex 57 l holding tanks within the controlled environment room. Non-focal males were included in female acclimation tanks to prevent increased female receptivity that can result when female mosquitofish are isolated from males (Hughes, 1985). Acclimation tank temperatures were adjusted at a rate not exceeding 4 ºC per hour using both 150 W submerged heaters (Eheim, Deizisau, Germany) and controlled environment room temperature settings. Water temperatures were maintained at ± 0.1 ºC from the desired observation temperature using digital temperature monitors (InkBird ITC-308, London, England).

Behavioral observations were recorded in an aquarium (40 x 21 x 12 cm deep) filled with treated tap water (Hikari USA Ultimate Water Conditioner, Hayward, CA) (Fig A1.2). A camera (Olympus Stylus TG-4, Bethlehem, PA) was placed on a shelf above each tank to record behavior, with two 18 W lights affixed beneath the shelf to illuminate the field of view. To reduce behavioral impacts of external stimuli, observation tanks were lined with blue felt. Each tank also contained an artificial plant to provide structural complexity. To eliminate chemical cues released from previous mosquitofish, aquarium water was emptied and replaced before each observation.

Prior to observations, an opaque divider was placed in the aquarium to create two acclimation chambers in the observation tank. Two size-matched females were randomly selected and were placed on one side of the divider, with a focal male placed on the opposite side. Fish acclimated to the tank environment for 20 minutes before observations began. To initiate each observation, the opaque divider was lifted from the tank. Behavioral interactions were recorded for 20 minutes, after which all fish were removed from the aquarium and placed into a recovery tank. The temperature within recovery tanks was returned to 26 ºC at a rate not exceeding 4 ºC per hour. Once recovery tank temperatures reached 26 ºC, mosquitofish were returned to their original housing tank and their behavior was not measured again for at least five days.

Video analysis

To quantify reproductive behaviors, we analyzed video recordings of mosquitofish interactions using CowLog 3.0 behavioral coding software (Hänninen & Pastell, 2009). Videos in which females gave birth during the observation period were excluded from analysis because postpartum female cues can modify male behavior (Farr, 1989). We recorded the total number of courtship displays, copulation attempts, and copulations performed by each male during the 20-minute observation period. Courtship displays occurred when males arched their body into an sigmoid-shape characteristic of poecilid courtship behavior (Bisazza, 1993). Copulation attempts were counted when a male approached a female from behind and oriented beneath the female caudal peduncle, a position required for copulation in livebearing fish (Bisazza et al., 2001). Copulations occurred after a subset of attempts and were identified by the rapid twisting motion that accompanied the male removal of the gonopodium from the female gonoduct (Wilson, 2005). Video observers were trained to identify behaviors on a standardized set of videos before collecting data to ensure consistency among observations. Each video observer was blind to treatment and source population identity when identifying and counting behaviors.

Statistical analyses

To test for population differences in the plasticity of mating behavior based on historical temperature exposure, we used generalized linear mixed effect models (GLMMs) to determine the effects of population identity, source temperature, and ambient temperature on each behavior. Male identity was included as a random effect, as we had up to five repeated measures for each individual male (Zuur et al., 2009). Separate models were developed for displays, copulation attempts, copulations (Table 1.1).

Displays, copulation attempts, and copulations were modeled using zero-inflated negative binomial GLMMs, which account for excess zeros and over-dispersion in response variables (Zuur et al., 2009). Zero-inflated models, also known as mixture models, treat processes generating excess zeros as separate from count data. Thus, our response variable was modeled in two parts, a binary process which modeled the probability of measuring a zero (zero-inflated binomial model), and a count process (negative binomial model). In addition to a model for counts of copulations, we also included a model for mating efficiency, the proportion of mating attempts resulting in copulations (successful copulations/ attempted copulations). Mating efficiency was modeled using a binomial GLMM.

Models were fitted using maximum likelihood estimation, with random effects assumed to be Gaussian on the scale of the linear predictor and integrated using the Laplace approximation. Zero-inflated negative binomial GLMM estimates were performed using the *glmmTMB* library (M. E. Brooks et al., 2017) and binomial GLMM estimates were performed using the *lme4* library (Bates et al., 2015) in R (version 4.1.0) and RStudio (RStudio, Inc., Version 0.98.981). We implemented a backward selection approach to identify the most appropriate explanatory model for each response variable. First, we tested overall model significance by estimating a full model including all fixed and random effects and comparing it to an intercept-only model. We used Wald X^2 tests to perform backward stepwise comparisons to determine which parameters to include in our final best fit model. Effects that did not differ significantly from zero were excluded from the final model.

All GLMM full models for each behavior contained a fixed effect called ambient temperature, the temperature at which behavior was measured. An effect of ambient temperature on observed behavior indicated behavioral plasticity. To account for the curvature characteristic of plastic responses to temperature, ambient temperature was modelled using a set of orthogonal polynomials, which minimized the collinearity of fixed effects. Polynomials included quartic, cubic, quadratic, and linear effects in models for displays, copulation attempts, and copulations, and quadratic and linear effects in models for mating efficiency. Ambient temperature was scaled and centered to the mean to support convergence during model fitting.

To test for an effect of historical thermal regimes on the plasticity of mating behavior, the average source temperature of each pond was also included as a fixed effect in the full model for each behavior. As with ambient temperature, source temperature was scaled and centered to the mean to support convergence during model fitting. Inclusion of source temperature in the best fit model for observed behaviors suggested that differences among populations were driven by historical thermal regimes in each pond and may represent local adaptation in behavioral plasticity. Each full model also included an interaction term between source temperature and ambient temperature. A significant interaction term indicated differences in the overall shape of the plastic response among populations due to source temperature.

While source temperature can be a strong selective factor in shaping behavioral plasticity, we were also interested in whether differences in plasticity among populations existed that were unrelated to historical thermal regimes. To capture this effect, created a second GLMM for each behavior that replaced the fixed effect of source temperature with population identity, an unranked categorical variable that corresponded to each source population. If population identity was included in the final best fit model for observed behaviors, populations differed in the magnitude of behavioral plasticity, although the source of differences could not be identified. If significant effects of both population identity and source temperature were found for a mating behavior, we used Akaike information criterion scores to identify which model best explained observed behavior. We provide a detailed summary of model structures in the Appendix (Tables A1.3- A1.6).

To assess model assumptions (normality, linearity, and homogeneity of variance) we used a simulation-based approach using the *DHARMa* library in R, since interpretation of conventional residuals for GLMMs is often problematic (Hartig, 2021). Visual assessments of assumptions were performed using QQ plots and plots of the simulated residuals against fitted values. We used a Kolmogorov-Smirnov test to test for normality and Levene's test to test for homogeneity of variance.

While GLMMs answered the general question of whether behavioral responses to ambient and source temperatures differed among populations, we were also interested in identifying differences among populations related to specific thermal performance landmarks that are linked to predictions of how average source temperatures impact plasticity (Fig 1.1). Specifically, we were interested in whether maximum performance (the highest trait value observed across ambient temperatures) and thermal optima (the temperature at which maximum performance occurs) were correlated with average source population temperatures. We identified maximum performance for each male as the highest number of displays, copulation attempts, or copulations an individual performed across all 5 ambient temperatures. Each male's thermal optimum was estimated as the ambient temperature at which the maximum performance occurred. When maximum performance values were equal at several temperatures, the thermal optimum was calculated as the average between the temperatures. Individuals that were not measured at all 5 ambient temperatures were excluded from landmark analyses. We tested for differences in maximum performance among populations using a Kruskal-Wallis test. If differences among populations were detected, we used linear regression to test whether source temperature could explain the differences. Chi-squared tests were used to test for differences in thermal optima among populations.

RESULTS

The total number of males observed from each population at each ambient temperature ranged from 17 to 32 individuals (Table A1.2). Sample sizes varied among populations due to random differences in mortality caused by an outbreak of columnaris disease in the rearing of F2 fish. During the 20-minute observation period, the frequency of courtship displays, copulation attempts, and copulations performed by an individual male ranged from 0 to 16, 0 to 113, and 0 to 17, respectively (Fig A1.3- A1.5). Ambient temperature had a significant effect on courtship displays, copulation attempts, and copulations, indicating behavioral plasticity (Table 1.1, Fig 1.3A, Fig 1.4A, Fig 1.5A). The frequency of all three mating behaviors demonstrated broad thermal resilience characteristic of mosquitofish (Pyke, 2005), with behavior increasing between 15 and 20 ºC, plateauing between 20 and 32 ºC, and declining between 32 and 37 ºC. Note that the coefficient estimates for all models and figures described below, including model fits and raw data, can be found in the appendices (Table A1.3- A1.7, Fig A1.3- A1.7).

We found no effect of source temperature on the frequency of courtship displays ($\chi^2 = 0$, df = 1, p = 1), copulation attempts ($\chi^2 = 0.3072$, df = 1, p = 0.58), or copulations ($\chi^2 = 0.8245$, df = 1, p = 0.3639). However, population identity had a significant effect on the frequency of all three behaviors (courtship displays: $\chi^2 = 61.737$, df = 5, $p < 0.001$; copulation attempts: $\chi^2 = 14.225$, df = 5, $p < 0.05$; copulations: $\chi^2 = 12.071$, df = 5, $p < 0.001$), indicating that populations differed in mating behaviors due to reasons other than source temperature. None of the final best-fit models contained an interaction term, either between source temperature and ambient temperature or population identity and ambient temperature (Table 1.1). Thus, differences in behavioral responses to ambient temperature among populations were attributed to differences in overall elevation of the performance curves (i.e. differences in mean level of behaviors) rather than differences in the shape of the curves.

Observed mating efficiencies (i.e. the probability that a mating attempt was successful) spanned from 0 to 1, encompassing the entire possible range of values (Fig 1.5, A1.6). Ambient temperature, (χ^2 = 83.776, df = 2, $p < 0.001$), source temperature (χ^2 = 8.6819, df = 1, $p < 0.001$), and the interaction between ambient and source temperature (χ^2 = 11.361, df = 2, $p = 0.003$) affected mating efficiency. When population identity was substituted for source temperature in the full model, ambient temperature, (χ^2 = 83.776, df = 2, $p < 0.001$), population identity (χ^2 = 8.6819, df = 1, $p < 0.001$), and the interaction between ambient and population identity (χ^2 = 11.361, df = 2, $p = 0.003$) had significant effects on mating efficiency. Inclusion of an interaction term in these models indicated differences in the shape of thermal performance curves for mating efficiency among populations. While mating efficiency was highly responsive to ambient temperature in some populations, in other populations it was unaffected by ambient temperature (Fig 1.5A). Although the inclusion of source temperature in the best fit model suggested that source temperature had a significant effect on mating efficiency, Akaike information criterion scores revealed that the model containing population identity (AIC: 1732.6) was a better fit than the model containing source temperature (AIC: 1783.7). Thus, while source temperature could explain some of the difference between populations, population identity provided greater explanatory power than source temperature.

To test the assumptions of generalized linear mixed effects models (normality, linearity, and homogeneity of variance), we performed model diagnostics on simulated residuals, which identified departures from assumptions in all mixed effects models describing courtship displays, copulation attempts, copulations, and mating efficiency. Shapiro-Wilks tests on simulated residuals found that all final models violated the assumption of normality (Table A1.7). However, visual assessment of Q-Q plots showed only minor deviations from normality. We also found deviations from homogeneity in models for courtship displays and copulation attempts (Table A1.7). However, because linear mixed effects models are robust to violations of normality and small violations of homogeneity, observed deviations were unlikely to affect interpretation of the results (Schielzeth et al., 2020; Zuur et al., 2009).

To identify shifts in plasticity at a finer resolution and test for evidence of the specific theoretical predictions of thermal adaptation described in Figure 1.1, we also tested for population divergence in landmarks of thermal performance curves. Males that were not measured at all five ambient temperatures were excluded from all landmark analyses. Males that had at least one observation with zero mating attempts were by necessity also excluded from landmark analyses for mating efficiencies, since mating efficiencies could not be calculated when zero attempts were performed (mating efficiency = copulations / total copulation attempts). Due to the frequency with which there were no successful copulations in one of the behavioral assays, these conditions required over 75% of males to be excluded from landmark analyses for mating efficiencies, leaving insufficient data to compare mating efficiency landmarks among populations. For the remaining behaviors, sample sizes of males from each population ranged from 15 to 24 individuals (18.9 ºC: n = 21; 21.1 ºC: n = 15; 23.7 ºC: n = 21; 27.0 ºC: n = 21; 31.6 ºC: n = 23; 33.3 ºC: n = 24).

Landmark analysis comparing thermal optima among populations found that populations did not differ in the temperature at which maximum performance occurred for courtship displays (Pearson's Chi-squared, $\chi^2 = 33.002$, df= 30, p = 0.3225) (Fig 1.3B), copulation attempts (Pearson's Chi-squared, $\chi^2 = 17.392$, df= 20, p = 0.6274) (Fig 1.4B), or copulations (Pearson's Chi-squared, $\chi^2 = 42.211$, df= 35, p = 0.1874) (Fig 1.5B). However, we found differences among populations in the maximum performance of courtship displays observed (Kruskal-Wallis, $\chi^2 = 25.951$, df= 5, $p < 0.001$) (Fig 1.3C), although there was no correlation between source temperature and maximum performance (linear regression, $R^2 = -0.008$, $F_{1,123} = 0.014$, p= 0.907). We found no differences in maximum number of copulation attempts

(Kruskal-Wallis, $\chi^2 = 2.904$, df= 5, p =0.7148) (Fig 1.4C) or copulations (Kruskal-Wallis, $\chi^2 = 9.5205$, df= 5, p = 0.09002) (Fig 1.5C) among populations.

DISCUSSION

In this study, we used a rigorous common-rearing design to test whether thermal sensitivity in mating behavior differed among populations of mosquitofish spanning a broad thermal gradient. Mosquitofish courtship displays, copulation attempts, and copulations were plastic in response to ambient environmental temperatures, with behavior frequencies increasing from 15 to 20 ºC, plateauing between 20 and 32 ºC, then sharply declining at 37 ºC. Although we anticipated a link between source temperatures and plastic responses based on theoretical predictions (Angilletta, 2009), we found that differences in plasticity of mating behavior among populations could not be explained by source temperatures. We also tested for population differences in thermal optima and maximum performance for courtship displays, copulation attempts, and copulations and found differences in only in the maximum performance of courtship displays among populations, which were also unrelated to source population temperature. Our results represent a departure from the predictions of theory and previous empirical work suggesting that historical thermal regimes influence the evolution of thermal performance.

The plasticity of mating behavior observed in this study is consistent with previous findings on the temperature sensitivity of reproductive behavior in mosquitofish

(Laudien & Schlieker, 1981; Wilson, 2005). Mosquitofish possess one of the most extreme thermal ranges of reproductive activity in ectotherms (Wilson, 2005), and mating behaviors, particularly copulation attempts, were observed at all ambient temperatures used in this study. While mating behavior was dependent on ambient temperature, this effect was largely driven by the decline in performance at the extreme ends of the thermal spectrum. Thermal performance curves for each mating behavior were relatively flat, with no distinction between intermediate temperatures but large differences in performance between intermediate and extreme temperatures. The steep declines in performance at extreme temperatures and consistency of the response among populations suggest that behavioral plasticity in mosquitofish mating may be shaped by physiological constraints on activity levels in ectotherms (Angilletta et al., 2002; Gunderson & Leal, 2015). In ambient temperatures where physiology places no constraints on physical activity, selection may have favored high investment of energy toward mating behavior, since mosquitofish males face high levels of intrasexual competition for mates (Bisazza & Marin, 1995; Deaton, 2008; Mcpeek, 1992). Although the energetic demands of maintaining high mating performance may vary across intermediate temperatures due to temperature dependence of metabolic rates (Cano & Nicieza, 2006; Schulte, 2015), males may adjust their energy budgets to maintain constant mating activity by modifying other behaviors or aspects of their physiology. However, at high or low temperatures, mosquitofish are subject to heightened physiological stress and cannot compensate through other mechanisms, leading to a decline in mating behavior (Otto, 1973; Wilson, 2005).

While the general concave shape of plastic responses to temperature was consistent among populations, elevations of thermal performance curves for mating behavior differed. However, despite large divergence in average temperatures among populations, differences in the plasticity of mosquitofish courtship displays, copulation attempts, and copulations could not be explained source temperature, and landmark shifts did not adhere to theoretical predictions for how average temperatures impact thermal performance (Fig 1.1). While source temperature had a significant effect on mating efficiency, our model of population differences that excluded source temperature had higher predictive value than the model that included it. One reason why source temperature may not explain differences in the plasticity of mating behavior is that the strength of selection imposed by temperature may be weaker on mating behavior compared to other performance traits where local adaptation in plasticity has been observed. Traits such as thermal tolerance, locomotion, and growth rates, which are often the focus of studies on local adaptation in thermal performance, are directly tied to temperature through the thermal dependence of chemical reactions (Angilletta et al., 2002; Asbury & Angilletta, 2010). Consequently, temperature is expected to impose strong selective pressure on these traits, causing evolutionary divergence among populations with different average temperatures. However, evidence of local adaptation in these traditionally thermally-sensitive traits often contrasts with predicted expectations when tested in mosquitofish inhabiting our focal geothermal springs. While previous studies on mosquitofish from these same geothermal ponds have found evidence of local adaptation to source temperature in juvenile growth rate (Fryxell et al. 2020) and excretion rate (Benavente et al., 2022), differences in field-measured metabolic rates in wild fish were found to be driven by plasticity rather than evolutionary change, a surprising result considering metabolic rates are directly tied to the rate of chemical reactions (Benavente et al., 2022; Moffett et al., 2018). Benavente et al. (2022) suggested that evolutionary downsizing in populations of mosquitofish inhabiting warmer geothermal springs offset the increased metabolic demands associated with higher temperatures due to the allometric scaling of metabolism (Brown et al., 2004). This example highlights the need to consider suites of traits, rather than individual traits, when predicting how temperature influences evolutionary trajectories, and may offer a potential explanation for why behavioral plasticity was unaffected by source temperatures.

While average temperature is considered a key abiotic driver of evolutionary change, mating behavior relies not only on abiotic environmental conditions, but also on complex social interactions (Andersson, 1994). The outcome of mating depends on a suite of factors such as intrasexual competition (Booksmythe et al., 2013), mate choice preferences (Bisazza, 1993; Bisazza et al., 2001), female responses (Dadda et al., 2005, 2008), and sex ratios (Bisazza & Marin, 1995; Smith & Sargent, 2006), each of which contributes to the intensity of sexual selection (Andersson, 1994). Since there is little variation in other abiotic factors among geothermal ponds in this system (Table A1.1), sexual selection caused by social conditions may be the primary driver of divergence in mating behavior among populations. Future research will aim to disentangle how the many facets of the mosquitofish social environment influence population differences in mating behavior.

Overall, this study demonstrates that evolutionary responses to thermal regimes may be less predictable than theoretical expectations suggest. Temperature is often assumed to be a dominant abiotic driver of evolutionary processes and trait diversity. While we understand the proximate consequences of temperature on biochemical processes (Angilletta, 2009), theoretical predictions of how selection across a thermal gradient affects phenotypic plasticity far outpace empirical work, and as our study demonstrates, experimental tests of theory frequently deviate from expectations. In fact, a recent meta-analysis found limited evidence that local adaptation in plasticity occurs in response to historical thermal regimes, although the authors admit the lack of evidence may be due to the paucity of studies rather than the absence of the evolutionary phenomenon (Arnold et al., 2019). If we aim to identify and protect the most at-risk populations as climate warms, we must not only collect more data on thermally sensitive traits, but also ensure synergy between theory and empiricism by designing rigorous experiments that explicitly test theoretical predictions.

TABLES

Table 1.1. Results of generalized linear mixed effect models used to test for effects of source temperatures or population identity on mosquitofish mating behavior. The table lists all fixed effects considered in the starting models, with non-significant fixed effects removed sequentially in order from top to bottom through backward, stepwise model selection. Significance was determined using Wald χ^2 tests. Fixed effects retained in the final models are in bold and indicated by an asterisk. Coefficients of the final models are described in Appendix Tables A1.3- A1.6.

Response	Fixed Effect	χ^2	df	p value
Courtship displays (n=198, units = count)				
	Source temperature x Ambient temperature	7.7534	4	0.101
	Source temperature	0	1	1
	Ambient temperature	342.5	4	**<0.001***
Courtship displays (n=198, units = count)				
	Population ID x Ambient temperature	28.106	20	0.1069
	Population ID	61.737	5	**<0.001***
	Ambient temperature	370.64	4	**<0.001***
Copulation attempts (n= 198, units = count)				
	Source temperature x Ambient temperature	1.3708	4	0.8492
	Source temperature	0.2982	1	0.585
	Ambient temperature	112.83	4	**<0.001***
Copulation attempts (n= 198, units = count)				
	Population ID x Ambient temperature	21.037	20	0.395
	Population ID	14.225	5	**0.014***
	Ambient temperature	117.88	4	**<0.001***
Copulations (n= 198, units = count)				

Source temperature x Ambient temperature	3.5051	4	0.4771
Source temperature	0.8314	1	0.3619
Ambient temperature	112.55	4	**<0.001***
Copulations (n= 198, units = count)			
Population ID x Ambient temperature	25.26	20	0.192
Population ID	12.071	5	**0.034***
Ambient temperature	114.86	4	**<0.001***
Mating efficiency (n=198, units= success/ failures)			
Source temperature x Ambient temperature	11.361	2	**0.003***
Source temperature	8.6819	1	**0.003 ***
Ambient temperature	83.776	2	**<0.001***
Mating efficiency (n=198, units= success/ failures)			
Population ID x Ambient temperature	33.371	10	**<0.001***
Population ID	25.05	5	**<0.001***
Ambient temperature	86.1	2	**<0.001***

FIGURES

Figure 1.1. Hypothetical shifts in thermal performance curves, thermal optima (T_{opt}), and maximum performance (T_{max}) among populations with different historical average temperatures. (A) If average source temperatures have no effect on behavioral plasticity, performance curves and landmarks will not differ among populations. Patterns of evolutionary divergence among populations related to average source temperature may include (B) a horizontal shift, (C) vertical shift, or (D) hotter-is-better shift.

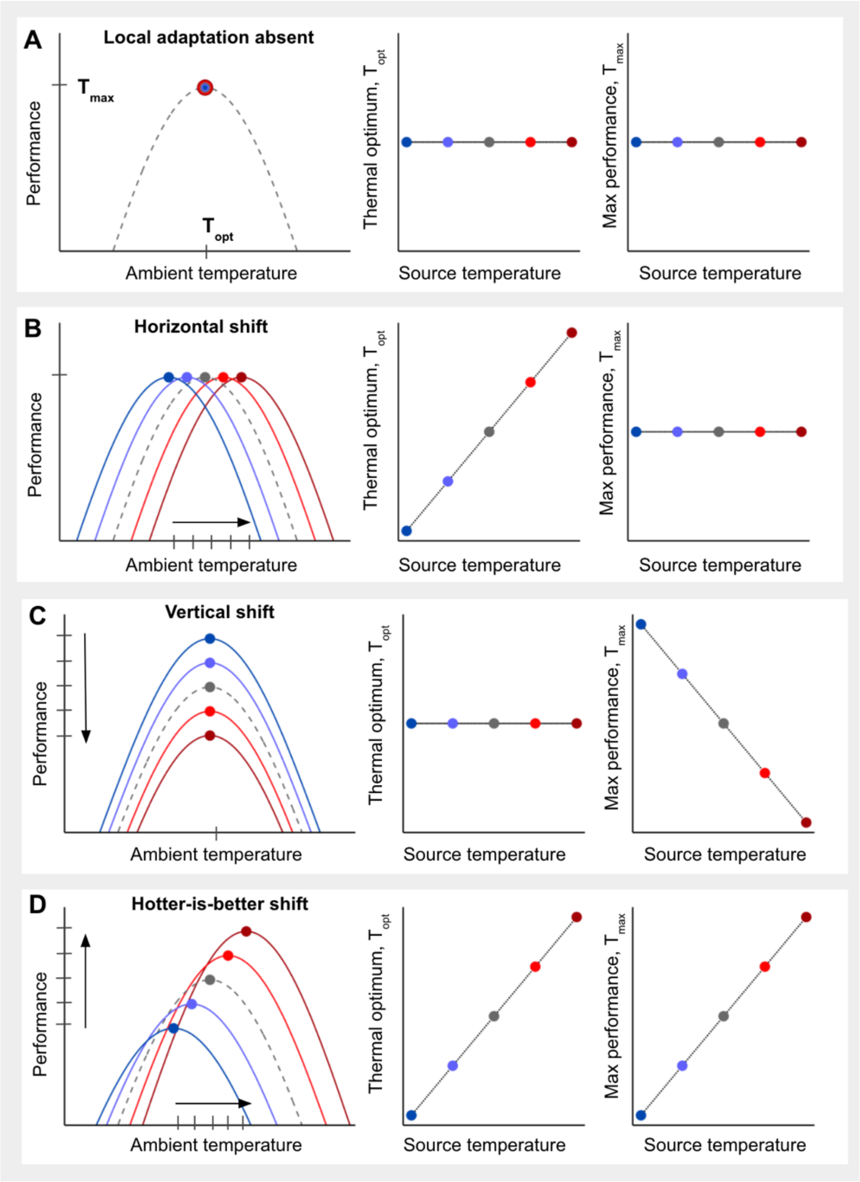


Figure 1.2. (A) Map of the geographic locations of geothermal spring ponds in Inyo and Mono counties, California, with colored points corresponding to the average source pond temperature. Black points indicate regional landmarks. (B) Temperature profiles of each geothermal spring measured at 15-minute intervals during spring 2014 (solid and colored lines). Dashed lines indicate average temperature during the sampling period. The daily average temperature measured at the Bishop, CA airport is plotted in black for reference. The temperature recorder at the warmest site (33.3 °C, “LHC”) failed on 25 February (adapted from Fryxell et al. 2020).

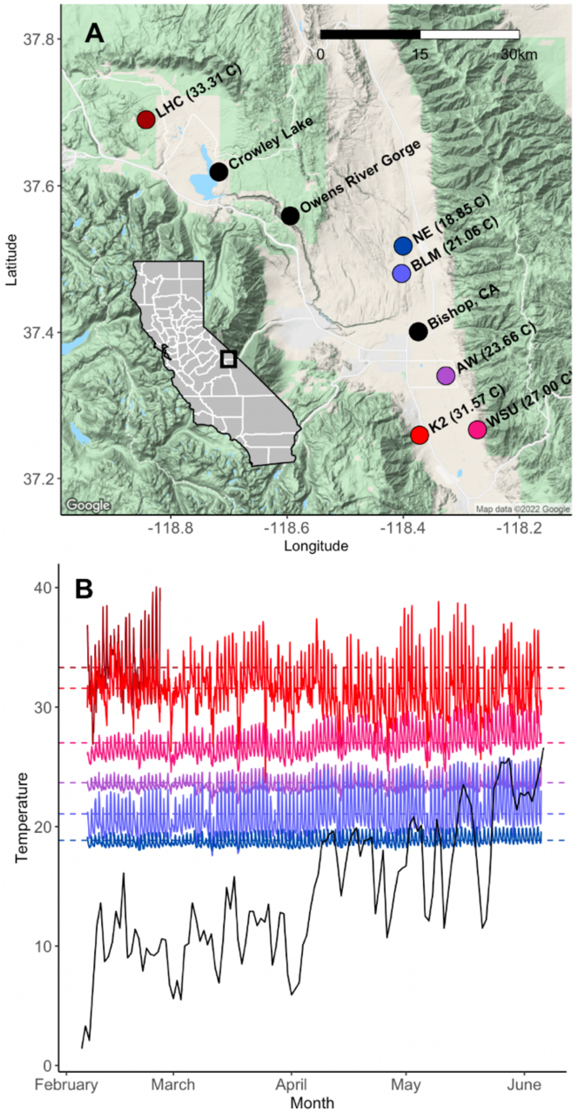


Figure 1.3. (A) Thermal performance curves for male courtship displays differed among populations of mosquitofish, but were unrelated to average source pond temperatures. Estimated marginal means at each ambient temperature are connected by solid lines. Error bars indicate +/- 2 SE from predicted estimated marginal mean values. (B) Thermal optima did not differ among populations. (C) Differences in maximum courtship displays among populations were unrelated to source pond temperatures (* = $p < 0.05$).

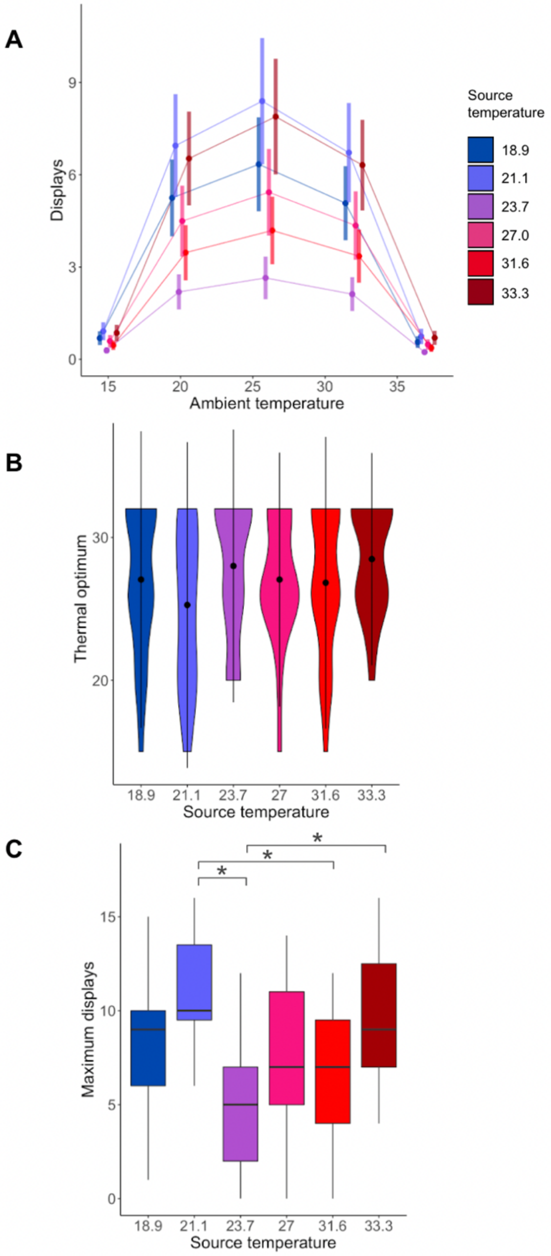


Figure 1.4. (A) Thermal performance curves for male copulation attempts differed among populations of mosquitofish but were unrelated to average source pond temperatures. Estimated marginal means at each ambient temperature are connected by solid lines. Error bars indicate +/- 2 SE from predicted estimated marginal mean values. Neither thermal optima (B) nor maximum copulation attempts (C) differed among populations.

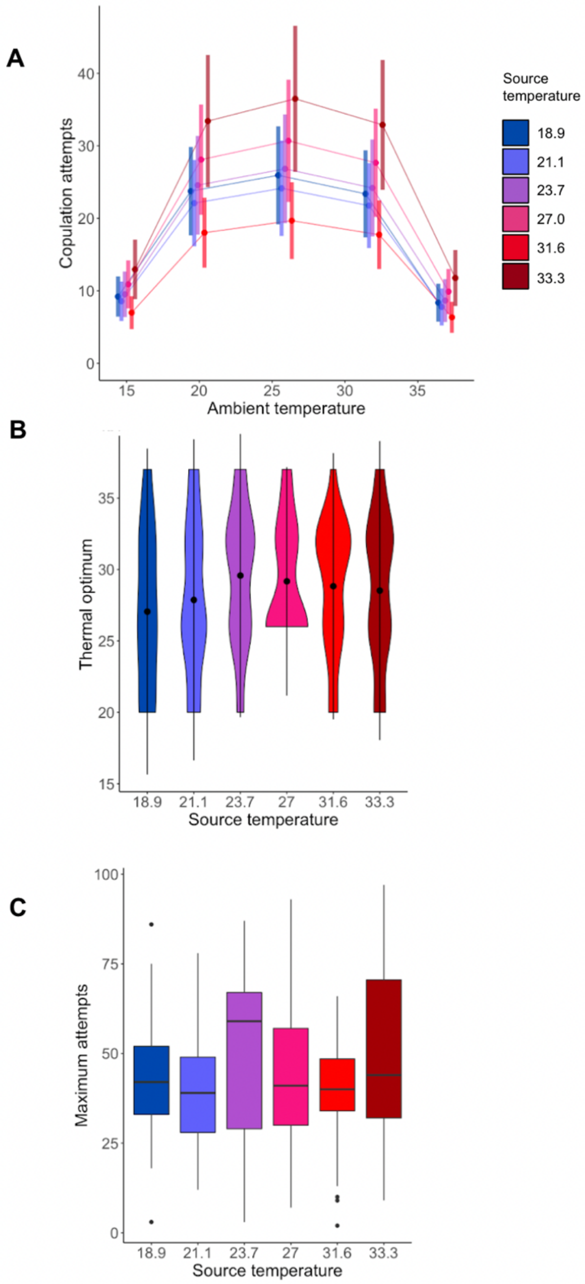


Figure 1.5. (A) Thermal performance curves for copulations differed among populations of mosquitofish but were unrelated to average source pond temperatures. Estimated marginal means at each ambient temperature are connected by solid lines. Error bars indicate +/- 2 SE from predicted estimated marginal mean values. Neither thermal optima (B) nor maximum copulations (C) differed among populations.

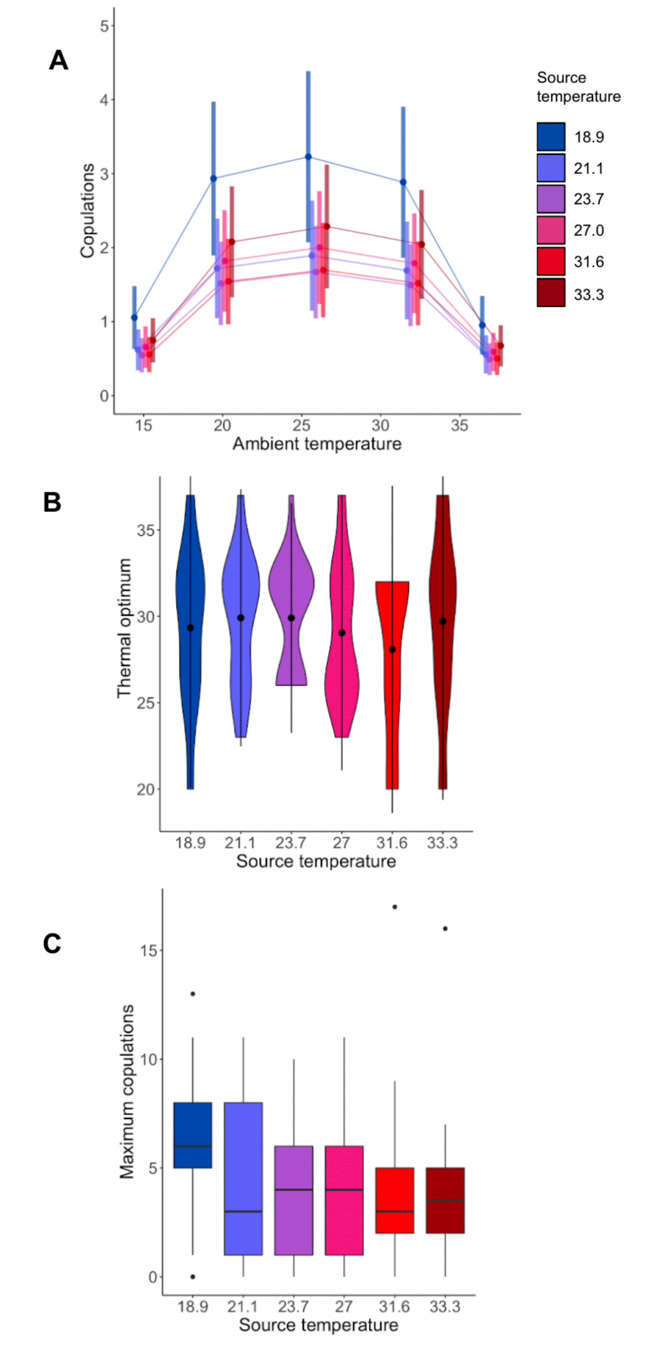
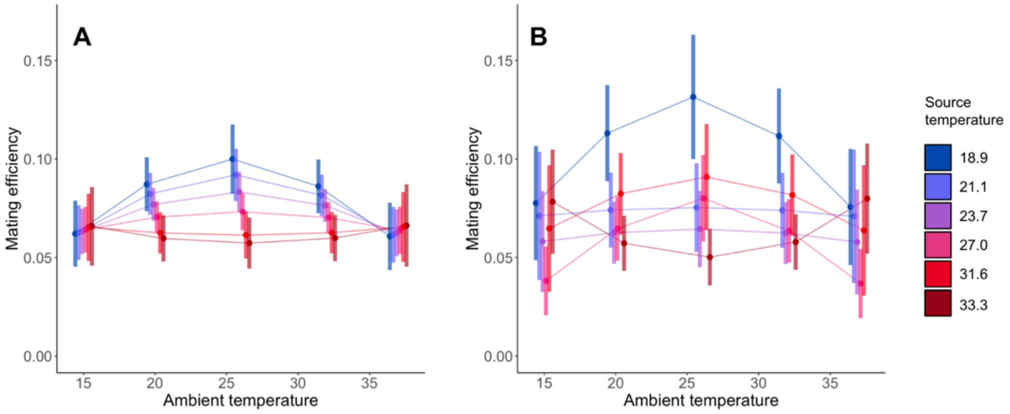


Figure 1.6. (A) Source temperature explained differences in thermal performance curves for mating efficiency. (B) However, Akaike information criterion scores indicated that differences could be better explained using population identity, an unranked categorical variable corresponding to each source population. Estimated marginal means at each ambient temperature are connected by solid lines. Error bars indicate +/- 2 SE from predicted estimated marginal mean values.



CHAPTER 2: Geographic variation in female gravid spots and male mate choice in western mosquitofish (*Gambusia affinis*)

INTRODUCTION

Since Darwin authored the *Descent of Man* over 150 years ago, the dominant narrative of sexual selection theory has emphasized how indiscriminate, ornamented males compete to mate with selective females (Andersson, 1994; Darwin, 1871). Indeed, empirical evidence and evolutionary theory have repeatedly demonstrated how female mate choice drives evolutionary change in male sexual signals. However, in the last few decades, this dichotomous narrative has shifted to consider the more nuanced and dynamic nature of sex roles (Fritzsche et al., 2021; Hare & Simmons, 2019). While females are typically considered the choosier sex due to their greater investment in offspring (Bateman, 1948; Trivers, 1972), male mate choice can emerge in some conditions, such as when female encounter rates are high, female quality is variable, and/or males incur costs to pursue mating opportunities (e.g. energetic costs of finding/chasing mates or courting) (Edward & Chapman, 2011; Nakahashi, 2008). Instances of males distinguishing and selecting among potential mates have been identified across diverse taxa (fishes: Sargent et al., 1986; Schlupp, 2018; birds: Hill, 1993; Jones et al., 2001; Sæther et al., 2001; lizards: Swierk et al., 2013; crustaceans: Reading & Backwell, 2007; insects: Bonduriansky, 2001). Still, our understanding of male mate choice and female sexual signaling is relatively limited, especially regarding which female traits are the target of male choice (but see: Rooker & Gavrilets, 2018; Tobias et al., 2012).

Male mate preferences are often based on cues of female fecundity. Body size is a common target of male choice due to the close link between female size and the quantity or quality of her offspring (Blueweiss et al., 1978; Bonduriansky, 2001). However, other traits, such as those indicating a female's reproductive state, may also affect male mate choice and modulate their response to female body size (e.g. humans: Haselton & Gildersleeve, 2016; primates: Deschner et al., 2004; Rooker & Gavrilets, 2018; birds: Cornwallis & Birkhead, 2007; reptiles: Hager, 2001). In species with cyclic female receptivity, mating when females are most receptive may result in a higher likelihood of fertilization and paternity. Conversely, avoiding copulations when females are unreceptive may prevent males from wasting energy pursuing mating attempts that will not contribute to paternity. Thus, perceiving and choosing among traits that indicate female reproductive status may confer a fitness advantage to males, enabling them to preferentially invest energy in pursuing mates when their likelihood of fertilization is highest.

While the evolution of male mate choice based on fecundity cues is relatively straightforward, the evolution of female sexual signals represents somewhat of an evolutionary puzzle (Hosken et al., 2016). Females that signal their quality may incur high costs associated with increased male attention (e.g. increased aggression or traumatic copulation increasing infection risk), so in order to evolve, these signals must offer fitness benefits that offset this cost (Hosken et al., 2016; Rooker & Gavrilets, 2018). One scenario in which female quality signaling may be evolutionarily favorable is if signals fluctuate with female reproductive status, offering a visual indicator of physiological receptivity. While this type of signaling may increase male attention when female fertility peaks, it could also reduce male harassment during periods of low receptivity. The evolution of fluctuating female signals depends both on the signaling of other females, as well as male responses to signals: females will temporarily experience decreased harassment during a limited window of low receptivity only if males divert their attention toward other, more receptive females during this period. This type of signaling may also have the added benefit of increasing male-male competition during fertile periods, which can reduce harassment costs if males compete directly with each other for mating opportunities or engage in mate guarding (e.g. Bisazza & Marin, 1991). Thus, female fecundity signaling may be evolutionarily favorable in some circumstances.

Gravid spots in livebearing fishes are promising candidates for examining the behavioral and coevolutionary dynamics of female sexual signaling and male mate choice. Gravid spots are conspicuous regions of dark pigmentation on the female ovarian sac that are visible near the genital opening (Norazmi-lokman et al., 2016). Since this pigmentation does not appear to be functionally linked to fecundity (e.g. it is not a direct artifact of embryonic development, and there is no obvious “reason” the ovarian sac would be pigmented), it may have evolved as a signal of receptivity. Gravid spot size has been linked to the development stage embryos, as well as clutch size in some livebearing species (Norazmi-lokman et al., 2016), and could thus serve as a reliable indicator of reproductive status and fecundity for males. Yet gravid spots are highly conspicuous, which may increase female vulnerability to visual predators; thus, we might expect gravid spots to confer fitness benefits that outweigh this cost. In some species, there is evidence suggesting that male mate choice underlies the existence of gravid spots in females. For example, in Western mosquitofish (*Gambusia affinis*), males only attempt to mate with female fish models possessing gravid spots and do not approach those lacking spots (Peden, 1973). However, male responses to gravid spots can be inconsistent, even within species. For example, other studies of Western mosquitofish have found that males prefer smaller gravid spots (Deaton, 2008). Thus, our understanding of male choice of gravid spot traits remains incomplete.

There are many potential explanations for the inconsistencies surrounding male responses to female gravid spots. One possibility is that male mate choice targets female gravid spot size relative to other females rather than absolute gravid spot size. Facing male harassment, female livebearers have been observed to respond by shoaling with other females, forcing males to select among individuals in a group (Agrillo et al., 2006; Dadda, 2015). Assuming that males benefit from mating but do not have unlimited time or energy to pursue all potential mates equally, they may allocate mating effort among females in proportion to female traits that indicate mate receptivity or fecundity. This pattern may explain why studies that present males with an individual female have different outcomes than those offering a simultaneous choice among females (Dougherty & Shuker, 2015).

Intraspecific inconsistencies in male responses to gravid spots may also be due to population divergence in male preferences and female signaling. Mate preferences and signaling are not only shaped by social interactions between sexes, but also by the ecological setting in which interactions take place (Endler & Houde, 1995). For example, in populations with high predation risk, conspicuous signals of mate quality may be less likely to evolve if they decrease the survival of the bearer (Endler, 1980). Females within some populations may also lack gravid spots due to other evolutionary factors and historical effects (e.g. small introduced populations may lack female gravid spots due to bottleneck effects). In populations where female signals aren't present, male mate preferences within these populations will be unlikely to evolve. Thus, divergent selection in different environments may generate systematic intraspecific differences in both female signaling and male mate choice, trends that can only be revealed by examining male and female traits in several populations.

In this study, we aimed to help unravel the mystery surrounding gravid spots by measuring male responses to female gravid spots in different populations of western mosquitofish (*Gambusia affinis*). First, we identified two focal populations among a set of isolated wild mosquitofish populations based on female gravid spot traits. Specifically, we sought populations in which females differed in gravid spot traits but did not differ in other potentially confounding traits that affect male mate choice, such as female length. Then, to understand whether differences in female gravid spots among populations could be linked to male mate preferences, we measured how males from each focal population responded to female gravid spots. We used a free-swimming paired choice design to determine whether males allocated mating effort (measured as copulation attempts and copulations) based on differences in female gravid spot sizes. Our analysis considered whether males responded to absolute gravid spot size and spot size relative to other females during behavioral assays. For each population, we asked (i) whether male mosquitofish mating attempts and copulations depended on the **absolute** size of a female's gravid spot; (ii) whether males allocated mating attempts based on a female's gravid spot size **relative** to other females, and (iii) whether male mating attempts depended on both **absolute** and **relative** gravid spot size.

METHODS

Study system

Mosquitofish (*Gambusia affinis)* from Texas were introduced to California in 1922 and have since been distributed throughout the state, although detailed records of their spread are rare (Dill and Cordone 1997). Focal populations used in this study were sourced from six geothermal spring ponds in Inyo and Mono counties, CA.

These sites have been used as a model system to study the effect of local adaptation to divergent thermal regimes, since average source pond temperatures range from 18.8 to 33.3 C while other ecological conditions among populations are relatively similar (Chapter 1, Table A1.1). Although we did not focus on temperature effects in this study, we selected these populations for our work because fish in these ponds developed in isolation with limited gene flow among ponds, thus creating circumstances conducive to evolutionary divergence and the coevolution of male and female traits. While there is evidence of local adaptation in several traits among these populations (Benavente et al., 2022; Fryxell et al., 2020; also see Chapter 1), our understanding of behavioral differences among ponds is only beginning to emerge.

Fish collection and F2 rearing

Fish collection and rearing followed protocols described in (D. C. Fryxell et al., 2020) and summarized below. Wild-caught fish were collected from each source pond and transported to environmentally controlled greenhouses on the UCSC campus, where they were randomly assigned to 568 L tanks. We reared F2 fish to minimize differences in mate choice and female traits among populations due to rearing temperature or maternal effects. All tanks were maintained at 26 ^{o}C, the average of the highest and lowest source pond temperatures in the natural thermal gradient. Adult fish were fed Tetramin (Tetra Holding, Blacksburg, VA, USA) flake food *ad libitum* twice per day, and juveniles were fed additional Frystartr (Skretting Inc, Stavanger, Norway) once per day to encourage growth. Mosquitofish reproductive activity is sensitive to photoperiod (Pyke, 2005, p. 20), so to encourage reproduction throughout the rearing process, the greenhouse photoperiod was set to 14:10 h daylight: dark using overhead full-spectrum lighting. To maintain water quality and remove waste, half of the water in each tank was siphoned and replaced twice weekly.

Collection and rearing of the first generation of lab-reared fish (F1s) began one month after adult collection to ensure that offspring were not exposed to their parent's environment at their source ponds during development. Fry were collected from a fry retention device within each tank and were transferred to fry baskets hung in 57 L tanks to protect maturing fish from adult filial cannibalism. Approximately one month after collection commenced, when at least 90 F1 fish were collected from each population (representing estimated genetic contributions of at least 12 females per population, but likely many more, see supplemental material in Fryxell et al., 2020), F0 fish were euthanized, and their tanks were drained and reset with clean water. F1 fish were introduced to these tanks, and in November 2018, collection of second-generation laboratory reared offspring (F2s) began, following the fry collection process described above. Up to 10 newborn fish per population were collected each day to ensure representative genetic diversity in F2s. When at least 150 individuals were collected per population, F2 fish were transferred to 132 L tanks to provide additional room for growth, and were subsequently introduced to larger 568 L tanks as they matured.

Upon maturation, F2 fish were transferred from greenhouse tanks to 24 L aquaria in a controlled environment room (TriMark R. W. Smith, San Diego, CA, USA) in June 2019 for easier accessibility during measurements and behavioral assays. 14 fish were introduced to each aquarium in a 1:1 male: female sex ratio, with between 4 and 7 replicate tanks per population. To reduce animal stress and minimize disease risk, tanks were maintained with 1.25 g of aquarium salt (API Aquarium Salt, Chalfont, PA, USA) per liter and included artificial plants. Controlled environment rooms were set to an air temperature of 26 ± 0.5° C with a photoperiod of 14:10 h daylight: dark supplied by overhead lighting and lights above each aquarium. To maintain water quality and homogenize tank temperatures, each tank was equipped with an aquarium filter (Marina Power Filter, Hagen Inc., Mansfield, MA, USA). Waste was siphoned from tanks during one-third water changes once per week.

Focal population selection

To control for potential confounding effects of female size on male gravid spot preferences, we sought focal populations with similar female standard lengths but different gravid spot sizes. To measure these traits, F2 females from all six source populations were individually placed in a small transparent chamber and photographed on both lateral sides. Standard length, gravid spot area, and body area were measured using ImageJ. We calculated relative gravid spot size by dividing each female's gravid spot area by her body area. All measurements were averaged between both sides of each individual.

To minimize effects of temperature on male behavior, we focused our analyses on two candidate focal populations with source temperatures closest to the F2 rearing temperature (26 C) and tested whether female standard lengths were similar and gravid spot sizes differed among the populations. We used a Shapiro-Wilks test to determine whether female standard lengths, absolute gravid spot sizes, and relative gravid spot sizes deviated from normality and a Levene test to test for non-homogeneity of variances within our dataset. Upon finding evidence of non-normality and non-homogeneity in standard length, absolute gravid spot size, and relative gravid spot size, we employed non-parametric Mann-Whitney U tests to detect differences in these traits of interest among the two populations. We then assessed behavior of male fish from these two focal populations as described below.

Behavioral assays

Male mating responses to female gravid spots were measured using a free-swimming choice design in which one male was exposed to a pair of females. To standardize male social exposure prior to experiments, each male was isolated from other fish for at least 16 hours before behavioral measurements to prevent recent encounters with females or other males in their housing tank from affecting their behavior. In order to standardize female stimulus fish, the females used in these assays were obtained from a single non-focal source population (TMo Fishery, Sacramento, CA USA) and were housed in a large mixed-sex tank that was maintained following the protocols outlined above for wild-caught fish.

Behavioral assays occurred within an observation tank (40 x 21 x 12 cm deep) filled with treated tap water (Hikari USA Ultimate Water Conditioner, Hayward, CA) and maintained at 26 C. Each observation tank was lined with blue felt to reduce external stimuli, included an artificial plant to add structural complexity, and was illuminated with two 18 W lights overhead. Prior to each assay, a camera was affixed above each tank to record interactions (Olympus Stylus TG-4, Bethlehem, PA). Two size-matched females were selected at random and placed with a single male from study source populations. Since female gravid spots were not observed during female selection, males were exposed to pairs of females whose relative spot sizes differed: some female pairs had large differences in their gravid spot sizes, while other pairs had relatively similar spot sizes. Prior to observations, males and females were placed on opposite sides of an opaque divider within an observation tank, where they acclimated for 20 minutes. Behavioral observations began when the divider was lifted. Interactions were recorded for 20 minutes, after which all fish were removed from the aquarium and returned to their housing tank. To eliminate chemical cues released from previous assays, aquarium water was emptied and replaced before each observation. After each assay, fish were removed and photographed. Female standard length, gravid spot area, and body area were measured in ImageJ following protocols described for F2 females above.

Video analysis

Mosquitofish mating behavior in video recordings was analyzed using CowLog 3.0 behavioral coding software (Hänninen and Pastell 2009). We recorded the total number of copulation attempts and copulations a male performed toward each female during the 20-minute observation period. Copulation attempts occurred when a male approached a female from behind and oriented beneath the female caudal peduncle, a position required for copulation in livebearing fish (Bisazza et al. 2001). Copulations occurred after a subset of attempts and were identified by the rapid twisting motion that accompanied the male removal of the gonopodium from the female gonoduct (Wilson 2005). To ensure consistency among measurements and minimize viewer bias, video observers were trained to identify behaviors on standardized videos before collecting data and were blind to treatment and source population identity when analyzing behavior.

Several conditions warranted video exclusion from our dataset: First, we excluded videos in which females gave birth during the observation period because postpartum female cues can modify male behavior (Farr, 1989). We also excluded videos in which female size differences were larger than 10% of the smallest female's standard length to account for errors in visual size matching and avoid confounding effects of standard-length differences among pairs. Trials with males who made zero copulation attempts and did not engage with either female were excluded because inactive males could not demonstrate a preference for gravid spot traits among the pair of females.

Lastly, for analyses that required assignment of "large spot" and "small spot" female (e.g. when preference scores were calculated, see statistical analyses below), trials in which female gravid spot sizes differed less than 0.01 cm^2 were excluded because males likely could not distinguish between which gravid spot was larger when female spot size differences this small.

Statistical analyses

To determine whether males in each population had a preference for absolute gravid spot size, we tested for an effect of absolute gravid spot size on male copulation attempts and copulations toward each individual female using generalized linear mixed effect models (GLMMs) (Bolker et al., 2009). Since two females were presented to each male simultaneously, we incorporated the dependency among observations within the same trial by using male identity as a random intercept. Fixed covariates in each model included absolute gravid spot size (continuous), population (categorical with two levels), an interaction term between gravid spot size and population. We also included standard length as a fixed covariate, since females differed in size among trials and previous studies have shown male western mosquitofish prefer larger females (Deaton, 2008). Models for male copulation attempts were fit with a quasi-Poisson distribution, which is appropriate for overdispersed count variables, and a log link function (Ver Hoef & Boveng, 2007). Models for copulations were fit with a zero-inflated negative binomial distribution and a log link function due to the high frequency of observations with zero copulations (Zuur et al., 2009). To model the probability that a copulation attempt was successful, we used a logistic GLMM with a binomial distribution and canonical logit link function (Zuur et al., 2009). To determine whether any covariates affected each response, we first tested for overall model significance by comparing a full model with all fixed and random effects to an intercept-only model. If this comparison was significant, we used Wald χ^2 tests to determine which parameters had a significant effect on male behavior.

We were also interested in whether relative spot sizes of the two paired females in each assay affected male mating behavior. To estimate whether males preferred large or small gravid spots, we calculated a preference score by subtracting the number of copulation attempts directed toward the female with the smaller spot from the number of attempts toward the female with the larger spot (Dosen & Montgomerie, 2004). A preference score of 0 indicated no preference, while positive scores indicated a preference for larger spots and negative scores indicated a preference for smaller spots. We first tested whether preference scores deviated from normality or homogeneity of variance using Shapiro Wilk tests and Levene test, respectively. If deviations were not found, we analyzed preference scores using parametric statistics, including one-sample t-tests to determine whether preference scores differed from zero within populations and two-sample t-tests to compare preference scores among populations. If significant deviations from normality and non-homogeneity were found, we used nonparametric tests. Specifically, we used one-sample Mann-Whitney

U tests to determine whether preference scores in each population differed from zero, and two-sample Mann-Whitney U tests to determine whether preferences differed among populations.

Recognizing that preference strength may be proportional to the difference in trait values, we predicted that male preference scores may also depend on the magnitude of the difference in paired female gravid spot sizes. During assays, each male was exposed to a unique pair of females with different spot sizes, with some males presented with females with highly divergent spot sizes and others exposed to females with similar spot sizes. To test whether the magnitude of difference in female gravid spot sizes affected male preference scores, we used linear regression. We also included population identity as a predictor to determine whether populations differed in preference scores, as well as an interaction term between gravid spot size differences and population identity to determine whether male sensitivity to spot size differences was population-specific.

Lastly, we were interested in understanding the combined effects of both absolute and relative spot size on male behavior. To determine whether relative spot size (i.e. spot size difference), absolute spot size, or population identity affected the probability that a male would attempt to mate with the small- or large-spot female, we used logistic regression, with male responses to each female described in separate models with identically structured explanatory factors. Each model included all combinations of interaction terms among the three variables. Significant interaction terms between either spot size difference or absolute spot size and population indicated that source populations differed in how relative and absolute gravid spot sizes affected the probability that a male would attempt to mate with the focal female. A significant three-way interaction between spot size difference, absolute spot size, and population indicated that source populations differed in the way that relative spot size modified the effect of absolute spot size on the probability that a male would mate with the focal female. To determine whether any factors of interest affected a male's probability of mating with the large- or small-spot female, we first tested for overall model significance by comparing a full model with all predictors to an intercept-only model. If this comparison was significant, we used Wald χ^2 tests to determine which specific factors affected the probability that a male would mate with the small- or large-spot female. Pairwise contrasts of estimated marginal means were used to determine which responses differed from each other, with Bonferroni corrections applied for multiple comparisons.

RESULTS

Population selection

As explained above, since differences in sexual signals can correspond to differences in mate preferences among populations, we sought two populations in which females displayed different gravid spot sizes but lacked differences in other variables that may affect male behavior, such as differences in source temperature or female body size.

Our initial set of mosquitofish study sites included six populations with average temperatures ranging from 18.9 to 33.3 C (Fig 2.1). To minimize differences in male behavior related to source population temperatures, we focused on comparing female traits in two populations with average source temperatures nearest to the F2 rearing temperature (26 C), Artesian Well (AW, 23.7 C) and Warm Springs Upper (WSU, 27.0 C). A Shapiro-Wilk test revealed significant deviations from normality in female standard length (W= 0.96, p=0.007), absolute gravid spot size (W= 0.91, p< 0.001), and gravid spot size relative to body area (W=0.91, p< 0.001) in both populations. Additionally, we found evidence of non-homogeneity of variance in all three traits using Levene test (standard length: df= 1, f= 8.49, p= 0.006; absolute spot size: df= 1, f= 9.52, p<0.001; relative spot size: df= 1, f= 8.84, p=0.003). Consequently, we used non-parametric Mann-Whitney U tests to compare female traits among AW and WSU. Females from WSU had significantly larger absolute gravid spot sizes (W= 511, p=0.004) (Fig 2.1A) and gravid spot sizes relative to their body area (W= 5.12.5, p=0.004) (Fig 2.1B) compared to females from AW. We also tested for differences in female size among populations, since males often prefer larger females and this may act as a confounding factor in the evolution of male mate preferences. However, we found no differences in female standard length among populations (W= 960.5, p= 0.17) (Fig 2.1C). Thus, we proceeded to compare male responses to female gravid spots in AW and WSU, which we will now refer to as the “small-spot population” and “large-spot population,” respectively.

Male responses to absolute female gravid spot size

Comparing intercept-only and full models to determine whether any model predictors had a significant effect on male behavior, we found no evidence that absolute gravid spot area, source population identity, or female standard length affected male copulation attempts (χ^2 = 3.55, df= 4, p= 0.47) (Fig 2.2A) or copulations (χ^2 = 7.59, df= 4, p= 0.11) (Fig 2.2B). However, at least one of these predictors had a significant effect on the probability that a copulation attempt would result in successful copulation (χ^2 = 9.55, df= 4, p= 0.048). The best fit model for the probability of successful copulation included only absolute gravid spot size, with larger spots associated with an increased likelihood that a male copulation attempt would result in successful copulation (χ^2 = 8.58, df= 1, p= 0.003) (Fig 2.2C). There was no effect of population identity (χ^2 = 1.19, df= 1, p= 0.27), population identity x gravid spot size (χ^2 = 0.76, df= 1, p= 0.41), or standard length (χ^2 = 0.27, df= 1, p= 0.60) on the probability that a copulation attempt resulted in successful copulation. Parameter estimates for the best fit model are shown in Table 2.1.

Male responses to relative gravid spot sizes

We found significant deviations from normality in male preference scores (Shapiro-Wilk test: W=0.91, p=0.001) as well as non-homogeneity of variance (Levene test: df= 1, F= 9.52, p= 0.003), and thus used non-parametric analyses for comparing preference scores within and among populations. Males from the small-spot population showed no evidence of a preference based on the relative spot sizes of the females they encountered: preference scores in males from this population did not differ from 0 (Mann-Whitney U: V= 150.5, p=0.23) (Fig 2.3A). In contrast, male preference scores in the large-spot population were significantly less than zero (Mann-Whitney U: V= 74, p= 0.03), indicating that when choosing between two females, these males attempted to mate more with the female possessing the smaller spot. Comparing the two populations directly, we found that males from the small-spot population also had lower preference scores than those of the large-spot population (Mann-Whitney U: V=369.5, p=0.02). A regression model containing population identity, gravid spot size difference, and an interaction term did not predict male preference scores better than an intercept-only model (df= 3, F= 1.75, p= 0.17) (Fig 2.3B).

Male responses to both absolute and relative gravid spot size

To understand how males allocated mating effort toward each female based on their gravid spot size, we modeled the probability that a male would attempt to mate with the small-spot female or large-spot female, including the focal female's absolute spot size, the difference between paired female spot sizes, and the male's source population identity as explanatory factors. Comparisons between an intercept-only model and the full model containing all explanatory variables and possible combinations of interactions found that at least one predictor affected the probability that a male would attempt to mate with the small-spot female (df= 7, deviance= 61.67, p< 0.001). Since Wald χ^2 tests revealed a significant interaction between the female's absolute spot size, paired female spot size differences, and male source population (df= 38, deviance= 4.455, p= 0.03), all three terms were included in the best fit model describing the probability that males would attempt to mate with the small-spot female. Parameter estimates for this model can be found in table 2.2.

The effect of absolute spot size on male behavior toward the small-spot female depended on both the difference between paired female spot sizes and the male's source population. Although relative spot size differences were measured and modeled as a continuous effect, we constrained our post-hoc analyses to three biologically relevant levels of spot size differences (small, medium, large) to facilitate calculations of estimated marginal means and perform pairwise comparisons of male behavior. Categorical spot size differences were based on observed differences in paired female gravid spot sizes during the experiment. Small spot difference (0.01 cm^2) was the smallest difference between two female spot sizes observed during the experiment, medium spot size difference (0.05 cm^2) was the mean spot size difference, and large spot size difference (0.1 cm^2) was the largest difference between two females.

For both populations, the probability that a male would attempt to mate with the small-spot female was unaffected by absolute spot size if the difference between female spot sizes was small (small-spot population: Z= -0.92, $p > 0.99$; large-spot population: Z= -0.73, $p > 0.99$) (Fig 2.4A). As the difference between paired female spot sizes increased, absolute spot size had a significant effect on the probability that a male would attempt to mate with the small-spot female in both populations. Specifically, the probability that males would mate with the small-spot female declined as her absolute spot size increased, but only if spot size differences between paired females were medium (small-spot population: $Z= 4.14$, $p < 0.001$; large-spot population: $Z= 2.53$, $p=0.03$) or large (small-spot population: $Z= 3.81$, $p < 0.001$; large-spot population: $Z= 2.83$, $p=0.01$) (Fig 2.4B, 2.4C). The effect of absolute spot size on the probability that a male would attempt to mate with the small-spot female also differed among populations. As absolute spot size increased, males from the small-spot population were less likely to attempt mate with the small-spot female compared to males from the large-spot population (p-values shown in Table 2.3). Note that since females were presented to males in pairs, small-spot females whose absolute spot size was on the larger end of the small-spot size spectrum must have been paired with large-spot females with even bigger absolute spot sizes. Thus, based on male responses to the small-spot female, it appears that male mosquitofish from both populations preferred extreme gravid spot traits– when the small-spot female had a very small gravid spot, males were more likely to mate with her than the other female, but when both gravid spots were large, the larger of the two gravid spots was preferred– although males from the small-spot population demonstrated a weaker preference.

We also considered whether the same set of explanatory factors– the focal female's absolute spot size, the difference between female spot sizes, and male source population– affected the probability that a male would attempt to mate with the large-spot female and found evidence that at least one factor included in the model influenced male behavior (df= 7, deviance= 75.20, p< 0.001). Similar to the model for male responses to the small-spot female, we found a significant effect of the interaction between the female's absolute spot size, relative difference between female spot sizes, and male source population identity on the probability that a male would mate with the large-spot female (df= 38, deviance= 7.29, p=0.007). Subsequently, all explanatory factors were included in the best fit model for the probability that a male would mate with the large-spot female. Parameter estimates for this model are shown in table 2.4.

The probability that a male attempted to mate with the large-spot female was affected by both absolute gravid spot size and the difference between female spot sizes, but only for males from the small-spot source population. For these males, the probability of mating with the large-spot female increased with absolute spot size if the spot size differences between females was medium (Z= -4.34, p< 0.001) or large (Z= -5.63, p< 0.001) (Fig 2.5). In contrast, for males from the large-spot population, absolute spot size had no effect on the probability that a male would mate with the large-spot female when the spot size difference between females was small (Z= 0.02, p= 0.99), medium (Z= -1.29, p=0.59), or large (Z= -1.61, p= 0.32). Consequently, males from the large-spot population were less likely to mate with the large-spot female than males from small-spot population when the absolute spot size was at the largest end of the spectrum (Table 2.5).

DISCUSSION

In this study, we aimed to better understand the evolutionary mystery of why female gravid spots have evolved in Western mosquitofish by determining if male mate choice could explain between-population differences in female gravid spot size. First, we measured and identified differences in female gravid spot sizes among six isolated mosquitofish populations. To determine whether coevolutionary dynamics between female traits and male preferences contributed to these differences, we selected two focal populations for further study of male mate choice. Assays of male behavior in response to standard females revealed that male preferences for gravid spot size differed among populations. However, we found mismatches between male mate preferences and female gravid spot sizes within populations, suggesting the evolutionary dynamics of male mate choice may deviate from that of female mate choice, which predicts correlated evolution between preferences and traits.

Predominant models describing the evolution of mate preference– including Fisherian selection, direct and indirect benefit selection, and sensory drive– predict that mate preferences and sexual traits will be correlated among populations (Edwards, 2000; Houde, 1993; Jennions & Petrie, 1997). These predictions have been upheld by empirical work examining geographic variation in preferences and traits (Houde & Endler, 1990; Ryan, 1988). If correlated evolution occurred between female gravid spots and male preferences in mosquitofish, we would expect parallel variation in these traits across populations, with males sourced from large-spot populations preferring larger spots and vice versa. Our results are inconsistent with these predictions. When selecting among a pair of females, males from the large-spot focal population performed more mating attempts toward females with smaller spots. Additionally, males from this population were more likely to mate with the smaller-spot female if her absolute spot size was small. However, the probability that males from the large-spot population mated with the large-spot female was unaffected by her absolute gravid spot size, suggesting that males from the large-spot population allocated mating effort based solely on their evaluation of females possessing smaller gravid spots. Males from the small-spot source population demonstrated different responses to female gravid spots. Although they had a similar preference for mating with the small-spot female when her absolute spot size was small, when both females had larger spots, (i.e., if the spot size of the small-spot female was relatively large), they were more likely to mate with the female possessing the larger spot. Essentially, males from the small-spot source population had a bimodal preference for either very small or very large spots. Given the observed lack of correspondence between male preferences and female gravid spots traits within and among populations, complex interactions among different sexual selection processes are likely at play in this system.

Despite differences in female gravid spot sizes among sources, we found evidence that males from both populations preferred small female gravid spots. Male preferences for small gravid spots are likely maintained by the direct benefits of mating with small-spot females. Gravid spot sizes in close relatives of western mosquitofish fluctuate throughout gestational stages, and small gravid spots are associated with early embryonic development and the presence of unfertilized eggs (Norazmi-lokman et al., 2016). Consequently, selection could favor male preferences for small spots, as copulating with females during this stage would more likely result in successful fertilization than mating when female gravid spots are larger. Previous studies of western mosquitofish have also found that males prefer smaller female gravid spots, suggesting this preference may be generally favorable for males in this species (Deaton, 2008).

If male preferences for small gravid spots were the dominant factor shaping selection on female signals, we might expect that directional selection would favor reduced gravid spots in all populations, but this was not the case in our study. We found that males originating from the source population with larger female gravid spots had a stronger preference for small spots than those from the small-spot source population, directly contradicting these predictions. One explanation for the mismatch between preferences and trait values in our focal populations could be that the evolutionary dynamics of male mate choice and female signaling are unique from the reverse scenario. In models describing female mate choice and male signaling, which predict correlated evolution of these traits, males with preferred signals benefit from increased female attention and mating opportunities because their mating success is proportional to the number of mates they can obtain (Bateman, 1948; Kokko et al., 2006). When sex roles are reversed, with choosy males and signaling females, these assumptions no longer hold: female reproductive success is not limited by the quantity of male mates they can obtain, and females signaling attractive traits may suffer negative fitness consequences due to increased male harassment (Hosken et al., 2016). Since male mosquitofish harassment is among the most intense of livebearing species (Plath et al., 2007), consequences of male attention may be especially severe, resulting in decreased feeding efficiency, increased disease transmission, and lower fitness (Jerry & Brown, 2017; Magurran & Seghers, 1994; Maklakov et al., 2005; Pilastro et al., 2003).

Given the costs of signaling mate quality, secondary sexual characteristics may serve a different function in females than they do in males. While male sexual signals increase mating opportunities, selection may favor female signals that reduce the costs of male harassment (Hosken et al., 2016). For example, in females with cyclic fertility, selection could favor sexual signals that fluctuate according to receptivity (Rooker & Gavrilets, 2018). These fluctuating signals would enable females to isolate harassment to periods when fertilization could occur. Heeding these signals also would be evolutionarily advantageous to males, who could maximize their reproductive success by allocating more energy to pursuing and mating with fertile females. Interestingly, in female mosquitofish, gravid spots are largest and most conspicuous when females are least fertile (Norazmi-lokman et al., 2016). Thus, gravid spots may have evolved as a "stop sign" analogous to aposematic coloration, conspicuously signaling infertility to males to prevent harassment and its associated costs. If gravid spots evolved to deter harassment in some populations, we would expect large-spot females to co-evolve with males that avoid large spots, a prediction consistent with female traits and male preferences in the large-spot source population.

In the small-spot focal population, we also found a mismatch between male preferences and female traits. Within this population, females had smaller gravid spots, and males were more likely to prefer larger gravid spots than males from the other focal population. The cost of male sexual harassment for females could also explain these patterns. First, male preferences for large gravid spots could have evolved within this population if males experienced reproductive benefits of mating with large-spot females that offset the reduced likelihood of fertilization during this period. Male reproductive success from copulation is a combination of the probability of fertilization and the total number of eggs fertilized. Although the probability of fertilization is lowest when female gravid spots are largest, large gravid spots can also be associated with higher female fecundity (Norazmi-lokman et al., 2016). Since female mosquitofish are capable of storing sperm, males that copulate during this reproductive stage may sire offspring in future broods (Pyke, 2005). Thus, preferences for large-spot females could be evolutionarily favorable if the benefits of mating with a highly fecund female offset the low probability of fertilization at the time of copulation. If elevated harassment is costly for females (see previous paragraph), directional selection may have favored smaller gravid spots in populations where males aggressively harassed large-spot females, generating a mismatch between male preference and female trait values.

Although we can only speculate regarding the evolutionary mechanisms underlying observed patterns of male preferences and female traits, our study is a useful complement to previous work on male mate choice of gravid spots in western mosquitofish. Previous research on male mosquitofish mate choice has found conflicting preferences based on gravid spot size, with some identifying a preference for reduced gravid spots (Deaton, 2008) and others finding that males prefer more conspicuous gravid spots (Peden, 1973). While these findings may seem contradictory, our results demonstrate that intraspecific differences in mate preferences can emerge among populations within the same species. Additionally, our study highlights the importance of considering mate choice based on absolute versus relative trait values. When choosing a potential mate, males may discriminate based on absolute trait values, evaluate female traits relative to other females, or employ a combination of both strategies. Theoretical models of mate choice often assume that individuals choose mates independently based on a fixed function that assigns a particular value to specific traits (Jennions & Petrie, 1997; Real, 1990). However, in species that encounter several potential mates simultaneously, mate choice may be based on the comparative evaluation of potential mates relative to others in a group (Bateson & Healy, 2005; Zandberg et al., 2020). Mosquitofish behavior facilitates simultaneous comparisons of many potential mates: Females facing male harassment approach other females and form groups to dilute harassment effects, and males prefer to approach groups of females rather than isolated individuals (Agrillo et al., 2006, 2008). Thus, it is unsurprising that in this study, we found evidence for the importance of relative gravid spot sizes in mate choice rather than preferences based solely on absolute trait values. By enabling males to choose among two females simultaneously, our design revealed how the relative gravid spot sizes of both potential mates modulated the effect of absolute spot size, more closely mimicking male behavior in natural settings. Our findings highlight the importance of intentional design and consideration of a species' social and ecological context when examining the existence and evolution of mate preferences (Zandberg et al., 2020).

Our study contributes to growing evidence that, while research on mate preferences overwhelmingly centers female choice, males can also be choosy when evaluating and pursuing potential mates. We found that male preferences and female gravid spot traits were geographically variable, although preferences and traits were not correlated among populations in ways that models of female mate choice and male signaling might predict. These findings highlight how sex-specific differences in selection on sexual signals could underly the unique evolutionary dynamics of male mate choice compared to female mate choice, although we can only speculate regarding the underlying evolutionary mechanisms shaping observed patterns. Our results may offer potential explanations for conflicting findings regarding male preferences for female gravid spots in previous studies, yet unraveling the causes of intraspecific variation in gravid spot preferences and traits with certainty remains a challenge for researchers hoping to understand the nuances of mate choice in mosquitofish. Mating behavior is complex, subject to both evolutionary and plastic influences, as well as the unique social and ecological contexts in which mating occurs. We recommend future studies explore correlations among female traits and male preferences using several populations to better infer how sexual and natural selection shape male reproductive success and female signaling.

TABLES

Table 2.1. The best fit model for the probability that a copulation attempt would result in copulation included only absolute gravid spot size, with larger gravid spots associated with an increased probability of copulation. Since two females were presented to each male simultaneously, the dependency among observations within the same trial was incorporated using male identity as a random intercept. Parameter estimates were produced using restricted maximum likelihood (REML) estimation and are based on a model fit using the small-spot population as the baseline. Model fitting was performed using the *glmmTMB* package in R.

Variable	Estimate	Standard Error	t-value	p-value
Intercept	-3.09	0.23	-12.95	<0.001
Absolute gravid spot size	8.25	3.06	2.70	0.007

Table 2.2. When males were paired with two females with different gravid spot sizes, the best fit model for the probability that a male would attempt to mate with the small-spot female included the female's absolute gravid spot size, the difference between the two female spot sizes, the male's source population identity, and all combinations of interaction terms. Parameter estimates were produced using maximum likelihood estimation and are based on a model fit using the small-spot population as the baseline. Model fitting was performed using *glm* in R. Variables and interactions with significant effects are in bold text.

Variable	Estimate	Standard Error	z-value	p-value
Intercept	-0.55	0.36	-1.51	0.13
Small spot size	18.67	10.38	1.80	0.07
Spot size difference	**29.07**	**11.03**	**2.64**	**0.01**
Population	**0.95**	**0.47**	**2.02**	**0.04**
Small spot size x spot size difference	**-1164.90**	**341.65**	**-3.41**	**<0.001**
Small spot size x population	-11.43	11.86	-0.96	0.33
Spot size difference x population	**-24.86**	**11.97**	**-2.08**	**0.04**
Large spot size x spot size difference x population	**782.72**	**372.17**	**2.10**	**0.04**

Table 2.3. Results of z-tests comparing model predictions for the probability that a male would mate with the small-spot female among populations at each level of spot size difference and absolute spot size. Absolute and relative spot sizes used in pairwise comparisons represent the minimum, maximum, and mean value for gravid spot traits that male fish were exposed to during behavioral assays. Estimates are tested against the standard normal distribution, using Bonferroni corrections for multiple comparisons. Significant differences among populations are in bold text.

Paired female spot differences	**Absolute spot size**	**z-value**	**p-value**
Small (0.01 cm)	Small (0 cm)	-1.86	0.06
	Medium (0.05 cm)	**-2.34**	**0.02**
	Large (0.1 cm)	-0.59	0.57
Medium (0.05 cm)	Small (0 cm)	0.86	0.39
	Medium (0.05 cm)	**-4.30**	**<0.001**
	Large (0.1 cm)	**-3.25**	**0.001**
Large (0.1 cm)	Small (0 cm)	1.80	0.07
	Medium (0.05 cm)	**-2.74**	**0.006**
	Large (0.1 cm)	**-2.56**	**0.01**

Table 2.4. When males were paired with two females with different gravid spot sizes, the best fit model for the probability that a male would attempt to mate with the large-spot female included the female's absolute gravid spot size, the difference between the two female spot sizes, the male's source population identity, and all combinations of interaction terms. Parameter estimates were produced using maximum likelihood estimation and are based on a model fit using the small-spot population as the baseline. Model fitting was performed using *glm* in R. Variables and interactions with significant effects are in bold text.

Variable	Estimate	Standard Error	z-value	p-value
Intercept	**0.58**	**0.29**	**2.02**	**0.04**
Large spot size	1.54	5.44	0.28	0.78
Spot size difference	**-56.21**	**11.05**	**-5.09**	**<0.001**
Population	-0.79	0.56	-1.41	0.16
Large spot size x spot size difference	**458.26**	**85.94**	**5.33**	**<0.001**
Large spot size x population	-2.60	7.74	-0.34	0.73
Spot size difference x population	**44.84**	**14.97**	**3.00**	**0.003**
Large spot size x spot size difference x population	**-344.02**	**128.38**	**-2.68**	**0.007**

Table 2.5. Results of z-tests comparing model predictions for the probability that a male would mate with the large-spot female among populations at each level of spot size difference and absolute spot size. Absolute and relative spot sizes used in pairwise comparisons represent the minimum, maximum, and mean value for gravid spot traits that male fish were exposed to during behavioral assays. Estimates are tested against the standard normal distribution, using Bonferroni corrections for multiple comparisons. Significant differences among populations are in bold text.

Paired female spot differences	Absolute spot size	z-value	p-value
Small (0.01 cm)	**Small (0.05 cm)**	**2.72**	**0.006**
	Medium (0.1 cm)	**2.68**	**0.007**
	Large (0.15 cm)	1.88	0.06
Medium (0.05 cm)	Small (0.05 cm)	-1.53	0.13
	Medium (0.1 cm)	**3.07**	**0.002**
	Large (0.15 cm)	**3.44**	**<0.001**
Large (0.1 cm)	**Small (0.05 cm)**	**-2.48**	**0.01**
	Medium (0.1 cm)	0.03	0.97
	Large (0.15 cm)	**3.80**	**<0.001**

FIGURES

Figure 2.1. (A) Absolute gravid spot size, (B) gravid spot size relative to body area, and (C) female standard length in F2 fish from all source populations. AW and WSU were initially explored as potential focal populations because average temperatures in these sources were closest to rearing temperatures (26 C). Females from these populations had different absolute and relative gravid spot sizes (Mann-Whitney U, * in figure indicates $p < 0.05$) but similar standard lengths ($p > 0.05$). Since we hypothesized that interpopulation differences in female gravid spot sizes could be explained by differences in male mate preferences, we selected AW and WSU as focal populations to further examine male mate choice.

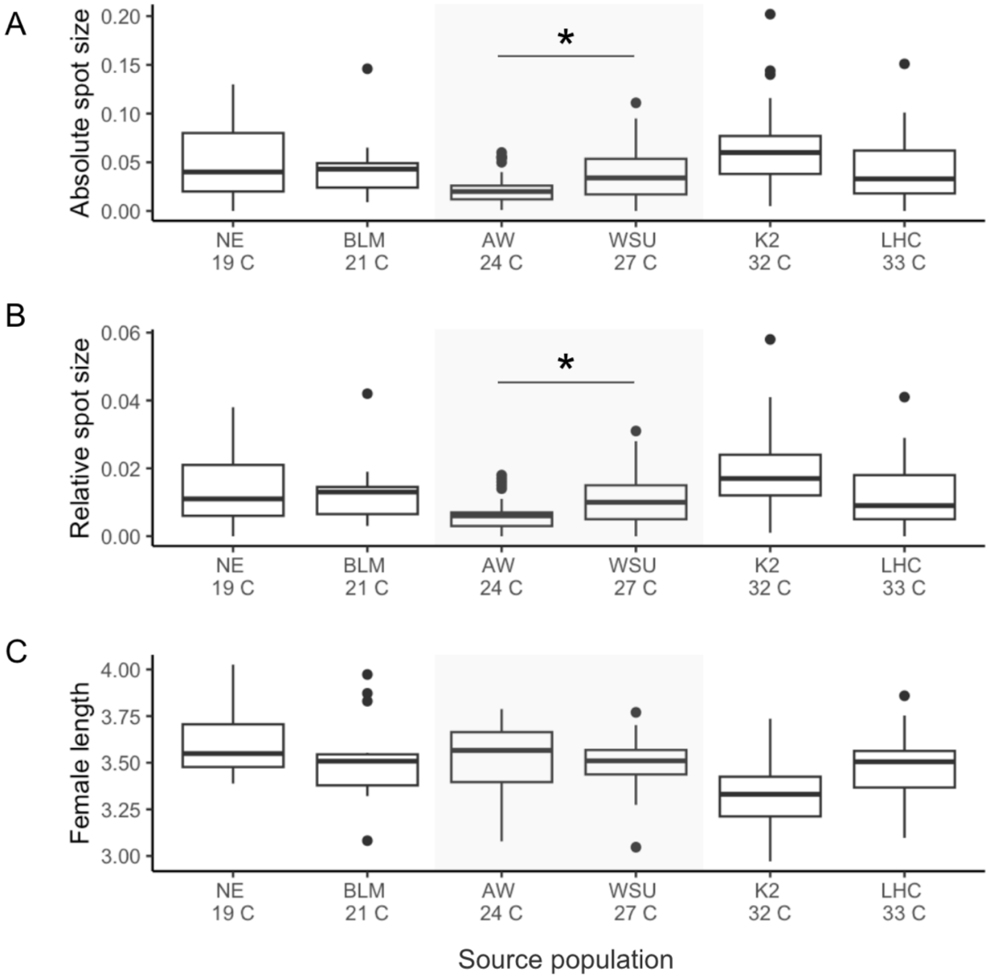


Figure 2.2. (A) Absolute gravid spot size did not affect male copulation attempts (χ^2 = 3.55, df= 4, p= 0.47) (B) or copulations (χ^2 = 7.59, df= 4, p= 0.11) in either the small-spot or large-spot population. (C) The probability that an attempt was successful increased with absolute gravid spot size, regardless of source populations (χ^2 = 8.58, df= 1, p= 0.003) (black line indicates model fit).

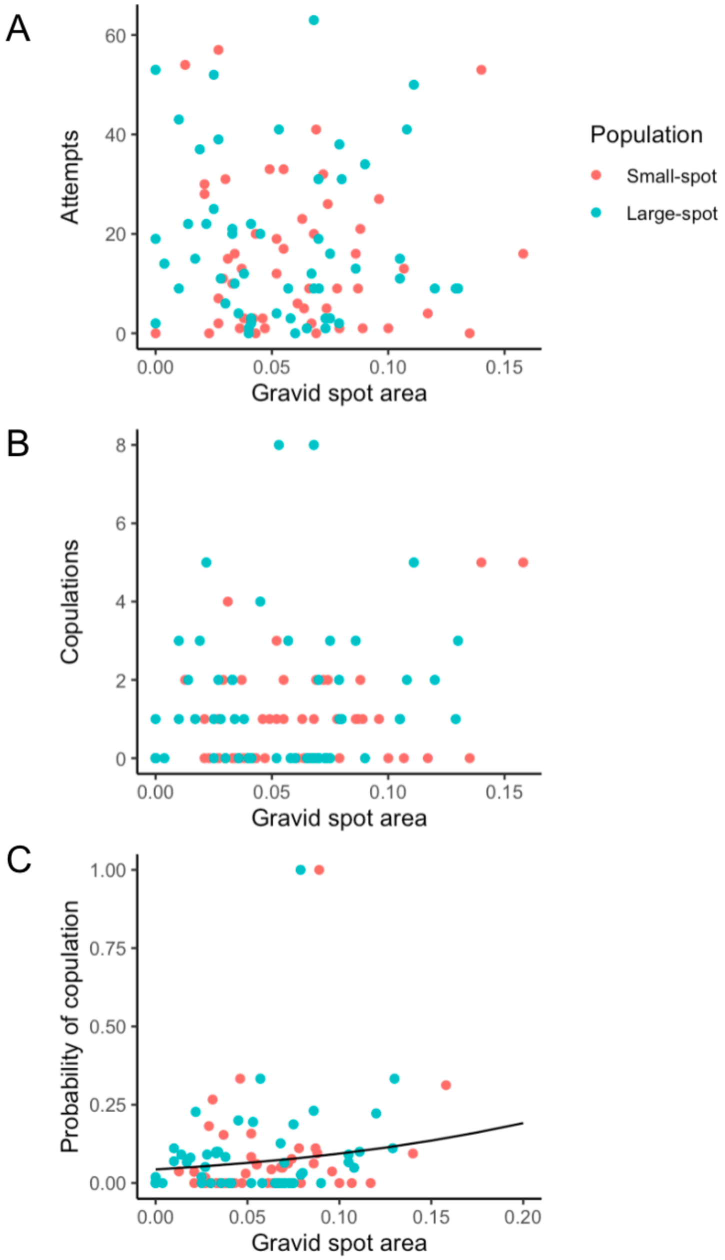


Figure 2.3. (A) Male preference scores (attempts toward large spot female - attempts toward small spot female) in the large-spot source population were significantly less than zero (One-sample Mann-Whitney U: V= 74, p= 0.03) and were lower in the than the small-spot population (Two-sample Mann-Whitney U: V=369.5, p=0.02), indicating that males in this population attempted to mate with the small-spot female more than large-spot female. (B) In both populations, there was no relationship between the difference in female gravid spot sizes and male preference scores (df= 3, F= 1.75, p= 0.17).

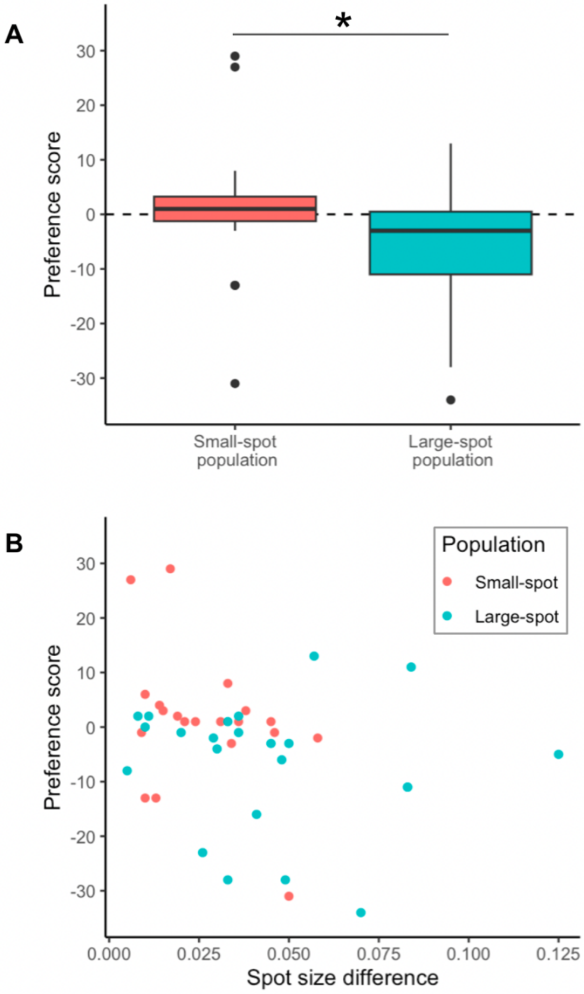


Figure 2.4. Model predictions for the probability of males attempting to mate with the small-spot female, estimated at (A) small (0.01 cm^2), (B) medium (0.05 cm^2), and (C) large (0.1 cm^2) spot size difference between females. Absolute and relative spot sizes used in pairwise comparisons represent the minimum, maximum, and mean value for gravid spot traits that male fish were exposed to during behavioral assays. Shaded region indicates where males were more likely to mate with the small-spot female rather than the large-spot female. Error bars indicate +/- 2 SE from predicted values. See Table 2.4 for parameter estimates and statistical significance.

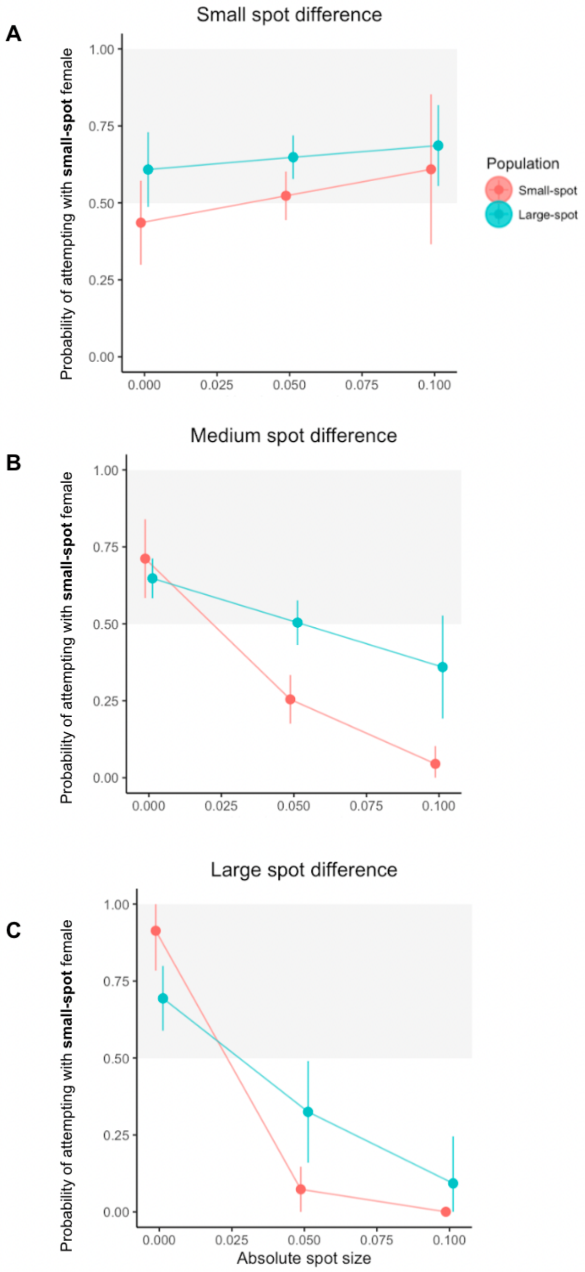
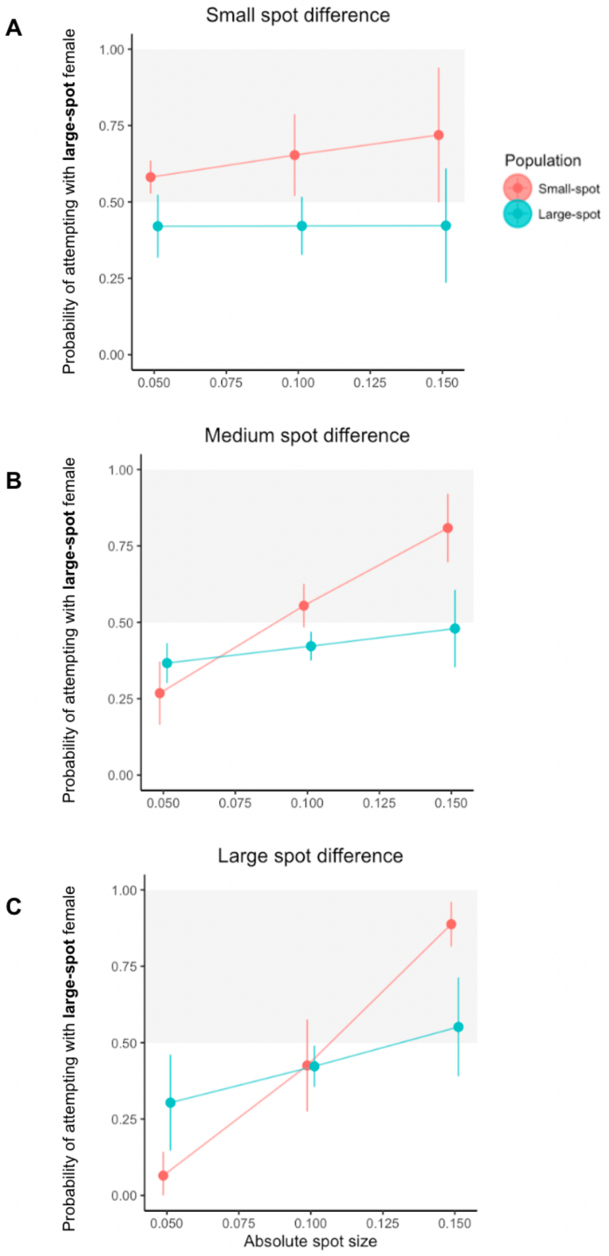


Figure 2.5. Model predictions for the probability of males attempting to mate with the large-spot female, estimated at (A) small (0.01 cm^2), (B) medium (0.05 cm^2), and (C) large (0.1 cm^2) spot size difference between females. Absolute and relative spot sizes used in pairwise comparisons represent the minimum, maximum, and mean value for gravid spot traits that male fish were exposed to during behavioral assays. Shaded region indicates where males were more likely to mate with the large-spot female rather than the small-spot female. Error bars indicate +/- 2 SE from predicted values. See Table 2.5 for parameter estimates and significance.



CHAPTER 3: Intraspecific variation in mating behavior modulates the effects of mosquitofish introduction on prey communities

INTRODUCTION

Studies of eco-evolutionary dynamics have identified important links between ecological and evolutionary processes. One key revelation emerging from this work is that the ecological effects of intraspecific trait variation can be comparable to or greater than the effects of species incidence and abundance (Bolnick et al., 2011; Des Roches et al., 2018). Thus, whether and how a species affects its environment depends on the phenotypes of individuals. While this finding has been replicated in diverse systems, most work on the ecological consequences of intraspecific variation has focused on traits shaped primarily by natural selection, such as feeding morphology (Palkovacs & Post, 2009). The role of sexual selection (i.e., selection on traits that increase mating or fertilization success; Andersson, 1994) in shaping communities and ecosystems has been largely overlooked (Alpedrinha et al., 2019; Giery & Layman, 2019; Svensson, 2019).

Sexual selection is a ubiquitous and potent driver of intraspecific variation that affects a variety of ecologically-relevant traits (Alpedrinha et al., 2019; Giery & Layman, 2019; Svensson, 2019). Sex-specific differences in reproductive strategies can lead to sexual dimorphism in morphological, physiological, and behavioral traits, with potentially widespread ecological implications (Carlson et al., 2011; D. C. Fryxell et al., 2019; Hedrick & Temeles, 1989). Sexual selection can also generate phenotypic diversity within sexes, as demonstrated by the exaggerated phenotypes shaped by mate choice (Ryan & Keddy-Hector, 1992) and the evolution of alternative reproductive tactics (Gross, 1996; Oliveira et al., 2008). Many of these sexually selected traits are directly linked to ecological processes. For example, changes in morphology that enhance success in male-male combat can also affect prey capture (e.g. enlarged canines (Morris & Carrier, 2016); mandibles (Mills et al., 2016); chela (Valiela et al., 1974)), with consequences for prey community composition and abundance. In addition to sexually selected morphological traits, behavioral traits that affect mating outcomes may also have ecological implications (Mittelbach et al., 2014; Sih et al., 2012). For example, in species with coercive mating systems where males actively pursue mating opportunities from unreceptive females, sexual harassment can alter feeding rates (Magurran & Seghers, 1994), which may have top-down effects on prey communities. While ecological consequences of sexually selected traits are likely, empirical tests of these effects are rare.

In this study, we test for the ecological effects of intraspecific variation in sexual harassment behavior in western mosquitofish (*Gambusia affinis*). Mosquitofish mating is dominated by sexual harassment, in which males constantly harass unreceptive females for mating attempts (Plath et al., 2007). Evolved intraspecific differences in the intensity of harassment have been measured among populations (see Chapter 1). Short-term laboratory experiments (i.e. on the scale of hours) in mosquitofish and related species have revealed that harassment can reduce feeding efficiency as individuals allocate less time to foraging while pursuing or avoiding mating (Arrington et al., 2009; Pilastro et al., 2003). Based on these observations, one might expect that high-harassment populations would feed at lower rates than low-harassment populations. However, over longer, ecologically relevant timescales, the opposite outcome could occur as organisms consume more prey to sustain the energetic demands associated with maintaining or avoiding continuous harassment. Regardless of the directionality of effects, mosquitofish are voracious planktivores, so changes in their feeding rates will likely have strong top-down effects on zooplankton abundance in aquatic ecosystems (Fryxell, 2015; Hurlbert et al., 2017; Hurlbert & Mulla, 1981; Pyke, 2008) (Fig 3.1).

Changes in foraging behavior due to harassment will likely not only affect prey abundance, but also prey phenotypes. In aquatic ecosystems, planktivores like mosquitofish selectively target larger, more conspicuous prey, a trend which can shape the size structure and relative abundance of zooplankton species (Bence & Murdoch, 1986; J. L. Brooks, 1968; Hurlbert & Mulla, 1981). Independent of whether harassment intensity leads to either an increase or a decrease in mosquitofish feeding, populations with higher consumption rates are predicted to experience corresponding declines in the relative size of zooplankton compared to populations with lower consumption rates. This may be reflected by declines in body size both within and among prey species. For example, as if high harassment causes consumption rates to rise, larger prey types may be selectively removed from the population first. Consequently, remaining prey in high-harassment populations may be comprised of smaller, less conspicuous individuals compared to prey in low-harassment populations (Fig 3.1).

Consequences of population differences in mosquitofish sexual harassment may also extend to other ecosystem effects. Among populations with divergent harassment levels, differences in zooplankton size or abundance caused by direct consumer-prey interactions may have indirect top-down cascading effects (Ripple et al., 2016). For example, if high-harassment consumers cause larger declines in prey abundance compared to low-harassment consumers, producers in high-harassment populations will experience a greater release from grazing. Consequently, we might expect higher chlorophyll concentrations in high-harassment populations compared to those with lower harassment (Fig 3.1). In aquatic ecosystems, increased productivity due to trophic cascades may cause greater water turbidity, increasing thermal absorption and causing temperatures to rise (Fryxell, 2015; Paaijmans et al., 2008). Harassment differences may also directly affect water chemistry. Raised activity levels in high-harassment populations will increase respiration and release more CO_2 relative to low-harassment populations (Killen et al., 2016). As CO_2 reacts with water to form carbonic acid, differences in harassment levels may be reflected in pH (Fryxell, 2015). Many of the above ecosystem effects of intraspecific variation in mosquitofish have been shown to result from traits shaped primarily by natural selection (Wood et al., 2019, 2022). Here, we examine these same effects, but with a sexually selected trait.

In this study, we used a pond mesocosm experiment to compare the community- and ecosystem-level consequences of divergent mosquitofish harassment levels. First, we artificially manipulated mosquitofish harassment rates by modifying their exposure to females, producing high- and low-harassment male phenotypes. To compare the community and ecosystem effects of high- and low-harassment mosquitofish introduction, we introduced each male type into mesocosm tanks with female mosquitofish and measured changes in biotic and abiotic conditions. We measured zooplankton community composition, abundance, and traits to characterize the effect of harassment on mosquitofish prey communities. To identify ecosystem effects of harassment, we sampled abiotic factors including productivity, ecosystem respiration, pH, and temperature.

METHODS

Harassment Treatments & Measurement

Mosquitofish were obtained from the Sacramento-Yolo County Mosquito Vector Control District (Elk Grove, CA) and transported to the University of California, Santa Cruz. Only sexually mature fish, identified by the presence of a female gravid spot or male elongated gonopodium, were selected for use. Each fish was injected with a single visible implant elastomer tag (Northwest Marine Technology, Inc., Anacortes, WA, USA) adjacent to their dorsal fin for individual identification. All fish were fed Tetramin flake food (Tetra Holding, Blacksburg, VA, USA) *ad libitum* twice daily until the mesocosm experiment began.

Previous studies have shown that in mosquitofish and closely related species, housing males in the absence of female conspecifics can increase sexual behavior and time spent near females upon re-exposure to mixed-sex environments (Field & Waite, 2004; Hughes, 1985). Thus, to generate high- and low-harassment phenotypes, male fish were randomly assigned to single-sex or mixed-sex tanks, respectively, where they were housed for 22 days. Males within each treatment tank were visually size matched to minimize aggressive intrasexual interactions. Single-sex treatments were established in seven 132 L tanks containing 24 +/- 1 males per tank. Mixed-sex treatments were established in 568 L tanks with a 1 male: 2 female sex ratio, with 23 (+/- 1) males and 52 (+/- 1) females per tank. Both single-sex and mixed-sex tanks contained aerators to maintain oxygenation and heaters set to 26 C to maintain similar temperatures among tanks.

Mesocosm Setup

We established 52 experimental ponds (1136 L stock tanks, 0.6 m deep, 1.5 m in diameter; Rubbermaid, Sandy Springs, Georgia, USA) in a 4 x 13 array on the University of California, Santa Cruz Coastal Science Campus (Santa Cruz, CA, USA). Tanks were filled with municipal water on 5 August 2020 and a single 20 x 20 x 20 cm^3 hollow concrete cinderblock was placed in the center of each tank to provide shelter for mosquitofish. After a 48-hour offgassing period for chlorine evaporation, we added equal amounts of a homogenized phytoplankton aliquot to each tank and stimulated productivity by adding 264 ug L of nitrogen/ L (as NaNO3) and 27 ug of phosphorus/L (as KH2PO4), resulting in N:P molar ratio of 22.

Each mesocosm was randomly assigned one of four treatments. Harassment effect treatments included either high- (n= 20) or low-harassment males (n= 20) with an established zooplankton community (see next paragraph). To test for the overall presence of a trophic cascade, we included a fishless reference treatment ("fish absent", n=7) in which zooplankton were added but fish were not, and a treatment without fish or zooplankton added ("zooplankton-absent", n=8).

On 17 August 2020, we introduced a 355 mL aliquot of a homogenized zooplankton sample from Westlake Pond (36°58'36.1"N 122°02'43.8"W), Santa Cruz, CA into all tanks except the zooplankton-absent reference treatment. Initial observations of the zooplankton aliquot revealed few cladocerans, which are a common species in most freshwater zooplankton communities and a common mosquitofish prey. Consequently, we supplemented each tank containing zooplankton with an even aliquot of a homogenized mixture of *Daphnia magna* on 19 August 2020 (AquaPlantWorld, Elk Grove, CA, USA). All tanks were covered with 1.9 cm^2 mesh netting throughout the experiment to prevent interference from avian and mammalian interactions while allowing for exposure to other natural physical, chemical, and biotic elements.

Mosquitofish were introduced to the mesocosms on 30 August 2020 in a density of 14 fish per tank (7M: 7F). One male was randomly selected from each housing tank (i.e. each mesocosm had one fish from each tank) to reduce tank-specific effects during the experiment and ensure even size distribution among tanks. Size-matched female mosquitofish were randomly selected from the mixed-sex treatment tanks and added to each mesocosm. The density of mosquitofish used in this study reflected natural population densities and was similar to that of prior mesocosm experiments that detected ecological consequences of mosquitofish introduction (Fryxell et al 2016, 2016; Fryxell & Palkovacs 2017).

Harassment behavior

Measurements of mosquitofish harassment behavior were collected the day after mosquitofish were introduced to tanks (day 2), and on days 4, 7, 10 and 13 of the experiment. Observers were rigorously trained to identify harassment behavior and measurement consistency was tested prior to data collection to minimize observer effects. Mesh netting was removed from all tanks at the beginning of observation days to enhance fish visibility. To begin an observation, observers stood motionless next to a tank for one minute, allowing fish to acclimate to their presence. After this period, the observer located and watched three pre-assigned males, identified by their elastomer tag color, for three minutes each. Stopwatches were used to record the time a male spent chasing a female during the observation period. Chasing behavior occurred when a male oriented his body toward a female and swam rapidly in her direction as the target female swam away. If a male became obscured from view during an observation, the observer stopped the timer until the focal fish could be relocated, when they would resume timing and complete the observation. Observers were blind to treatment while collecting all behavioral data.

Harassment times for all three males within a tank were added to produce a tank-level harassment score, which we compared among treatments to determine whether harassment phenotype manipulations were successful. We used this score as a response in a generalized linear mixed-effects model (GLMM) to identify differences in harassment intensity among treatments and over time. Our model structure included *harassment treatment* (high or low), *timepoint*, and an interaction term as fixed effects to account for changes in harassment over time. We also included *tank identity* as a random effect to account for differences within tanks over time. The model was fit using a gamma distribution with a log link function, which is appropriate for continuous, positive-skewed data (Zuur et al., 2009). We tested overall model significance by estimating a full model including all fixed and random effects and comparing it to an intercept-only model, then used Wald X^2 tests to determine which specific parameters had significant effects on harassment. We then performed pairwise comparisons using estimated marginal means with Bonferroni corrections to compare harassment levels over time and among treatments. All analyses were completed in RStudio using the packages “glmmTMB” (M. Brooks E. et al., 2017) and “emmeans” (Lenth, 2021).

Zooplankton community effects

Zooplankton community characteristics in each mesocosm were sampled the day prior to mosquitofish additions (Day 0) to ensure all treatments began with similar conditions. We found no differences among high-harassment, low-harassment, and fish-absent treatments in zooplankton communities prior to fish introduction (Kruskal Wallis test, $p > 0.05$). Subsequent zooplankton collections were made on days five, eight, and fourteen after fish introduction. Collections took place on days following behavior observation to minimize effects of sampling on fish behavior. Zooplankton were collected from a 6 L depth-integrated water sample that was filtered through 80-micron mesh. Specimens were preserved in 70% ethanol and were identified and counted under a microscope. Since we were also interested in potential shifts in prey size, we photographed and measured a subset of up to 50 *Daphnia* in each zooplankton sample and measured them to the nearest 0.01 mm using ImageJ.

Our analysis considered the direct effect of high- and low- harassment mosquitofish introduction on zooplankton communities from two perspectives. First, we were interested in comparing high- and low-harassment tanks to each other to determine whether the strength of harassment induced different ecological responses in populations containing mosquitofish. We were also interested in understanding whether harassment strength modulated the effect of fish introductions, which we tested by comparing high- and low-harassment treatments to the fish-absent control treatment. Thus, we included high-harassment, low-harassment, and fish-absent treatments in all zooplankton analyses. Zooplankton community responses for each analysis included the total number of zooplankton counted in a sample to determine overall community effects of harassment, as well as an analysis of each dominant zooplankton species to determine specific effects of harassment on community composition.

Although male harassment levels differed between treatments throughout the course of the experiment, harassment levels in both treatments changed over time (see results). Since harassment was strongest overall at the first timepoint, then declined, we tested for harassment treatment effects at each timepoint independently. We performed Kruskal Wallis tests at each timepoint to detect differences among high-harassment, low-harassment, and fish-absent treatments. If differences were found, we employed Dunn tests with Benjamini-Hochberg corrections for multiple comparisons to identify which treatments differed from each other. If any effect of harassment treatment on zooplankton was found, we calculated Hedge's g effect sizes for all responses to compare the magnitude of these effects.

Ecosystem effects

Ecological characteristics in each mesocosm were measured on the same day as community characteristics. We used a handheld fluorometer (Aquaflash handheld active fluorometer, Turner Designs, San Jose, CA) to measure pelagic chlorophyll from a 2 L depth-integrated homogenized water sample collected from the north side of each tank. To measure net primary production (NPP), ecosystem respiration (ER), and gross primary production (GPP), we used a handheld probe to measured dissolve oxygen concentrations (mg/l) in each tank at dawn, dusk, and dawn the following morning (YSI Pro 2030). NPP was calculated as the increase in oxygen between dawn and dusk. ER was calculated as the amount of oxygen consumed between dusk and dawn. GPP was calculated by adding the oxygen consumed overnight (ER) to the total oxygen produced during the day (NPP) (Harmon et al., 2009). pH was measured using a handheld probe (HI98191, Hanna Instruments). We measured surface temperatures at dusk to capture potential differences caused by heat absorption accumulated during the day (YSI Pro 2030). Prior to fish introductions, we found no effect of treatment on chlorophyll concentrations, pH, NPP, GPP, ER, or temperature, indicating that all mesocosm tanks began with similar conditions (Kruskal Wallis test, $p > 0.05$).

Our analysis of ecosystem effects paralleled that of community effects, including Kruskal-Wallis tests for each response at each timepoint. However, for both analyses, we also included the zooplankton-absent treatment. Including this control allowed us to test for effects of the overall trophic cascade, including indirect effects of differences in harassment. As with zooplankton analyses, if any effect of harassment treatment was found, we calculated Hedge's g effect sizes for all ecosystem responses to compare the magnitude of these effects.

RESULTS

Harassment treatments

As expected, we found a significant effect of *treatment* (low- versus high-harassment; X^2= 4.52, df= 1, p=0.034) and *timepoint* (week of the experiment; X^2= 58.80, df= 5, p< 0.01) on harassment scores within each tank. At all timepoints, high-harassment treatments had higher male harassment levels than low-harassment treatments (df= 231, t= 2.13, p= 0.035) (Fig 3.2). However, overall harassment levels declined over the course of the experiment: within each treatment, harassment levels on day 2 were higher than harassment levels on all subsequent observation days (Table 1). Harassment levels on days 4-13 did not differ from each other. We found no effect of a *treatment x timepoint* interaction (X^2= 1.23, df= 5, p= 0.94), indicating that the magnitude of the difference between high- and low-harassment treatments did not change over time.

Zooplankton community effects

We identified seven dominant zooplankton taxonomic groups within our samples: *Daphnia, Ceriodaphnia, Calanoid copepod, Cyclopoid copepod, Bosmina, Chydoridae,* and mosquito larvae. Rare taxa (fewer than 500 individuals counted across all samples) were excluded from the analysis because their effect on overall zooplankton communities was negligible. We highlight results from the earliest sampling timepoint below, as this was when harassment was strongest during the experiment, and later sampling points showed few effects of harassment. Analyses for all timepoints can be found in the appendix (Fig A3.1).

Overall, our results suggest that harassment intensity modulated the effects of fish introduction on *Daphnia*, *Ceriodaphnia*, *Calanoid*, and *Chydorid* abundance as well as *Daphnia* size phenotype. Specifically, fish introduction caused lower abundances in these species and smaller *Daphnia* sizes, but only if introduced fish had a high-harassment phenotype. Since harassment intensity was strongest at the beginning of the experiment, we focused on comparing community responses among treatments on day 4, the earliest sampling point (pairwise comparisons for all timepoints can be found in the appendix, Fig A3.2). Harassment treatment differences had no effect on total zooplankton abundance (X^2 = 1.59, df = 2, p-value = 0.45) (Fig 3.3), However, *Daphnia*, *Ceriodaphnia*, and *Calenoid* abundances were significantly lower in high-harassment treatments compared to fish-absent treatments (*Daphnia*: Z= -2.53, p=0.04; *Ceriodaphnia*: Z= -2.86, p= 0.01; *Calanoid*: Z= -2.96, p=0.01), and *Chydorid* abundance and *Daphnia* size were marginally lower in high-harassment treatments compared to fish-absent treatments *Chydorid:* Z= -2.22, p= 0.08; *Daphnia* size: Z= -2.29, p=0.07) (Fig 3.3). *Calenoid* abundance was also marginally lower in high-harassment than low-harassment treatments (Z= -1.93, p=0.08), although no other differences between high- and low-harassment treatments were found. For each of these community responses, high-harassment fish induced “large” negative effect sizes (meaning decreased zooplankton abundance, consistent with increased feeding) compared to fish-absent treatments (i.e. effect sizes less than -0.8 (Hedges & Olkin, 1985)) (Fig 3.4).

In contrast to high-harassment treatments, low-harassment treatments were not associated with declines in these same zooplankton responses: we found no differences between low-harassment and fish-absent treatments in *Daphnia*, *Ceriodaphnia*, *Calenoid*, or *Chydorid* abundance or *Daphnia* size (*Daphnia:* Z= -1.19, p=0.23; *Ceriodaphnia:* Z= -1.86, p= 0.10; *Calenoid:* Z= -1.57, p=0.12; *Chydorid:* Z= -1.50, p=0.20; *Daphnia* size: Z=-1.19, p= 0.23). Additionally, effect sizes of introducing low-harassment fish were medium, small, or negligible (Fig 3.4).

We found different patterns of treatment effects in other zooplankton taxa. Mosquito larvae were less abundant in both high-and low-harassment treatments compared to fish-absent treatments (high: Z= -2.69, p= 0.01; low: Z= -3.66, p= 0.001), with no differences between high- and low-harassment tanks (Z= 1.34, p=0.18) (Fig 3.3). The effect sizes of introducing high- and low-harassment fish on mosquito larvae were both large and negative (Fig 3.4). These results indicate that fish introductions reduced mosquito larvae abundance, regardless of their harassment phenotype. We also found no effect of treatment on *Bosmina* and *Cyclopoid* abundance (p > 0.10 for all pairwise comparisons), indicating that fish introductions had no effect on these populations.

Ecosystem effects

We found no evidence that fish addition affected any ecological factor prior to day 12 of the experiment (Appendix, Fig A3.2). Consequently, we focus our analyses of ecosystem effects on Day 12, because prior to this time point the presence of fish had no significant effect (pairwise comparisons for all timepoints can be found in Fig A3.2). At this timepoint, we found treatment effects of fish addition in both chlorophyll concentrations and pH, but not in NPP, ER, GPP, or temperature (Fig 3.5). Chlorophyll concentrations were lower in fish-absent treatments compared to high-harassment (Z= 3.68, p< 0.01), low-harassment (Z= 3.50, p< 0.01), and zooplankton-absent treatments (Z = -4.15, p< 0.01), a pattern consistent with trophic cascades. However, high- and low-harassment treatments did not differ from each other (Z= 0.24, p= 0.81), indicating that harassment intensity did not affect the strength of the cascade. pH was lower in both high- and low-harassment treatments compared to fish-absent (high: Z= -2.45 p= 0.03 ; low: Z= -2.38, p=0.03) and zooplankton-absent treatments (high: Z= -3.49, p= 0.003 ; low: Z= -3.44, p=0.002). Note that initial pH levels in the experiment were elevated in all tanks (range: 8.87 to 9.63) due to the CZU Lightning Complex Fires in 2020, which deposited ash into mesocosms. Since no effect of harassment treatment was found in any ecosystem responses, effect size calculations are not included in our results.

DISCUSSION

The consequences of intraspecific variation for ecological patterns and processes are widely established (Bolnick et al., 2011; Des Roches et al., 2018; Raffard et al., 2019), but few studies have explored the ecological implications of variation in sexually selected traits (Giery & Layman, 2019; Svensson, 2019). Here, we show that phenotypic divergence in reproductive behavior (sexual harassment of females by males), a sexually selected trait, can have important yet nuanced ecological consequences. We found that mosquitofish introductions had large negative effects on the abundance of several dominant zooplankton species, but only in high-harassment populations. Harassment had a similar effect on prey phenotypes: we found that *Daphnia* were marginally smaller in treatments with high-harassment fish compared to treatments with low-harassment fish or no fish. However, harassment-based differences were constrained to prey communities and had no effect on cascading ecosystem effects. Mosquitofish introductions increased chlorophyll concentrations and decreased pH compared to treatments without fish, yet this effect occurred regardless of harassment treatment.

Our results suggest that variation in reproductive behavior can mediate the scope and strength of consumer effects on communities and ecosystems. Specifically, sexual harassment phenotypes of mosquitofish can govern whether their introduction causes detectable changes in prey community composition and abundance. In our study, zooplankton communities were affected by fish addition only if mosquitofish harassment levels were high: while high-harassment fish reduced the abundance of *Daphnia*, *Ceriodaphnia*, *Calenoids*, and *Chydorid* relative to treatments with no fish, zooplankton communities in treatments with low-harassment fish were indistinguishable from those without fish. This finding is surprising, since mosquitofish are well-known for their ecological effects, earning the name "plague minnow" due to the destructive impact of their feeding behavior on native freshwater communities (Pyke, 2005, 2008). Previous work has demonstrated that effects of mosquitofish introduction are modulated by intraspecific trait diversity, yet effects of fish introduction on community structure and ecosystem function are found consistently, regardless of fish phenotypes (D. C. Fryxell & Palkovacs, 2017; Wood et al., 2019, 2022).

That high-intensity sexual harassment was linked to declines in zooplankton abundance is consistent with our hypothesis that fish in high-harassment environments expend more energy and consequently must consume more prey to fuel metabolic demands. In livebearing species closely related to mosquitofish, stress caused by male presence is insufficient to illicit an increase in female metabolic rates, although males in these studies did not directly chase or interact with females (Köhler et al., 2011). Energetic costs of sexual harassment have been documented in numerous other species (Córdoba-Aguilar & González-Tokman, 2011; Watson et al., 1998). Although direct energetic costs of harassment are unmeasured in mosquitofish, their harassment is among the most intense of livebearing species (Plath et al., 2007).

Maintaining or escaping this level of harassment requires prolonged bouts of activity that are likely metabolically taxing and demand increased feeding to replenish energy. Although we did not directly measure mosquitofish feeding rates, greater declines in some prey species in high-harassment tanks compared to low-harassment tanks suggest that high-harassment fish consumed more prey. This finding may appear to contradict previous laboratory studies that show male harassment in livebearers decreases female feeding efficiency (Dadda et al., 2005; Magurran & Seghers, 1994; Pilastro et al., 2003). However, reduced female feeding efficiency may occur simultaneously with an increase in overall consumption rates– females in high-harassment treatments may be less efficient consumers (i.e. they have a lower rate of prey capture compared to females encountering less harassment), but over long time periods may have higher energetic demands and thus consume more prey overall. This result demonstrates the importance of considering timescales when applying patterns found in laboratory experiments to ecological settings.

Effects of harassment on zooplankton prey differed among species, a finding consistent with diet selectivity in mosquitofish predation (Angeler et al., 2002; Bence, 1988; Crivelli & Boy, 1987; Hurlbert & Mulla, 1981). In our study, the preferred targets of mosquitofish predation appear to be *Daphnia*, *Ceriodaphnia*, *Calanoids*, and *Chydorid* zooplankton, which were found in lower abundances in high-harassment treatments compared to low-harassment treatments. In contrast, we found no effect of treatment on *Bosmina* or *Cyclopoid* abundance. Mosquitofish diet selectivity is likely related to our finding that total zooplankton abundance did not differ among high-harassment, low-harassment, and fish-absent treatments. As mosquitofish selectively consumed some prey types, compensatory responses in non-target prey that were released from competition may have masked overall effects on total prey abundance (Tessier & Woodruff, 2002). Indeed, since *Bosmina* were the most numerically dominant species in our samples, their extremely high relative abundance in the total zooplankton count likely masked the comparatively small reductions among other species, resulting in similar total zooplankton abundance among treatments.

Our findings related to mosquito larvae and *Daphnia* size structure are also consistent with diet selectivity playing a key role in shaping harassment effects on the zooplankton community. In both cases, mosquitofish selectively targeted larger, more conspicuous prey. Mosquito larvae were less abundant in treatments containing fish, regardless of their harassment intensity. Mosquito larvae are a common target of mosquitofish predation (Bence, 1988) and were likely consumed first in both harassment treatments due to their larger size and increased visibility relative to other prey types. As mosquito larvae abundance decreased and energetic demands in high-harassment tanks increased, fish may have begun consuming other species, leading to reductions in other prey types that were not found in low-harassment treatments. This prediction is consistent with observed changes in daphnia size among treatments: high-harassment fish significantly reduced daphnia size relative to treatments without fish, although there was no difference in daphnia between low-harassment and fish-absent treatments. This outcome suggests that larger daphnia were selectively consumed (see also Bence & Murdoch, 1986).

Effects of harassment on community composition were temporary, appearing at the beginning of the experiment but disappearing at later sampling points. The transience of community responses paralleled changes in overall harassment levels. Although harassment rates differed in high- and low-harassment treatments throughout the experiment, harassment was significantly higher at the first sampling point compared to later sampling points. Given that harassment effects were highest early in the experiment, it is unsurprising that consequences of harassment were most prominent during the first sampling point. The parallel decline in overall harassment levels and ecological effects of harassment that occurred over the course of the experiment could be seen as supportive evidence that harassment caused observed ecosystem differences. However, it is also possible that harassment effects on ecological responses were strongest immediately after fish introduction but waned over time. These two possible explanations are inextricable in our experiment. Future studies on the ecological consequences of harassment should prioritize methods that sustain phenotypic differences over time in order to disentangle possible explanations for temporal trends (e.g. by using different male morphs, or chemically manipulating behavior).

While harassment effects were evident in community responses (i.e. zooplankton), we found no evidence for harassment effects on trophic cascades (i.e. phytoplankton) or abiotic ecosystem characteristics. However, we found effects of fish introduction on chlorophyll concentrations in general. At the final sampling point, chlorophyll was higher in treatments containing fish compared to those without fish. Effects of fish introduction on chlorophyll are well established and are typically due to two non-mutually exclusive mechanisms, trophic cascades and fish excretion (Attayde & Hansson, 2001; Persson, 1999). Trophic cascades are driven by the indirect consequences of fish predation, which suppresses large zooplankton grazers such as daphnia that would otherwise diminish producer populations (Persson, 1999). Mosquitofish also directly enrich productivity by releasing nutrients through excretion, stimulating phytoplankton growth (Matveev et al., 1994). While we cannot separate the relative importance of each mechanism stimulating productivity in our experiment, both processes were likely involved in the patterns we observed.

Mosquitofish are notorious for their impact on aquatic ecosystems (Pyke, 2005, 2008), but our findings indicate that these effects can be modulated by sexual harassment phenotypes. Specifically, mosquitofish modified zooplankton prey communities only when males exhibited high sexual harassment phenotypes. These results extend the scope of intraspecific ecological effects beyond those shaped by natural selection to also include traits shaped by sexual selection. Other sexually selected traits may also have important implications for communities and ecosystems, such as physical habitat modification through nest construction or use of display sites (Giery & Layman, 2019). We encourage future investigations on the implications of intraspecific variation in sexually selected traits using other study systems with strong ecosystem interactors (e.g. keystone species, ecosystem engineers). Such work can further inform our understanding of the nuanced ways that sexual selection can influence ecological patterns and processes.

TABLES

Table 3.1. Pairwise comparisons of estimated marginal mean harassment levels between timepoints, with Bonferroni corrections applied for multiple comparisons. Estimated marginal means were generated from best fit generalized linear mixed-effect model describing tank harassment times predicted as a function of harassment treatment (low- vs. high-harassment) and timepoint. Note that because there was no significant interaction term between timepoint and treatment in the best fit model, pairwise comparisons between timepoints are identical for both high- and low-harassment treatments.

Timepoint contrast	**df**	**t-ratio**	**p value**
Day 2 / Day 4	231	1.57	**< 0.001**
Day 2 / Day 7	231	1.62	**< 0.001**
Day 2 / Day 10	231	1.86	**< 0.001**
Day 2 / Day 13	231	1.92	**< 0.001**
Day 4 / Day 7	231	1.03	> 0.99
Day 4 / Day 10	231	1.19	> 0.99
Day 4 / Day 13	231	1.22	0.79
Day 7 / Day 10	231	1.15	> 0.99
Day 7 / Day 13	231	1.19	> 0.99
Day 10 / Day 13	231	1.03	> 0.99

FIGURES

Figure 3.1. Competing hypotheses for the effects of harassment on grazer communities and producer abundance. High harassment may increase (solid black arrow) or decrease (dashed black arrow) consumption rates and prey selectivity in mosquitofish compared to low-harassment populations. These differences may cascade to lower trophic levels, with producer abundance inversely proportional to the abundance of grazers (grey arrows).

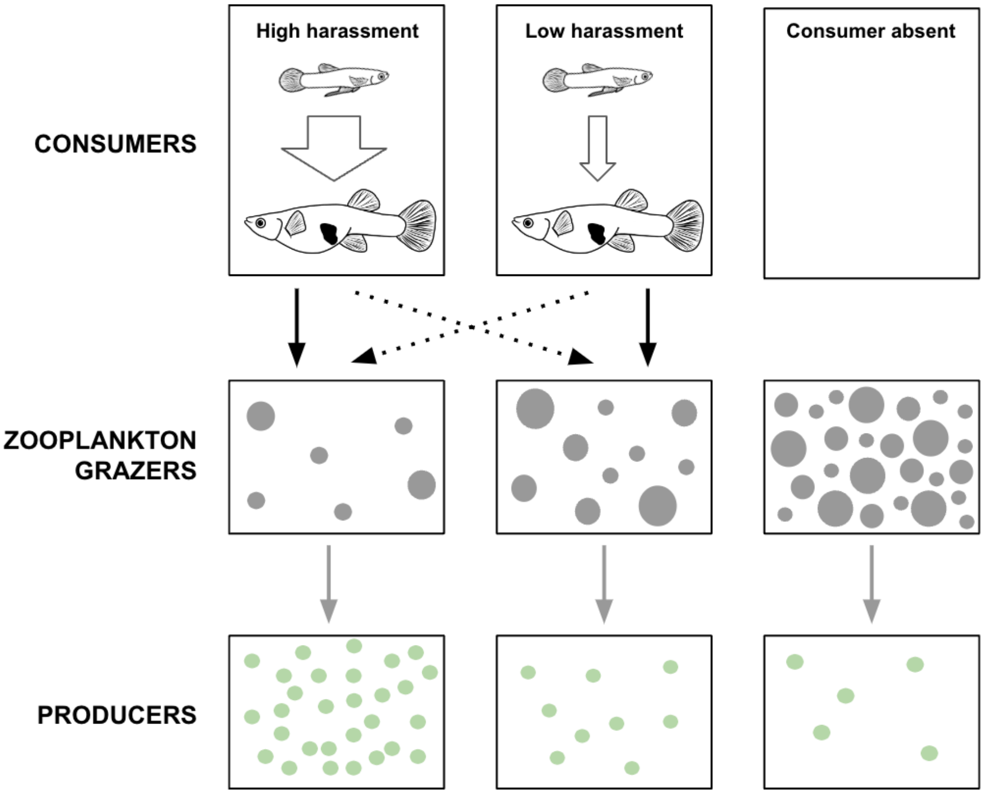


Figure 3.2. Harassment behavior was elevated in high-harassment treatments compared to low-harassment treatments throughout the experiment. However, harassment levels were highest overall on Day 2 compared to later observation days in both treatments. Error bars indicate +/- 2 SE from predicted values.

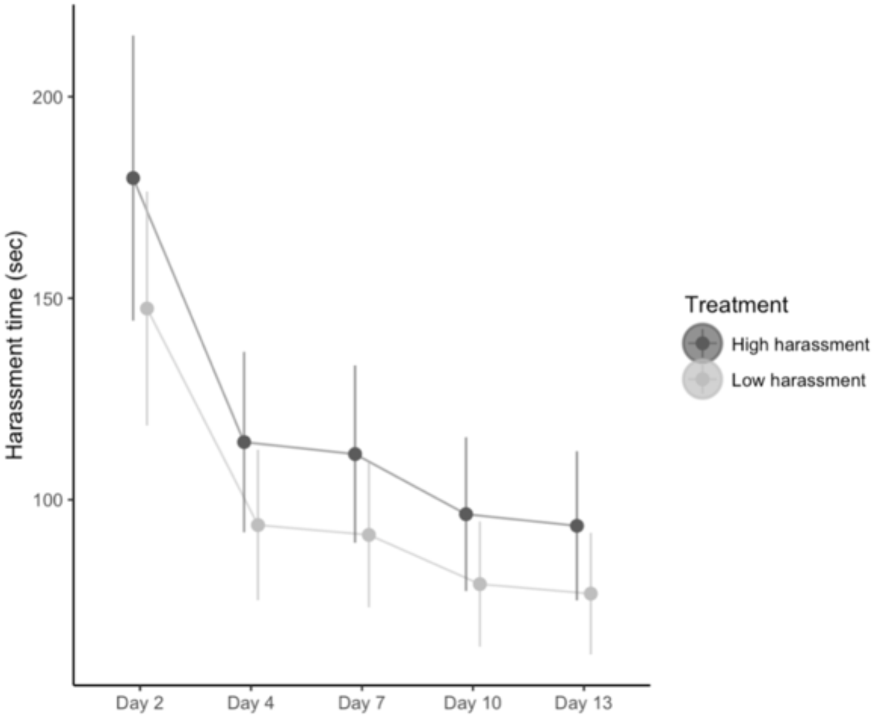


Figure 3.3. Effects of high-harassment, low-harassment, and fish-absent treatments on zooplankton community abundance and traits on day 4 of the experiment. Compared to tanks in which fish were absent, tanks with high-harassment fish had lower daphnia, calanoid, ceriodaphnia, and chydorid abundance, as well as smaller daphnia sizes. Tanks with high-harassment fish also had lower calanoid abundance than those with low-harassment fish. Fish introductions decreased mosquito larvae abundance regardless of their harassment. Significance symbols from Dunn pairwise comparison tests with Bonferroni corrections ($p<0.05$*, $p<0.01^{+}$).

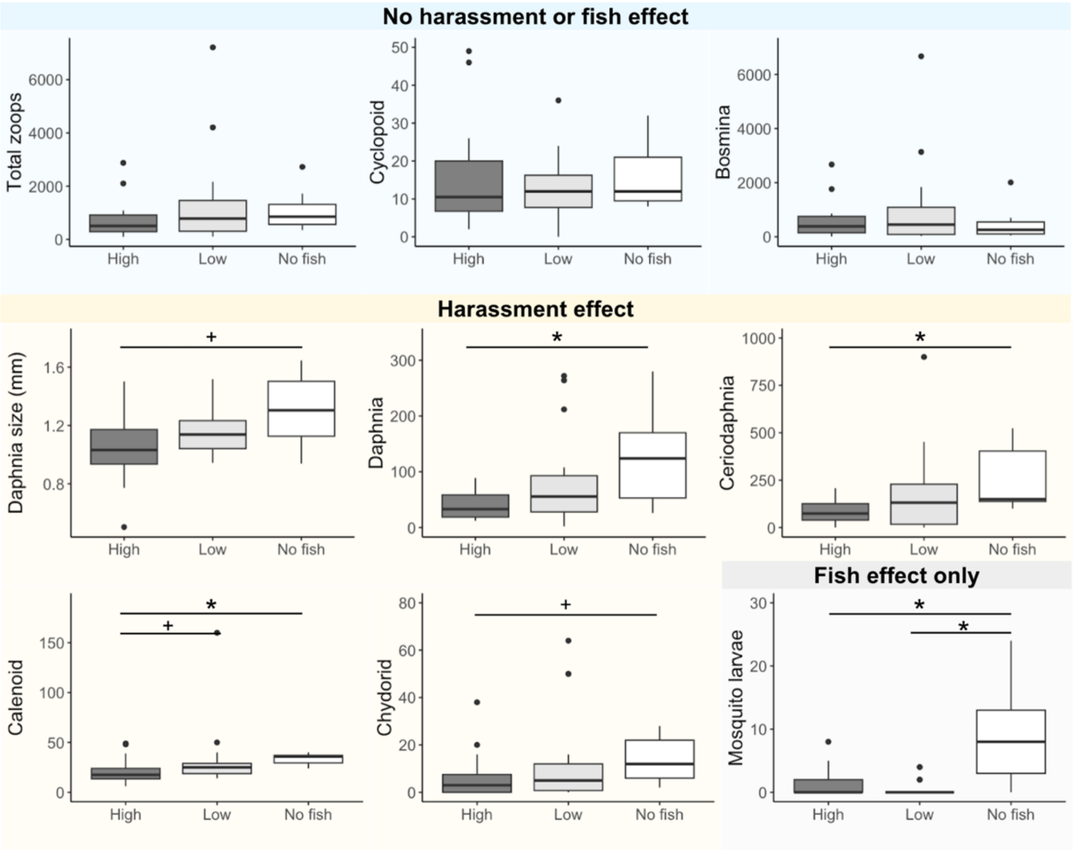


Figure 3.4. Comparisons of the effects of high- and low-harassment fish introduction on zooplankton community abundance and traits. Significance symbols indicate that responses in high- or low-harassment treatments differed from treatments with no fish (Dunn tests with Bonferroni correction, $p< 0.05^{*}$; $p< 0.10^{+}$). High-harassment treatments had a large negative effect on daphnia, ceriodaphnia, calenoid, and chydorid abundance and daphnia size. Both high- and low-harassment fish had large negative effects on mosquito larvae abundance.

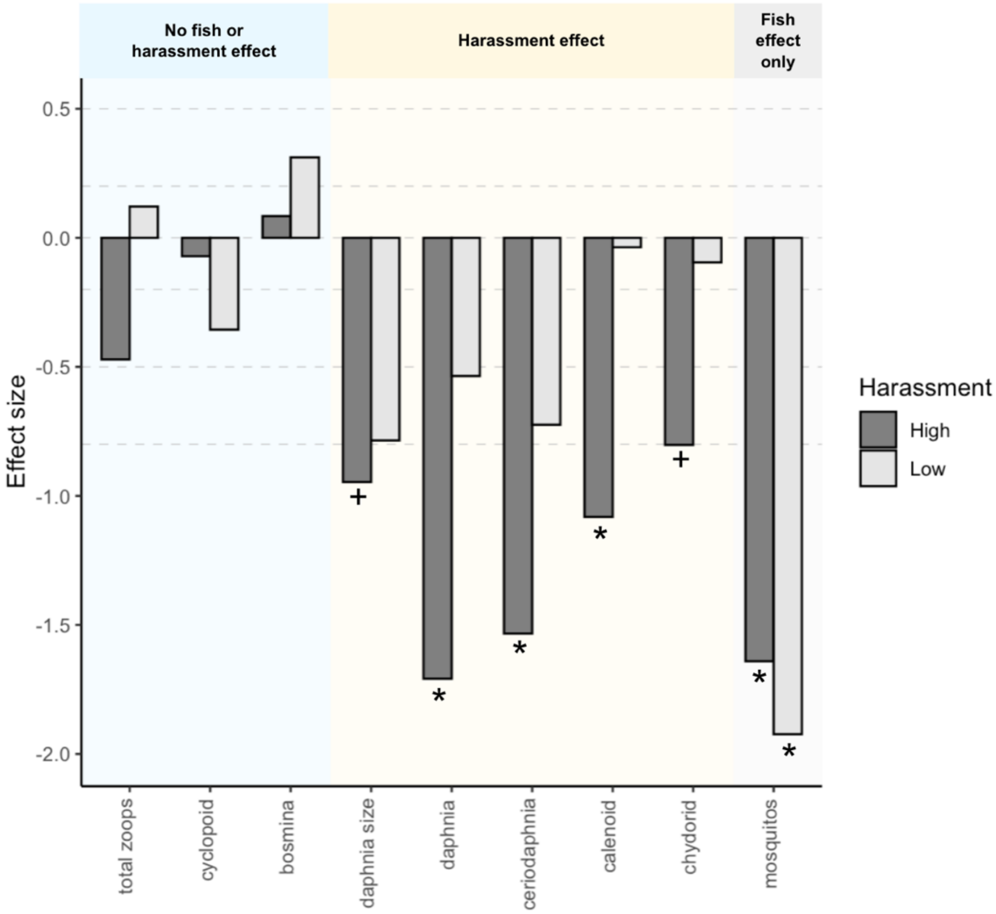
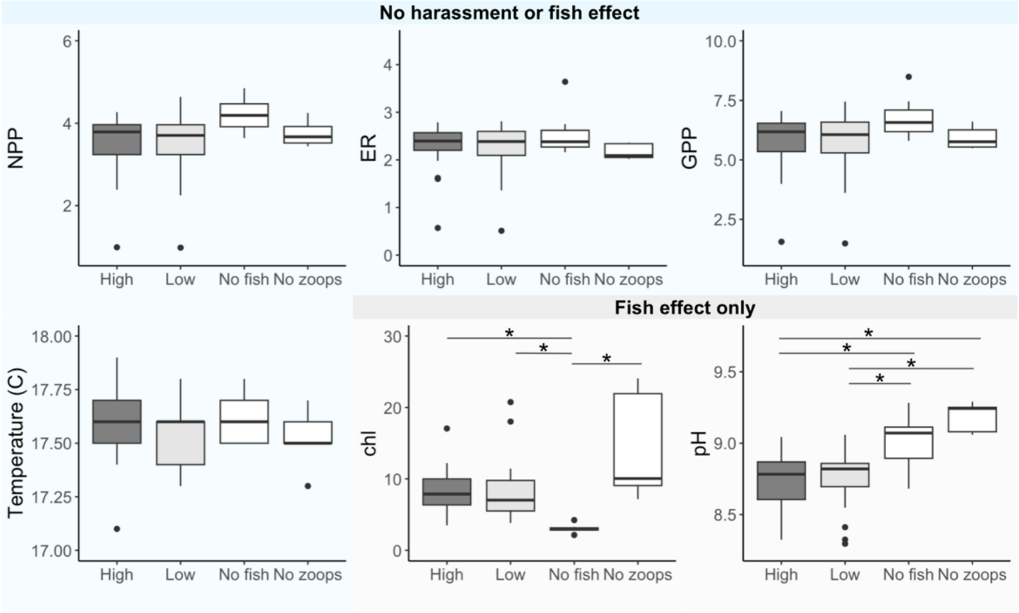


Figure 3.5. Effects of high-harassment, low-harassment, fish-absent (“No fish”), and zooplankton absent (“No zoops”) treatments on various ecological factors on day 12 of the experiment. Ponds with fish had higher chlorophyll concentrations than those with no fish but did not differ from tanks without zooplankton added. However, this effect did not differ among high- and low-harassment tanks. Tanks with fish also had lower pH compared to fish-absent and zooplankton-absent treatments, regardless of harassment levels. Significance symbols from Dunn pairwise comparison tests with Bonferroni corrections (p<0.05*).



SYNTHESIS

The field of eco-evolutionary dynamics is rapidly elucidating the many ways that ecological and evolutionary processes are intertwined (Palkovacs & Hendry, 2010). Countless studies have revealed how ecological settings influence natural selection on diverse phenotypic traits and identified how evolutionary changes in traits can then feedback to affect ecological outcomes (Carlson et al., 2011; Harmon et al., 2009; Palkovacs & Post, 2009; Wood et al., 2022). However, the role of sexual selection in eco-evolutionary dynamics remains relatively unexplored (Svensson, 2019; Svensson & Gosden, 2007). My dissertation paves the way for greater integration of sexual selection into the eco-evolutionary framework by exploring sources of intraspecific variation in sexually selected traits and the ecological consequences of such variation, using western mosquitofish (*Gambusia affinis*) as a model system. Collectively, this body of work demonstrates that evolved differences in sexually selected traits may be pervasive, although the causes of such variation may be more elusive than expected based on theoretical predictions. Despite this ambiguity in identifying the drivers of observed intraspecific variation, I found that differences in mating behavior can have important consequences for community ecology.

In **Chapter 1**, I asked whether persistent differences in average temperatures among populations of mosquitofish generated intraspecific diversity in male mating behavior, a sexually selected trait. Temperature is often considered one of the most important abiotic factors in organismal biology because it governs the rate of biochemical processes, which scales to affect physiology, morphology, and behavior (Angilletta, 2009). Thus, I predicted that selection in populations with divergent thermal regimes would generate systematic, temperature-based differences in mating behavior (Angilletta et al., 2002; Angilletta, 2009; Angilletta et al., 2010; Huey & Kingsolver, 1989). Using common rearing techniques and behavioral assays, I measured evolved differences in copulation attempts, copulations, and courtship displays in male mosquitofish. Although these behaviors were sensitive to ambient temperature, peaking at intermediate temperatures and declining at thermal extremes, I found no evidence that divergent source temperatures could explain observed behavioral differences among populations. These findings highlight how evolutionary responses to temperature may be less predictable than theoretical expectations suggest, especially in sexually selected traits, which may be more strongly influenced by social context than abiotic conditions.

In **Chapter 2**, I aimed to explain the evolutionary mystery of intraspecific variation in female mosquitofish gravid spots. Gravid spots in livebearers correspond to the developmental stage of embryos, as well as female fecundity (Norazmi-lokman et al., 2016). I predicted that males use gravid spots as a sexual signal, and that differences in male preferences could explain variation in female gravid spot sizes among populations. Theory describing the evolution of sexual signaling and mate preferences predicts that sexual signals and behavioral responses will coevolve, leading to correlated traits and responses across populations (Edwards, 2000; Houde, 1993; Jennions & Petrie, 1997). While this pattern has previously been identified with male signals and female preferences (e.g. Houde & Endler, 1990), few have considered evolutionary outcomes when sex roles are reversed. To test whether male preferences could explain variation in female sexual signals, I identified two focal populations with different average gravid spot sizes and compared male preferences for gravid spots among these populations using a free-swimming paired choice behavioral assay. I found that male preferences for gravid spots differed among populations, but patterns of variation in female traits directly opposed theoretical predictions: female gravid spots were smallest in the population in which males demonstrated a stronger preference for larger gravid spots. One possible reason for this outcome is that the dynamics of coevolution of male mate choice and female signals may be distinct from those of female mate choice and male signaling due to the sex-specific selection. While males often benefit from increased mating opportunities obtained by signaling their mate quality, females may endure excess sexual harassment, which can be costly for their fitness. Although we can only speculate on the specific factors driving diversity in female gravid spots among populations, this study again demonstrated the prevalence of intraspecific variation in sexually selected traits.

In **Chapter 3**, I used a mesocosm experiment to determine how intraspecific variation in male mosquitofish sexual harassment affected aquatic ecosystem dynamics. I generated phenotypic diversity in male behavior by isolating males from females to temporarily increase harassment rates, then measured and compared the ecological consequences of mosquitofish introductions among high-harassment, low-harassment, and fish-absent treatments. I found that harassment phenotypes modulated the effect of mosquitofish introductions on the abundance of several zooplankton species, including *Daphnia*, *Ceriodaphnia*, *Calenoids,* and *Chydorids.* Specifically, only high-harassment treatments reduced the abundance of these species relative to fish-absent treatments. We found no differences in zooplankton abundance between treatments with low-harassment fish and treatments with no fish added. Additionally, only high-harassment mosquitofish reduced *Daphnia* body size phenotypes. Effects on zooplankton did not cascade throughout the ecosystem. These findings offer concrete empirical evidence of the importance of intraspecific diversity in sexually selected traits.

In summary, this dissertation demonstrates that traits under sexual selection are fertile ground for exploring the interplay of ecological and evolutionary processes. Using mosquitofish as a model system, I identified notable intraspecific variation in both behavioral and morphological sexually selected traits among populations, as well as concrete ecological consequences of this variation. Although the research completed in this body of work focuses on a single species, sexual selection is ubiquitous in nature, and intraspecific variation in other sexually selected traits, such as physical habitat modification through nest construction or use of display sites, may also have important implications for communities and ecosystems (Giery & Layman, 2019).

Thus, there is immense potential in expanding the study of eco-evolutionary processes to incorporate sexually selected traits. Identifying which ecological contexts drive particular evolutionary outcomes in sexually selected traits remains an elusive challenge, creating an exciting opportunity for future research to expand our understanding of eco-evolutionary dynamics.

APPENDICES

CHAPTER 1

Table A1.1. Focal site characteristics, ordered by mean site temperature from the coolest site to the warmest site (adapted from Fryxell, 2020). We approximated site area by drawing Google Earth polygons and calculating its area at http://earthpoint.us/shapes.aspx. We measured pH with an Oakton PT Testr and measured dissolved oxygen and specific conductance with a YSI Pro DO. We measured water PO4 and NOx concentrations from previously filtered (0.7 µm) then frozen site water using standard manufacturer methods on a LaChat flow injection analyzer. We determined pelagic chlorophyll a concentration after acetone extraction using the non-acid module on a Turner Designs Trilogy Fluorometer. Bullfrogs are nonnative American bullfrogs (Lithobates catesbeianus), crayfish are nonnative red swamp crayfish (Procambarus clarkii), and pupfish are native and endangered Owens pupfish (Cyprinodon radiosus). We gathered these data on the same dates for each characteristic, albeit on different dates across characteristics. Because these are stable, spring-fed systems, we expect these point-estimates to reflect largely consistent variation among sites. Pearson correlation values between each environmental variable and temperature are included.

Spring name	**Name code**	**Tmean (ºC)**	**Tvar (SD)**	**Max depth (m)**	**Site area (m^2)**	**pH**	**Specific cond (mS/cm)**	**Diss. Oxygen (mg/L)**	**[PO4] (ppm P)**	**[NO_x] (ppm N)**	**Chlorophyll a (µg/L)**	**Barrier-purpose**	**Other large animals**
Northeast Spring	NE	18.9	0.835	1.7	101	8.3	0.36	8.26	0.03	0.68	0.24	Manmade-unknown	Crayfish
BLM Spring	BLM	21.1	1.700	1.7	886	8.2	0.47	6.98	0.01	0.33	0.65	Manmade-pupfish	Pupfish, crayfish

Artesian Well	AW	23.7	0.627	1.7	350	7.4	0.45	4.49	0.03	0.17	1.66	Manmade-cattle	Crayfish
Warm Springs	WSU	27.0	1.230	2.0	435	7.8	0.51	7.91	0.01	0.11	0.40	Manmade-pupfish	Crayfish, Bullfrogs
Keough Hot Ditch	K2	31.6	2.124	1.2	228	8.4	0.86	5.31	0.01	0.16	0.36	Natural-NA	None
Little Hot Creek	LHC	33.3	2.263	1.0	127	8.2	2.04	6.11	0.05	0.04	1.81	Natural-NA	None
Cor (Tmean, Y)		1	0.66	-0.67	-0.31	0.18	0.79	-0.42	0.24	-0.72	0.26	0.26	

Table A1.2. Sample sizes for the number of mosquitofish from each source population temperature observed at each experimental temperature.

Source Temperature	Experimental Temperature				
	15	**20**	**26**	**32**	**37**
18.85	27	28	26	25	26
21.06	21	27	23	26	17
23.66	26	30	31	32	24
27.00	26	26	27	22	26
31.57	26	26	27	28	26
33.31	28	28	25	28	28

Table A1.3. Coefficient estimates for each of the best fit zero-inflated generalized linear mixed effect models explaining display behavior. Courtship displays were modeled in two parts, a binary process to determine the probability of measuring a zero (zero-inflated binomial model with a log link), and a count process (negative binomial model with a logit link). The table lists the coefficients and standard error for fixed effects, and the variance explained by the random effect of male identity (+/- standard deviation).

Response	**Fixed Effects**				**Random Effects**
	Effect	**Estimate (±SE)**	**Z value**	**p value**	**Variance (±SD)**
Displays: Population as source temp					
	Conditional Model				
	Intercept	01.01 (± 0.09)	11.89	**<0.001**	0.13 (± 0.36)
	Ambient temperature	-15.48 (± 2.42)	-6.41	**<0.001**	
	(Ambient temperature)^2	-23.53 (± 2.09)	-11.24	**<0.001**	
	(Ambient temperature)^3	-9.72 (± 1.53)	-6.37	**<0.001**	
	(Ambient temperature)^4	-6.12 (± 0.97)	-6.29	**<0.001**	
	Zero-inflation model				
	Intercept	-1.62 (± 0.44)	-3.66	**<0.001**	
	Ambient temperature	-1.85 (± 0.45)	-4.15	**<0.001**	
Displays: Population as population identity					
	Conditional Model				
	Intercept	1.12 (± 0.12)	9.27	**<0.001**	0.09 (± 0.29)
	Ambient temperature	-16.75 (± 2.27)	-7.37	**<0.001**	

(Ambient temperature)^2	-21.38 (±2 .02)	-10.59	**<0.001**
(Ambient temperature)^3	-10.90 (± 1.50)	-7.27	**<0.001**
(Ambient temperature)^4	-5.76 (± 1.02)	-5.63	**<0.001**
Population: BLM	0.19 (± 0.15)	1.25	0.21
Population: AW	-0.87 (± 0.16)	-5.49	**<0.001**
Population: WSU	-0.15 (± 0.16)	-0.98	0.33
Population: K2	-0.44 (± 0.16)	-2.80	**0.005**
Population: LHC	0.19 (± 0.15)	1.27	0.21
Zero-inflation model			
Intercept	-5.32 (± 0.77)	-6.95	**<0.001**
Ambient temperature	-5.25 (± 0.68)	-7.69	**<0.001**

Table A1.4. Coefficient estimates for each of the best fit zero-inflated generalized linear mixed effect models explaining copulation attempts. Attempted copulations were modeled in two parts, a binary process to determine the probability of measuring a zero (zero-inflated binomial model with a log link), and a count process (negative binomial model with a logit link). The table lists the coefficients and standard error for fixed effects, and the variance explained by the random effect of male identity (+/- standard deviation).

Response	**Fixed Effects**				**Random Effects**
	Effect	**Estimate (±SE)**	**Z value**	**p value**	**Variance (±SD)**
Copulation attempts: Population as source temp					
	Conditional Model				
	Intercept	2.56 (± 0.07)	34.70	**<0.001**	0.24 (± 0.49)
	Ambient temperature	16.58 (± 1.93)	8.58	**<0.001**	
	(Ambient temperature)^2	-20.72 (± 1.44)	-14.36	**<0.001**	
	(Ambient temperature)^3	4.10 (± 1.25)	3.27	**0.001**	
	(Ambient temperature)^4	-4.00 (± 1.15)	-3.49	**<0.001**	
	Zero-inflation model				
	Intercept	-1.89 (± 0.27)	-7.038	**<0.001**	
	Ambient temperature	0.13 (± 0.34)	0.376	0.707	
Copulation attempts: Population as population identity					
	Conditional Model				
	Intercept	2.65 (± 0.14)	18.67	**<0.001**	0.23 (± 0.48)
	Ambient temperature	17.01 (± 1.99)	8.56	**<0.001**	

(Ambient temperature)^2	-21.11 (±1.43)	-14.76	**<0.001**
(Ambient temperature)^3	4.06 (± 1.25)	3.26	**0.001**
(Ambient temperature)^4	-4.26 (± 1.16)	-3.67	**<0.001**
Population: BLM	-0.08 (± 0.20)	-0.40	0.69
Population: AW	-0.21 (± 0.20)	-1.05	0.29
Population: WSU	-0.01 (± 0.20)	-0.07	0.94
Population: K2	-0.47 (± 0.20)	-2.33	**0.02**
Population: LHC	0.20 (± 0.20)	1.02	0.31
Zero-inflation model			
Intercept	-1.98 (±0.33)	-5.96	**<0.001**
Ambient temperature	0.21 (±0.40)	0.53	0.60

Table A1.5. Coefficient estimates for each of the best fit zero-inflated generalized linear mixed effect models explaining copulations. Copulations were modeled in two parts, a binary process to determine the probability of measuring a zero (zero-inflated binomial model with a log link), and a count process (negative binomial model with a logit link). The table lists the coefficients and standard error for fixed effects, and the variance explained by the random effect of male identity (+/- standard deviation).

Response	**Fixed Effects**				**Random Effects**
	Effect	**Estimate (±SE)**	**Z value**	**p value**	**Variance (±SD)**
Copulations: Population as source temp					
	Conditional Model				
	Intercept	-0.31 (± 0.11)	-2.85	**0.004**	0.56 (± 0.75)
	Ambient temperature	30.42 (± 3.06)	9.95	**<0.001**	
	(Ambient temperature)^2	-23.59 (± 2.72)	-8.69	**<0.001**	
	(Ambient temperature)^3	4.58 (± 2.01)	2.28	**0.02**	
	(Ambient temperature)^4	-5.20 (± 1.37)	-3.79	**<0.001**	
	Zero-inflation model				
	Intercept	-8.71 (± 5.93)	-1.47	0.14	
	Ambient temperature	6.24 (± 4.16)	1.50	0.13	
Copulations: Population as population identity					
	Conditional Model				
	Intercept	0.15 (± 0.19)	0.79	0.43	0.54 (± 0.73)
	Ambient temperature	30.57 (± 3.06)	10.00	**<0.001**	

(Ambient temperature)^2	-23.70 (±2.71)	-8.73	**<0.001**
(Ambient temperature)^3	4.48 (± 2.01)	2.23	**0.03**
(Ambient temperature)^4	-5.27(± 1.37)	-3.84	**<0.001**
Population: BLM	-0.60 (± 0.26)	-2.26	**0.02**
Population: AW	-0.79 (± 0.26)	-3.11	**0.002**
Population: WSU	-0.42 (± 0.26)	-1.62	0.11
Population: K2	-0.66 (± 0.26)	-2.52	**0.01**
Population: LHC	-0.35 (± 0.26)	-1.35	0.18
Zero-inflation model			
Intercept	-8.66 (±5.74)	-1.51	0.13
Ambient temperature	6.21 (±4.03)	1.54	0.12

Table A1.6. Coefficient estimates for each of the best fit generalized linear mixed effect models explaining mating efficiency (successful copulations/ total copulation attempts). Mating efficiency was modeled with a binomial distribution and a log link function. The table lists the coefficients and standard error for fixed effects, and the variance explained by the random effect of male identity (+/- standard deviation).

Response	**Fixed Effects**				**Random Effects**
	Effect	**Estimate (±SE)**	**Z value**	**p value**	**Variance (±SD)**
Copulations: Population as source temp					
	Intercept	-2.84 (± 0.07)	-40.18	**<0.001**	0.26 (± 0.51)
	Ambient temperature	13.51 (± 1.65)	8.21	**<0.001**	
	(Ambient temperature)^2	-8.57 (± 1.45)	-5.89	**<0.001**	
	Source temperature	-0.03 (± 0.07)	-0.46	0.64	
	Ambient temperature x source temperature	-3.57 (± 1.51)	-2.35	**0.02**	
	Ambient temperature x (source temperature)^2	4.51 (± 1.38)	3.26	**0.001**	
Copulations: Population as population identity					
	Intercept	-2.49 (± 0.15)	-17.02	**<0.001**	0.22 (± 0.47)
	Ambient temperature	18.50 (± 3.47)	5.33	**<0.001**	
	(Ambient temperature)^2	-14.70 (± 3.17)	-4.64	**<0.001**	
	Population: BLM	-0.49 (± 0.25)	-2.01	**0.04**	
	Population: AW	-0.66 (± 0.24)	-2.71	**0.007**	
	Population: WSU	-0.39 (± 0.22)	-1.78	0.08	
	Population: K2	-0.17 (± 0.23)	-0.71	0.48	
	Population: LHC	-0.39 (± 0.20)	-1.91	0.06	
	Ambient temperature x BLM	4.25 (± 6.08)	0.70	0.48	
	(Ambient temperature)^2 x BLM	3.75 (± 5.35)	0.70	0.48	
	Ambient temperature x AW	-0.58 (± 6.12)	-0.10	0.92	

(Ambient temperature)^2 x AW	3.88 (± 5.33)	0.73	0.47
Ambient temperature x WSU	-16.13 (± 5.34)	-3.02	**0.003**
Ambient temperature)^2 x WSU	4.58 (± 4.68)	0.98	0.33
Ambient temperature x K2	-9.88 (± 5.59)	-1.77	0.08
Ambient temperature)^2 x K2	7.11 (± 5.24)	1.36	0.17
Ambient temperature x LHC	-7.23 (± 4.77)	-1.51	0.13
Ambient temperature)^2 x LHC	15.27 (± 4.31)	3.55	**<0.001**

Table A1.7. Model diagnostics table. Diagnostic statistical tests were performed on the final models for each response variable. Significant results indicated in bold.

Model	Shapiro-Wilks (normality)		Levene's Test (homogeneity of variance)		
	W	p	df	f	p
Courtship display models					
Source temp model	0.95068	**<0.001**	4	2.6423	**0.052**
Population identity model	0.95314	**<0.001**	4	3.4982	**0.007**
Copulation attempt models					
Source temp model	0.92935	**<0.001**	4	10.24	**<0.001**
Population identity model	0.93414	**<0.001**	4	11.501	**<0.001**
Copulation models					
Source temp model	0.94709	**<0.001**	4	1.0169	0.3977
Population identity model	0.95128	**<0.001**	4	0.7223	0.5768
Mating efficiency models					
Source temp model	0.94428	**<0.001**	4	0.2811	0.8903
Population identity model	0.94701	**<0.001**	4	0.7089	0.586

FIGURES

Figure A1.1. Photograph of fry retention devices floating within 568 L tanks containing wild-caught or F1 mosquitofish and their offspring (center). Fry were collected from fry retention devices using hand nets and were transferred to fry baskets contained within 57 L tanks (left).

Figure A1.2. Photographs of setup for behavioral video recordings. (A) Males were housed in individual fry baskets within temporary holding aquaria to acclimate overnight prior to behavioral observations. (B) Observation tanks, with recording cameras fixed above each tank. (C) A screenshot of a behavioral recording. The mosquitofish on the leftmost side within the group of three fish is a male, with a visible implant elastomer tag clearly visible on his back.

Figure A1.3. The relationship between ambient temperature and courtship displays in each source population. When population identity, ambient temperature, and their interaction were included in the full model explaining courtship displays, the best-fit model included both population identity and ambient temperature (solid line in each panel). When source temperature was included in the full model rather than population identity, the best fit model included only ambient temperature (dashed line). Error bars indicate +/- 2 SE from estimated marginal means. Observed data for courtship dislays is included for reference (open circles).

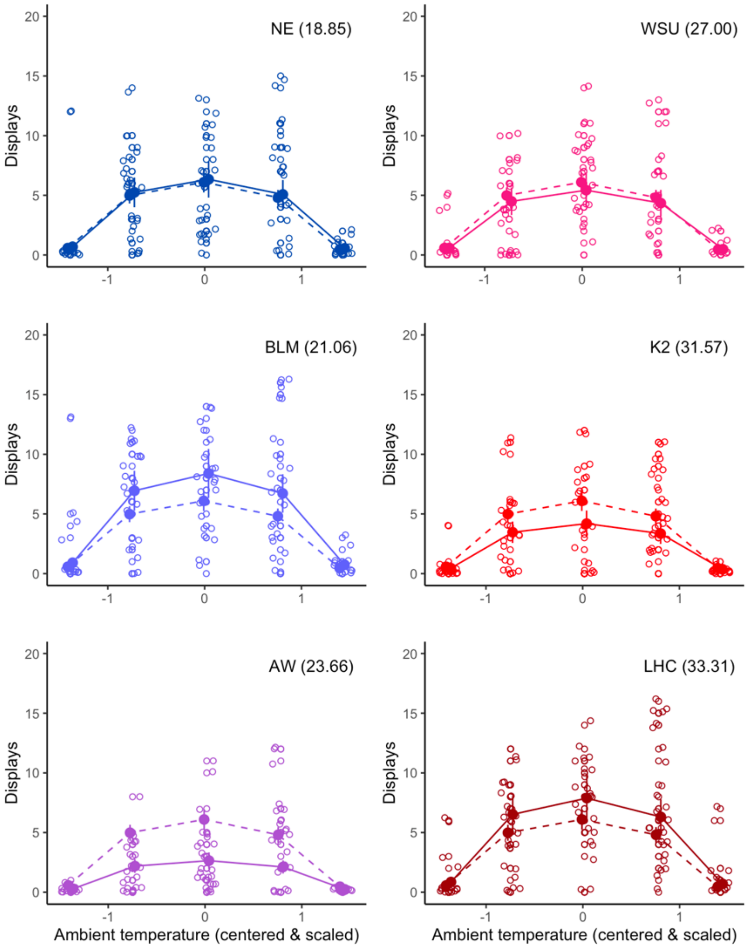


Figure A1.4. The relationship between ambient temperature and copulation attempts in each source population. When population identity, ambient temperature, and their interaction were included in the full model explaining copulation attempts, the best-fit model included both population identity and ambient temperature (solid line in each panel). When source temperature was included in the full model rather than population identity, the best fit model included only ambient temperature (dashed line). Error bars indicate +/- 2 SE from estimated marginal means. Observed data for copulation attempts is included for reference (open circles).

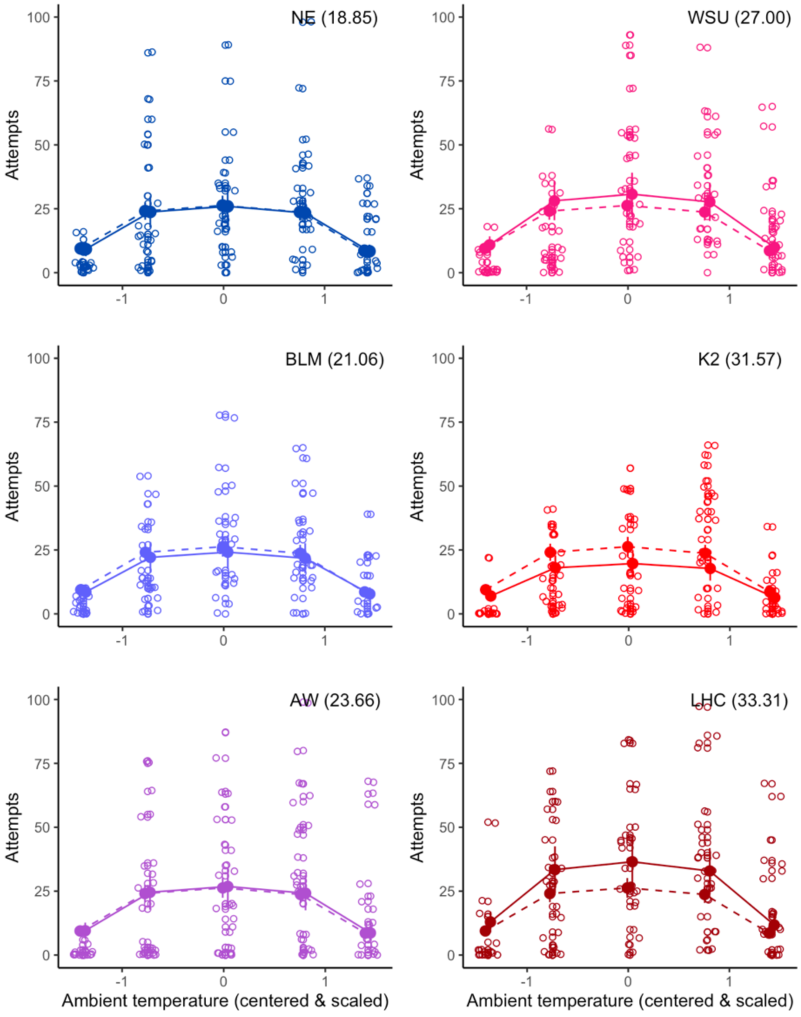


Figure A1.5. The relationship between ambient temperature and copulations in each source population. When population identity, ambient temperature, and their interaction were included in the full model explaining copulations, the best-fit model included both population identity and ambient temperature (solid line in each panel). When source temperature was included in the full model rather than population identity, the best fit model included only ambient temperature (dashed line). Error bars indicate +/- 2 SE from estimated marginal means. Observed data for copulations is included for reference (open circles).

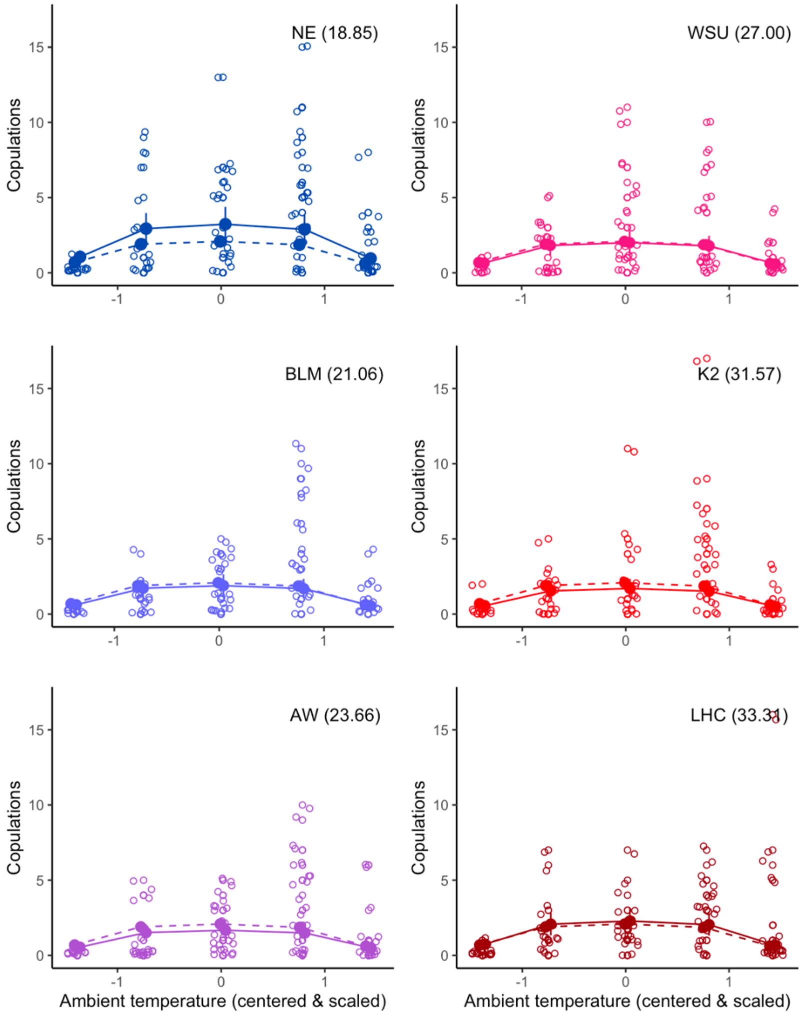
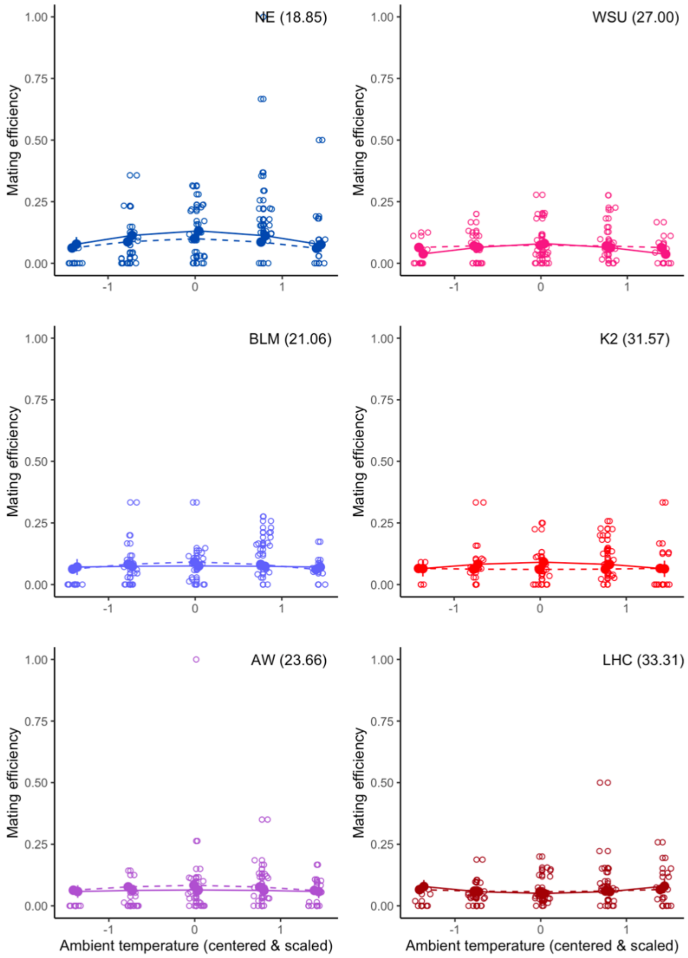


Figure A1.6. The relationship between ambient temperature and mating efficiency (copulations/ copulation attempts) in each source population. When population identity, ambient temperature, and their interaction were included in the full model explaining copulations, the best-fit model included both population identity and ambient temperature (solid line in each panel). When source temperature was included in the full model rather than population identity, the best fit model included both source temperature and ambient temperature (dashed line), but this model had a higher AIC score than the model containing population identity (AIC: 1732.6 vs. 1783.7). Error bars indicate +/- 2 SE from estimated marginal means. Observed data for mating efficiency is included for reference (open circles). Obervations in which zero total attempts were observed are excluded from observed data because mating efficiency could not be calclated.



CHAPTER 3

Figure A3.1. Effects of high-harassment, low-harassment, and fish-absent treatments on zooplankton community abundance and traits on days 4, 7, and 12 of the experiment. Significance symbols from Dunn pairwise tests with Bonferroni corrections for multiple comparisons ($p<0.05^*$, $p<0.01^+$).

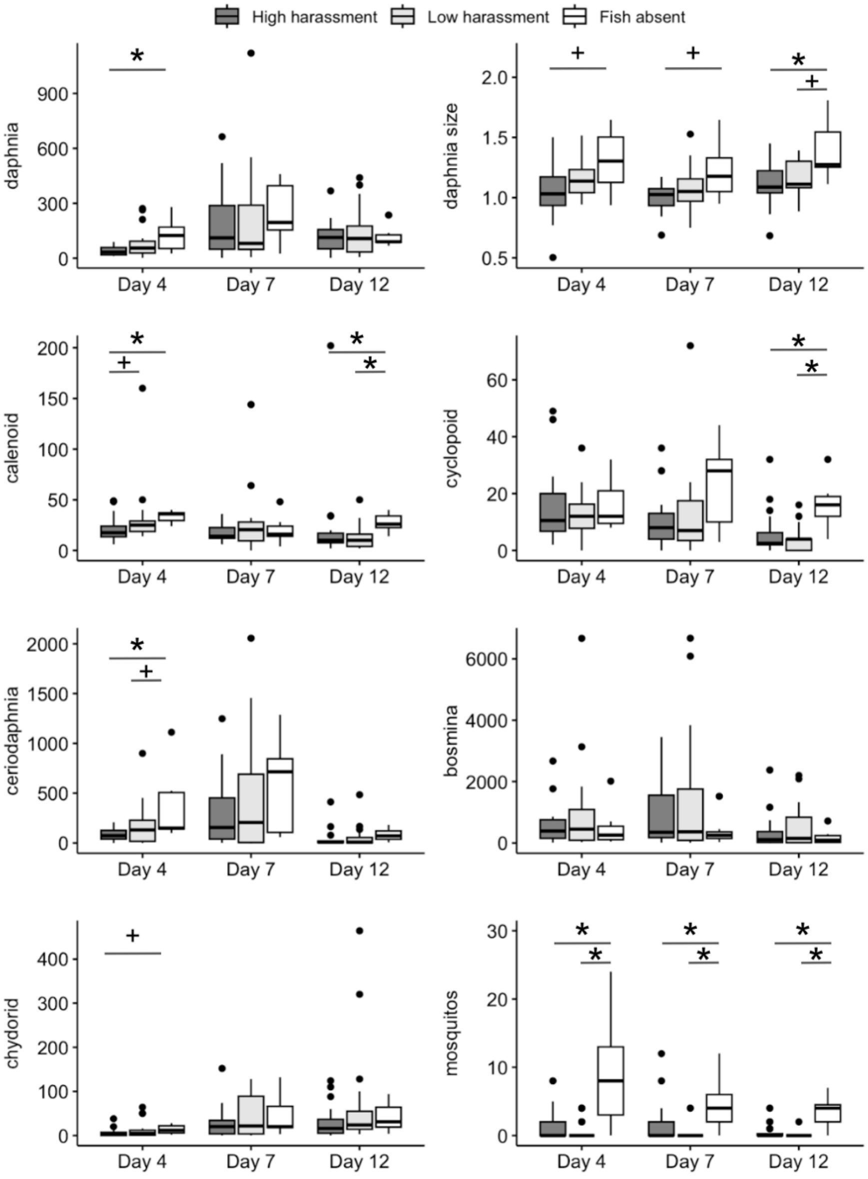
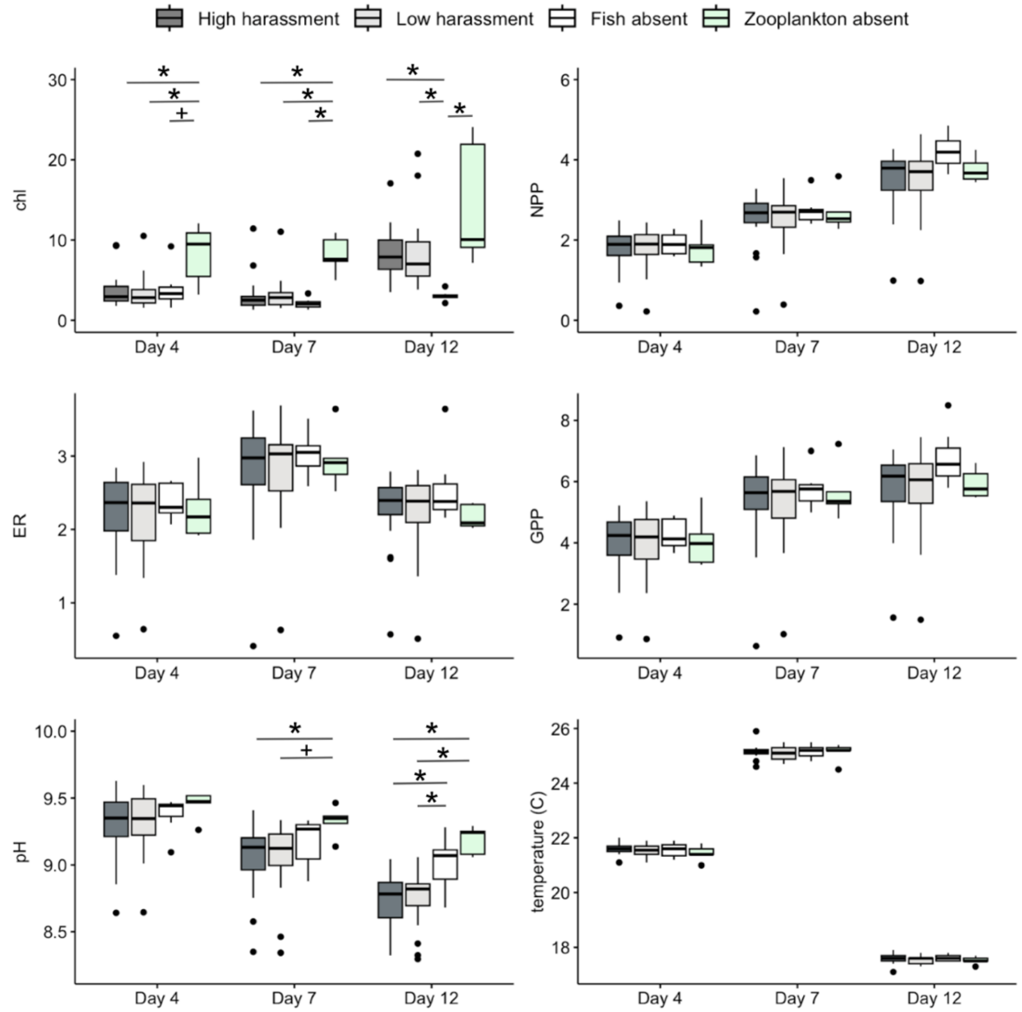


Figure A3.2. Effects of high-harassment, low-harassment, and fish-absent treatments on ecological factors on days 4, 7, and 12 of the experiment. Significance symbols from Dunn pairwise tests with Bonferroni corrections for multiple comparisons ($p<0.05$*, $p<0.01^+$).



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