UNIVERSITY OF CALIFORNIA
SANTA CRUZ

EXPLORING IMPORTANT YET LESSER-KNOWN ASPECTS OF POSTMATING SEXUAL SELECTION: SOCIAL ENVIRONMENT, TEMPERATURE, AND CRYPTIC FEMALE CHOICE

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

DOCTOR OF PHILOSOPHY

in

ECOLOGY AND EVOLUTIONARY BIOLOGY

by

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June 2024

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Contents

Figures and Tables

List of Figures

List of Tables

Abstract

Exploring important yet lesser-known aspects of postmating sexual selection: social environment, temperature, and cryptic female choice

by

Matthew Choi Kustra

The complex dynamics of reproductive interactions drive organismal diversity. Thus, understanding these dynamics is an important goal for evolutionary biology. Reproductive interactions after mating can result in postmating sexual selection, selection on traits affecting fertilization success. My dissertation provides novel insights into how postmating sexual selection is influenced by (1) the social environment, (2) temperature, and (3) cryptic female choice, a process where females bias fertilization to specific males after mating. In Chapters 1 and 2, I used behavioral and experimental approaches on the ocellated wrasse, a fish with external fertilization. This species has three male morphs: a large nesting male, an intermediate-sized satellite male, and a small sneaker male. Females have a strong mating preference for nesting males. Sneakers override this preference by releasing more sperm than nesting males. Female reproductive fluid in this species decreases the importance of sperm number but increases the importance of sperm velocity in determining fertilization success, effectively favoring nesting males. I looked at how the social environment and male behaviors influence the timing of sneaking by conducting underwater behavioral observations. I found that sneaking time delays decreased (likely higher fertilization success) with increasing nest activity for sneakers. In Chapter 2, I tested how temperature influences the effect of female

reproductive fluid on sperm performance. I found that cryptic female choice will be less effective at higher temperatures as female reproductive fluids no longer benefit nesting male sperm at high temperatures. For my final two chapters, I developed mathematical models to understand the evolutionary consequences of cryptic female choice. In Chapter 3, I found that incorporating cryptic female choice can drastically alter predictions of male reproductive investment compared to previous models that ignore cryptic female choice. I also found that male and female traits involved in cryptic female choice coevolve even with weak cryptic female choice. In Chapter 4, I found that this coevolution can help maintain reproductive isolation, helping the creation of new species. Overall, my dissertation demonstrates that postmating sexual selection can be influenced by both biotic and abiotic factors that are currently underappreciated.

Acknowledgements

First, I want to thank my PhD advisor, Suzanne Alonzo, whose mentorship and leadership made my dissertation possible. From the more menial skills of handling fish and dealing with the bureaucracy of academia to thinking about the "question" and how to try to answer the "question," Suzanne has helped me grow and develop as an independent scientist. I will forever strive to be as brilliant a scientist as Suzanne, who can somehow be equally emotionally intelligent. Not to pat myself on the back too much, but Suzanne is also a great judge of character. The people that Suzanne has put in her academic life and are thus in mine have all been amazing, wonderful people to work with.

My lab mates are amazing people who have made my PhD a positive experience. My senior lab mates, Dori Weiler and Sabrina Beyer, were great mentors who helped me navigate the labyrinth that is graduate school, especially during my first year and now in my final year trying to graduate and format this thesis. Louise Alissa De Morais and Megan Molinari have been wonderful to work with on the wrasses. It really made Corsica much more fun and enjoyable when I was no longer the only graduate student there. I want to thank Madi Gamble, who helped me navigate applying to postdoctoral fellowships, gave great feedback on applications, and introduced me to Wingspan.

The non-UCSC wrasse team: Kelly Stiver, Susan Marsh-Rollo, and Jennifer Hellmann are amazing collaborators and mentors. Before my first trip to Corsica, I had nearly zero experience in fish behavior and/or scientific diving. They all helped me get up to speed faster than I thought was possible. With her genetics expertise, Kelly Stiver has been extremely helpful in dealing with a missing chapter that only through her help may one day see the light.

Next, I would like to thank the dream team that is my committee: John Fitzpatrick, Bruce Lyon, Maria Servedio, and Barry "Yoda" Sinervo. Collectively, they have all contributed their expertise to make this dissertation as strong as possible. John has an amazing mind for experimental design and is the leading expert in postmating sexual selection. His comments have been immensely helpful, from designing studies to interpreting and framing the results. Bruce Lyon's amazing and, as he puts it, "overly picky" comments have greatly improved the clarity of my writing. Bruce has also been amazingly helpful in framing my papers to make them as strong as possible and bringing connections to parts of the scientific literature that I might have missed otherwise. As the leading expert in speciation theory, Maria Servedio played a crucial role in improving Chapters 3 and 4. But more generally, our email exchanges and conversations about these chapters have immensely improved my development as a theoretical biologist.

Barry, who sadly passed away in the third year of my PhD, played a pivotal role in my first few years. He taught me how to use the supercomputer and do parallel computing, which was an essential tool in all four of my chapters. He also welcomed me into his lab meetings, where I had my first experience of combining math and biology. In those meetings, I connected with Carla Sette, Regina Spranger, and Haley Ohms, whose conversations about the intersection of biology and math profoundly influenced me as a first-year PhD student.

Next, I would like to thank the EEB staff for keeping the department running so well. I especially want to thank Judy Straub and Stephanie Zakarian. Who have both helped me navigate the graduate program and UCSC. Without them, my PhD would have been much harder and more hectic.

I would next like to thank my cohort for making my PhD experience fun. I have

truly made lifelong friendships during my PhD. I especially want to thank Matt Glasenapp, Laura Goetz, Julia Harenčár, Rachel Pausch, Sushmita Poudel, Ryan Salladay, and Christa Seidl. Ryan and Rachel are amazing planners and are behind some of my favorite moments during my PhD. It has always been great to vent about the flaws of academia and share various tweets from science Twitter with other Matt. Besides being a fun and great person to chat with, Julia's writing group has kept me productive during my PhD, and exchanging drafts of postdoc proposals has been immensely helpful.

I'm fortunate to have a science community outside of UC Santa Cruz. I am so grateful for my first science mentor, Ariel Kahrl, who got me first involved in research and sperm competition when she was a graduate student. She also networked for me on my behalf, which helped me get into graduate school. I also want to thank Robert Cox, Ariel's advisor, who welcomed me into his lab at UVA and has been an important mentor then and now. I am also thankful for the rest of the Cox lab, who are all amazing folks: Aaron Reedy, Rachana Bhave, Tyler Wittman, Heidi Seears, and Cara Giordano. Such a great lab community made for a fantastic first research experience that made me want to continue to go on to graduate school and helped my development as a scientist. I am fortunate to still stay in touch with everyone there.

I would like to thank my friend Sid Ajith. Although he is in physics, talking to him about graduate school and pursuing a career in academia has helped me push through.

I would also like to thank Tyler Carrier, who's been distracting me from my PhD with side projects on microbial manipulators in marine invertebrates that I find equally interesting. Working with him has helped my writing, figure-making, and growth as an independent scientist.

I want to thank my family for raising me and supporting me. My mother, Bonnie Kustra, and father, Mark Kustra, raised me to be the hard worker and self-motivated person I am today. Because we moved around every few years, I lived a unique childhood that exposed me to diverse ecosystems that first sparked my interest in nature. My brothers John and Luke helped nurture that interest in nature with all the time we spent together playing outside and catching/observing animals. Throughout my PhD, having them in my life has made me happier. I have also been extremely fortunate to have extended family close by: my uncle Charles Choi, my aunt Lorrie Lieb, and my uncle Tim Lieb.

Finally, I want to thank my wife, Emma, for many reasons. First, she has always supported me through the ups and downs of my PhD experience. Second, she is the hardest and most intelligent person I know. Spending time with her has made me strive to meet her excellence. Third, she is one of the best naturalists I know. It is quite sad that I could have gone my entire PhD in Ecology and Evolutionary Biology as a "field biologist" without experiencing California's diverse flora and fauna. Emma has cured me of my plant blindness and connected me to nature by planning excursions to see rare plants and birds. Finally, as an almost lawyer and a printmaker, Emma has a keen eye for detail. Emma has been the final eye for every important thing I send to catch embarrassing typos, grammar mistakes, and aesthetic nightmares.

The work of this dissertation was supported by the US National Science Foundation via a GRFP award to M.C.K (Award number: DGE-1842400), an ARCS fellowship award to M.C.K., an American Society of Naturalists student research award to M.C.K., and a National Science Foundation grant awarded to S.H.A (Award number: IOS-1655297).

The text of this dissertation includes reprints of the following previously published material:

Chapter 1:

Kustra, M. C., Stiver, K. A., Marsh-Rollo, S., Hellmann, J. K., & Alonzo, S. H. (2023). Social Environment Influences the Temporal Dynamics of Sneak-Spawning in a Fish with Alternative Reproductive Tactics. *The American Naturalist*, *202*(2), 181–191

Chapter 3:

Kustra, M. C., & Alonzo, S. H. (2023). The coevolutionary dynamics of cryptic female choice. *Evolution Letters*, *7*(4), 191–202

The co-authors listed in these publications approve of this material to be used in my dissertation. My PhD advisor and co-author, Dr. Suzanne Alonzo, directed and supervised the research which forms the basis of my dissertation.

Introduction

§ 0.1 Broad context

The complex dynamics of reproductive interactions within and between the sexes can drive organismal diversity in fascinating ways, such as the elaborate display of the male peacock (*Pavo cristatus*). Charles Darwin first proposed that the evolution of such extreme ornamentation and elaborate behaviors that seem to oppose natural selection could be explained by selection acting on traits influencing reproductive interactions (i.e., sexual selection; Darwin, 1859, 1871). Historically, sexual selection was studied in the context of mating success, in other words, premating sexual selection (Birkhead, 2010; Shuker and Kvarnemo, 2021). However, reproductive interactions and, thus, selection, can occur not only before mating but also during and after mating (Figure 0.1A). The fact that sexual selection can continue to operate after mating via postmating selection, selection on traits affecting fertilization success, was not recognized until Geoff Parker's seminal work on sperm competition in insects (Parker, 1970). Initially, the field of postmating sexual selection focused on male-male competition, until over a decade later when Randy Thornhill coined the term cryptic female choice (Thornhill, 1983), a process where females bias sperm usage to favor specific males. Cryptic female choice did not gain much traction until the mid-90s after William Eberhard's influential book "Female Control: Sexual Selection by Cryptic Female Choice" (Eberhard, 1996). Despite growing evidence of cryptic female choice across a wide range of taxa, from plants to urchins to humans (reviewed in Firman et al., 2017; Gasparini et al., 2020), studies of cryptic female choice severely lag behind other topics of sexual selection (Ah-

King, 2022). This is especially true in the theoretical literature. My dissertation seeks to fill multiple gaps in our understanding of the important and widespread biological process of postmating sexual selection.

Most work on postmating sexual selection has focused on ejaculate-ejaculate interactions and ejaculate-female interactions (third stage in Figure 0.1A). However, selection in one stage of reproductive interactions may shape selection in earlier or later stages. For example, the absolute timing of mating can influence selection on ejaculate traits and fertilization success of both species with internal fertilization (i.e., differences in time between copulations of different males with the same female; Carleial et al., 2020; Manier, Lüpold, Pitnick, and Starmer, 2013; Manier et al., 2010; Pizzari et al., 2008; Smith, 2012) and external fertilization (i.e., differences in time between sperm release of different males and the same set of eggs; Egeland et al., 2015; Fitzpatrick, 2020; Ota and Kohda, 2015; Stoltz and Neff, 2006a, 2006b; Taborsky et al., 2018; Yeates et al., 2007). Additionally, there is growing evidence that female reproductive fluids change sperm competition dynamics (Alonzo et al., 2016; Gasparini et al., 2020), ultimately influencing the relative importance of the timing of mating and various ejaculate traits. However, our understanding of the connections between various stages of reproductive interactions is still in its infancy (Alonzo, 2010).

Reproductive interactions are not independent of the biotic or abiotic environment (Leith et al., 2022; Perry and Rowe, 2018; Reinhardt et al., 2015). For example, environmental conditions can influence the strength of reproductive interactions (Shuster and Wade, 2003) and the degree of multiple mating (West and Kodric-Brown, 2015), which ultimately shapes how selection acts on traits involved in reproductive interactions (Figure 0.1A). It is only recently becoming appreciated how temperature can influence

sexual selection (reviewed in Leith et al., 2022). So far, most of these studies have focused on premating sexual selection. Thus, we have a limited understanding of how temperature can influence postmating reproductive interactions. For my dissertation, I use a combination of empirical (Chapters 1 and 2) and theoretical (Chapters 3 and 4) research to expand our understanding of postmating sexual selection. In Chapter 1, I will broaden our understanding of how the social environment influences the timing of mating and build the link between "behaviors pre-ejaculation" (Stage 1; Figure 0.1A) and "ejaculate and female interactions" (Stage 3; 0.1A). I will accomplish this by analyzing behavioral observations across multiple years. In Chapter 2, I will improve our understanding of how temperature influences postmating female-male interactions. I will accomplish this by conducting an experiment measuring sperm performance at three temperatures relevant to current and future climate conditions (16℃, 22℃, and 28℃) with and without female reproductive fluid. For Chapters 1 and 2, I will use the ocellated wrasse (*Symphodus ocellatus*) as a study system, described below (Figure 0.1B). In Chapters 3 and 4, I will develop general theory, not specific to a particular organism, to focus on the evolutionary consequences of cryptic female choice (Figure 0.1C). In Chapter 3, I will develop a theoretical modeling framework to test how incorporating cryptic female choice changes current predictions on male ejaculate investment and influences the coevolution between female and male traits involved in postmating interactions. In Chapter 4, I will test how coevolution via cryptic female choice can maintain reproductive isolation and result in speciation.

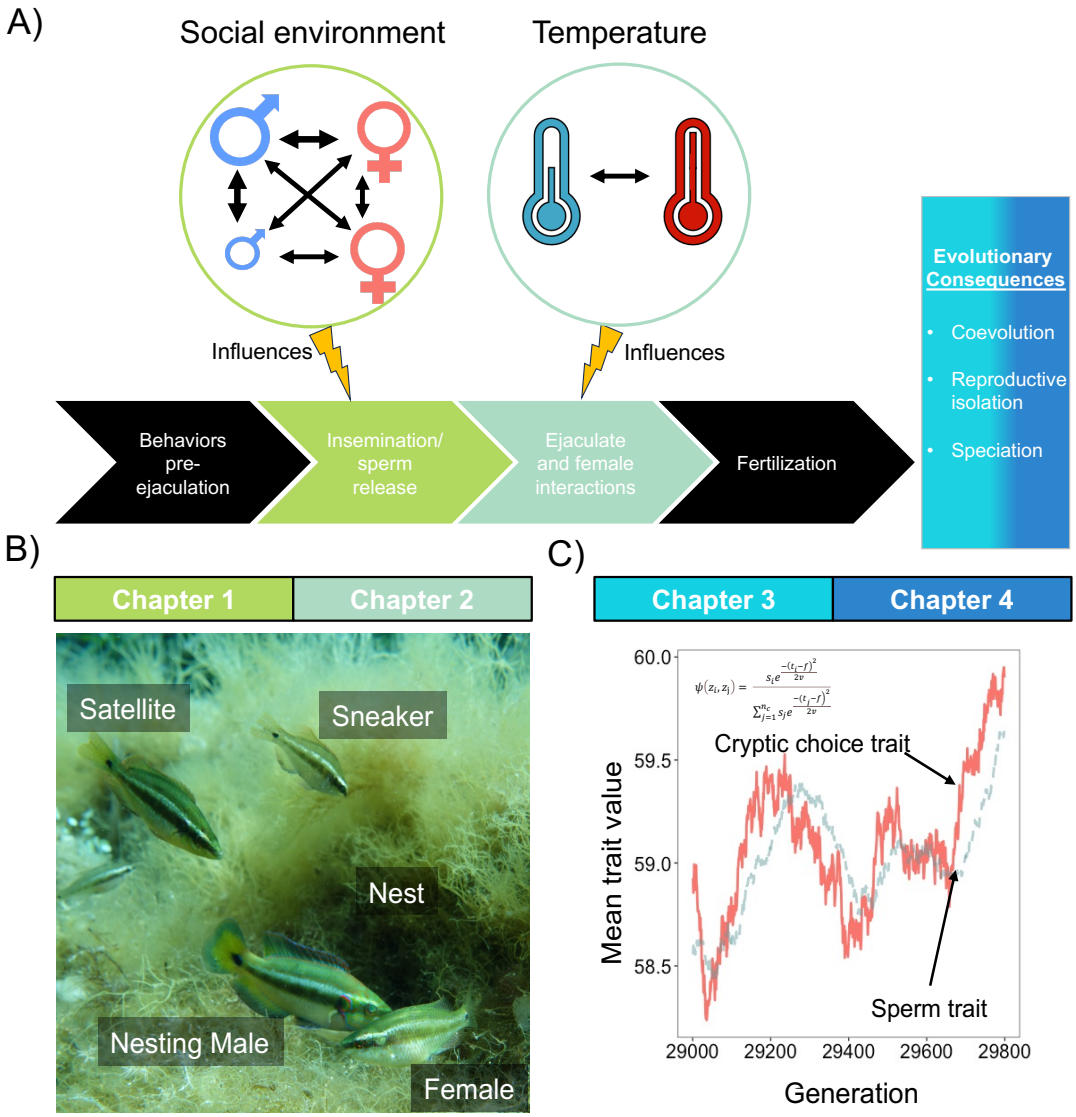

Figure 0.1: Dissertation summary.(A) Diagram of the successive steps to fertilization. Colors correspond to the focus of the chapters in my dissertation. (B) Chapters 1 and 2 are empirical work with the ocellated wrasse (*Symphodus ocellatus*). The nesting male builds nests, courts females, and provides parental care. Females have a strong mating preference for nesting males. Satellite males aid nesting males by chasing away sneakers and courting females, but also perform sneak spawns. (C) Chapters 3 and 4 are theoretical using individual based models to model the coevolution of male sperm traits and cryptic female choice traits and how this may result in the formation of new species. Photograph taken by Susan Marsh-Rollo.

§ 0.2 Study system: ocellated wrasse (*Symphodus ocellatus*)

The ocellated wrasse is a Mediterranean fish species with external fertilization (Lejeune, 1984). This species has three alternative male reproductive tactics: nesting males, satellite males, and sneaker males (Figure 0.1B; Alonzo et al., 2000; Lejeune, 1984; Taborsky et al., 1987). Nesting males make nests, chase away sneakers, court females, and provide all obligate parental care. Females strongly prefer mating with nesting males and release their gametes synchronously with nesting males. Sneaker males try to join nesting males and females during these spawning events and release their sperm after a time delay (Alonzo and Warner, 2000a). Satellite males pair up with nesting males and expend energy aiding the nesting male by chasing away sneakers and courting females. However, satellite males will also try to join mating events between nesting males and females. The male tactics are likely condition-dependent based on juvenile growth rates (Alonzo et al., 2000). Males with fast juvenile growth will be satellite males in their first reproductive season and nesting males in their second and final reproductive season. Males with slow growth will be sneaker males in their first year and satellite males in their second and final reproductive year (Alonzo et al., 2000).

The alternative male tactics differ in gonadal gene expression (Dean et al., 2017) and ejaculate characteristics (Alonzo and Warner, 2000a; Alonzo et al., 2016, 2021). Specifically, nesting males produce sperm with faster initial sperm velocity, while sneakers have sperm with longer longevity (Alonzo et al., 2016). During mating, sneakers release approximately three times as much sperm as nesting males (Alonzo and Warner, 2000a). Female reproductive fluid (ovarian fluid) that coats the eggs counteracts

this numeric advantage by decreasing the importance of sperm number but increasing the importance of sperm velocity in determining fertilization success, effectively favoring the nesting males (Alonzo et al., 2016). Because sperm velocity is the distance a sperm travels given a time interval, ovarian fluid may also increase the importance of the timing of mating. The ocellated wrasse is, thus, an ideal system to study postmating sexual selection and connections between various stages of reproductive interactions.

Chapter 1

Social environment influences the temporal dynamics of sneak-spawning in a fish with alternative reproductive tactics

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The text of this chapter is a reprint of the following published material, with permission from its co-authors:

Kustra, M. C., Stiver, K. A., Marsh-Rollo, S., Hellmann, J. K., & Alonzo, S. H. (2023). Social Environment Influences the Temporal Dynamics of Sneak-Spawning in a Fish with Alternative Reproductive Tactics. *The American Naturalist*, *202*(2), 181–191

§ 1.1 Abstract

Several predictions of sperm competition theory are not well supported empirically. One potential reason is that most current theory and empirical research ignore how the social environment influence the temporal dynamics of mating. We propose that understanding these dynamics is key to understanding sexual selection and improving the predictive power of theory. To demonstrate the importance of these dynamics, we quantify how males' social role, interactions among males, and current social environment influence the timing of mating in *Symphodus ocellatus*, a species with three alternative male reproductive tactics. Nesting males spawn synchronously with females; sneakers and satellites sneak-spawn with some time-delay. Satellites also cooperate with nesting males. We found that satellites have shorter sneak-spawning delays than sneakers, a benefit of their cooperation with nesting males. Sneak-spawning delays decreased with increasing nest activity for sneakers but not satellites, suggesting sneakers may benefit from increased sperm competition intensity. Current sperm competition models ignore this potential benefit which may be why the prediction that males should decrease investment when sperm competition involves more than two males is not well supported. Our study provides insight into mechanisms that drive variation in the timing of spawning, which could explain mismatches between theoretical and empirical results.

§ 1.2 Introduction

A central goal of biology is to develop theory that can explain and predict empirical patterns. When empirical observations frequently deviate from theoretical predictions, it is important to ask — what is current theory missing? Despite extensive theory and empirical research (Kahrl, Snook, and Fitzpatrick, 2021; Lüpold and Pitnick, 2018; Lüpold, Reil, et al., 2020; Parker, 1970; Parker and Pizzari, 2010), several predictions of sperm competition theory are not well supported empirically. One potential reason could be that most models ignore active roles of females (Parker and Pizzari, 2010, but see Alonzo and Pizzari, 2010; Ball and Parker, 2003; Bocedi and Reid, 2016; Requena and Alonzo, 2014). However, we propose that another often-overlooked aspect of post-mating intra-sexual selection theory is that the absolute timing of mating can influence selection on ejaculate traits and fertilization success. This is true for both species with internal fertilization (i.e., differences in time between copulations of different males with the same female; Carleial et al., 2020; Manier, Lüpold, Belote, et al., 2013; Manier et al., 2010; Pizzari et al., 2008; Smith, 2012) and external fertilization (i.e., differences in time between sperm release of different males and the same set of eggs;Egeland et al., 2015; Fitzpatrick, 2020; Ota and Kohda, 2015; Stoltz and Neff, 2006a, 2006b; Taborsky et al., 2018; Yeates et al., 2007). Thus, success in postmating intra-sexual selection is a product of at least two steps: (1) selection for traits and behaviors that decrease the time delay between multiple male mating events, and (2) selection on ejaculate traits that favor fertilization. While much research has focused on ejaculate traits (step two), selection on these traits is irrelevant unless the male can gain timely access to advantageous fertilization opportunities (step one). For example, sperm velocity is

unimportant if a male mates after the female's eggs have already been fertilized by males able to mate more quickly. Yet, little is known about how the immediate social environment influences the absolute timing of mating (step one). We argue that this limits our ability to develop postmating sexual selection theory that accurately predicts empirical patterns.

For example, sperm competition theory generally predicts that males should allocate more energy to an ejaculate during mating when the immediate risk of sperm competition (defined as the probability a female mates with more than one male) is greater, but decrease allocation to the ejaculate as the immediate intensity of sperm competition (defined as the number of males mating with the same female) increases (Parker and Pizzari, 2010). Meta-analyses have shown that predictions for plastic ejaculate allocation in response to the immediate risk of sperm competition are generally well supported (Immler et al., 2011; Kelly and Jennions, 2011). However, predictions about ejaculate allocation in response to immediate intensity of sperm competition are not well supported by the available data (Kelly and Jennions, 2011). This lack of support could result from most sperm competition theory ignoring how the social environment (e.g., number of competing males) affects the absolute timing or positioning of gamete release, which affects fertilization success in many species (Parker and Pizzari, 2010). Although previous empirical and theoretical work have focused on behaviors such as mate guarding Parker, 2020), they tend to focus on (1) how it decreases the risk/intensity of sperm competition, and (2) on relative mate-ordering affects. Consequently, there are few empirical studies quantifying the absolute timing of mating and how the social environment and behavioral interactions influence these temporal dynamics (Brattli et al., 2018; Stoltz and Neff, 2006a). Thus, empirical work on these dynamics is crucial

to help inform the development of future theory.

Systems with alternative reproductive tactics (ARTs) — discontinuous variation in behavior, physiology, or morphology within a sex to achieve reproductive success in alternative ways (Gross, 1996; Taborsky et al., 2008)— have been used extensively to test predictions of sperm competition theory and are therefore powerful systems for studying the effect of temporal dynamics in mating events. Male ARTs are common across taxa and often involve a dominant territory-holding tactic and a sneaker tactic that exploits the dominant male's mating opportunities (Gross, 1996; Taborsky et al., 2008). As sneakers are almost always in sperm competition, postmating sexual selection is strong, and the intensity of sperm competition is often high. Sneakers are also often at a temporal disadvantage which likely influences selection on ejaculate traits and on traits that influence the timing and position of mating (Kustra and Alonzo, 2020; Taborsky et al., 2018). Thus, the temporal dynamics of sneaking (mating with a female after a dominant male has mated) will also be important in the evolution and maintenance of alternative reproductive tactics. However, few studies have quantified observed variation in sneak-spawning times, and even fewer determined how factors, such as the social environment, influence the spatiotemporal dynamics of mating (Brattli et al., 2018; Ota, 2019; Ota and Kohda, 2015; Ota et al., 2010; Sørum et al., 2011; Stoltz and Neff, 2006a). For example, if many sneakers attempt to sneak simultaneously, they may interfere with each other. Their sneak-spawning delays—the difference between the time a dominant male and a subordinate male mate with a female—could increase, likely lowering the probability of fertilization. Alternatively, spawning situations with many males involved may prevent a dominant male from effectively defending against sneakers, resulting in shorter sneak-spawning delays for sneaker males. We argue that

understanding and quantifying how social interactions and the social environment affect the temporal dynamics of mating is essential for developing sperm competition theory with greater predictive power.

Here, we investigate how the social interactions between males of different tactics and the social environment influence the timing of mating. We study this in the ocellated wrasse (*Symphodus ocellatus*), a species with three ARTs: a large dominant nesting male, an intermediate-sized satellite male, and a small sneaker male (Figure 1.1; Alonzo and Warner, 2000b; Lejeune, 1984; Taborsky et al., 1987). These ARTs allow us to look at how the social role and social environment affect the timing of mating. The male tactics are likely condition-dependent based on juvenile growth rates (Alonzo et al., 2000). Males with fast growth will be satellite males in their first reproductive season and nesting males in their second and final reproductive season. Males with slow growth will be sneaker males in their first year and then satellite males in their second and final reproductive year (Alonzo et al., 2000). Sneaker males adopt a purely parasitic ART; satellites, on the other hand are partially cooperative with nesting males. Satellite males ally sequentially with various nesting males across the reproductive season and expend energy aiding the nesting male by chasing away sneakers and courting females (Taborsky et al., 1987). However, both satellite males and sneaker males primarily achieve reproductive success through sneak spawning (Stiver and Alonzo, 2013). Satellite males are socially dominant to sneaker males and are allowed closer to the nest by the nesting male. This may increase satellite male reproductive success via shorter sneak-spawning delays relative to sneaker males (Stiver and Alonzo, 2013; Taborsky et al., 1987). However, the benefits of being a cooperative satellite tactic over a pure sneaker tactic remain an open question.

We analyzed behavioral observations across multiple years to better understand the temporal dynamics of mating in this system. We first tested if satellite males have shorter sneak-spawning delays than sneakers and if this differed depending on the immediate sneaking situation ("single sneaker or satellite," "single sneaker and satellite," "multiple sneakers and a satellite"). We predicted that satellite males would have shorter sneak-spawning delays than sneaker males because they are allowed closer to the nest by the nesting male (as hypothesized in Taborsky et al., 1987). Next, we tested how (1) nest activity (e.g., number of female visits, number of spawning events, etc.) and (2) interactions between males (e.g., nesting male aggression to satellite males and sneakers, etc.) influence sneak-spawning delays. We predicted that sneak-spawning delays would increase with nest activity because more competition could cause interference among males. However, the opposite effect may happen if nesting males are distracted at busy nests, allowing sneakers to get closer and have shorter sneak-spawning delays. Finally, we predicted that nests with more aggressive nesting males would have longer sneak-spawning delays as both sneaker and satellite males may be forced farther away from the nest.

§ 1.3 Methods

1.3.1 Live observations

This research was conducted at the University of Liege Marine Station (STARESO) near Calvi, Corsica, France (42.5806°N, 8.7243°E) from mid-May to mid-June (the breeding season of S. ocellatus) in 2014 and 2016 – 2019. We performed ten-minute live behavioral observations using SCUBA on haphazardly selected *S. ocellatus* nests.

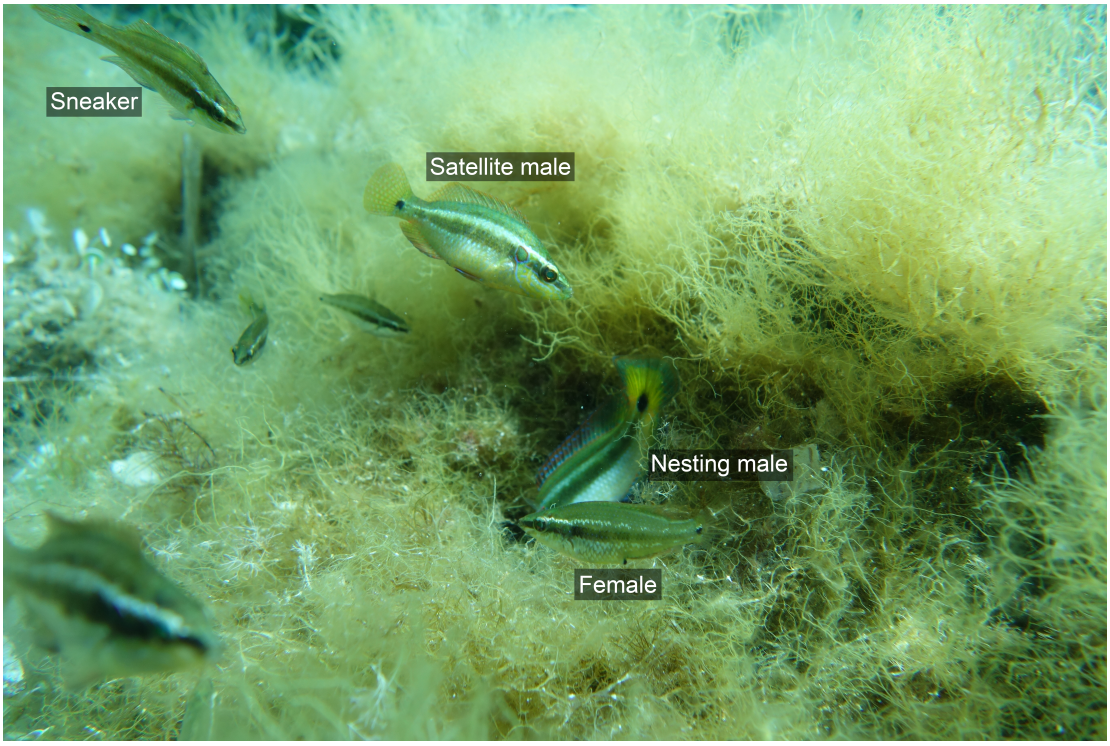

Figure 1.1: *Symphodus ocellatus* nest with all three male tactics and female. The nesting male (shown in his nest) builds nests, courts females, and provides parental care. Females have a strong mating preference for nesting males and spawn in synchrony with them. Satellite males aid nesting males by chasing away sneakers and courting females, but also perform sneak spawns. As shown above, satellite males are allowed closer to the nest than sneaker males. Photograph taken by Susan Marsh-Rollo.

We focused on nests with a spawning nesting male, at least two sneaker males, and a satellite male present for us to record sneaks from multiple males. During the observations, we quantified social interactions between male ARTs: the number of aggressive behaviors from the nesting male to sneaker males, the nesting male to the satellite male, and the satellite male to sneaker males. We also recorded the number of submissive behaviors of the satellite male to the nesting male. For a more in-depth description of these behaviors, see (Stiver and Alonzo, 2013). Every minute, we counted the number of sneakers within one meter of the nest (estimated visually) that were also oriented toward the nest or interacting with other individuals at the nest. To measure sneaker presence, we averaged the number of sneakers counted per minute in the ten-minute observations. We also recorded the total number of female visits, the total number of females that spawned, the total number of spawns that occurred, the number of sneaks from sneakers, and the number of sneaks from satellite males. We concurrently recorded a video of these live observations to quantify sneak-spawning delays—the difference between the time that the nesting male spawned and the time the subordinate male(s) spawned.

1.3.2 Sneak-spawning delay data collection

To quantify the sneak-spawning delay of sneaker and satellite males, we analyzed the ten-minute nest focal videos shot at 30 fps that were synchronized with the live observations described above using QuickTime 7 (2014 and 2016) and BORIS 7.11.1 (2017, 2018, and 2019; Friard and Gamba, 2016). For each sneak-spawning event (e.g., Figure 1.1), we recorded the time to the millisecond when the nesting male spawned with the female and the time that either a sneaker or satellite male completely entered

the nest to sneak spawn (Figure 1.1; Video S1.1). We calculated the sneak-spawning delay by subtracting these two times. To match observations recorded from QuickTime 7, which rounded observations to the nearest millisecond, we rounded all BORIS sneak-spawning delay observations to the nearest millisecond. We also recorded the number of males that sneaked in each sneak-spawning event and what type of males sneaked (i.e., sneakers and/or a satellite). To focus on natural spawning behaviors, we only included videos for which an experimental manipulation did not occur. We excluded videos that did not have at least one sneaker and one satellite sneak-spawning event because we wanted to compare the sneak-spawning delays of both male types. We also excluded nests with more than one satellite (typically, nests only have one satellite). We excluded any sneak-spawning delay observations if the video was too blurry to make the sneak-spawning delay difficult to measure accurately. This resulted in the following final nest sample sizes (n = 34): 2014 (n =10 nests); 2016 (n = 9 nests); 2017 (n = 4 nests); 2018 (n = 6 nests); and 2019 (n= 5 nests).

1.3.3 Statistical analyses

We first wanted to test how sneaker and satellite males differed in sneak-spawning delays and whether that was affected by the social makeup of specific sneaking events. We categorized all sneaking events into three sneaking categories: "single sneaker or satellite" (a sneaker or the satellite sneak-spawned alone; n = 34 satellite observations; n = 79 sneaker observations), "single sneaker and satellite" (one sneaker male and the satellite male sneak-spawned at the same sneaking event; n = 46 satellite sneaks; n = 46 sneaker sneaks), or "multiple sneakers and satellite " (multiple sneaker males and the satellite male sneak-spawned at the same sneaking event; n = 31 satellite sneaks; n = 80

sneaker sneaks). We did this grouping instead of using the number of parasitic males due to much lower sample sizes after more than 3 males (Figure S1.1). If a spawn fell into the "multiple sneakers and satellite" category, we used all sneak-spawning delay times in the analysis. We handled the non-independence of these observations with random effects (described below). We excluded sneaking events with multiple sneakers but no satellite male because there would be no satellite male observations in this category and the goal of this analysis was to look at differences between the tactics. This resulted in the total sample sizes of n = 111 satellite sneaks and n = 205 sneaker sneaks. To ensure any result was not simply due to number of males as opposed to presence of a satellite, we performed an analogous analysis with sneaker only observations (n = 191 sneaker sneaks).

We analyzed the data using a hierarchical Bayesian generalized linear regression with a Gamma family and log link (i.e., GLMM) using the "brms" package (Bürkner, 2018; Hobbs and Hooten, 2015). We chose a Gamma family because the response variable, sneak-spawning delay, is strictly positive and skewed. The fixed effects of this test were male type (satellite or sneaker), the category of sneaking event, and their interaction. The random effects were sneaking event nested within the fish nest of an observation nested within year. For this model, we used uninformative priors (Hobbs and Hooten, 2015). Specifically, we used: Normal (mean = 0, SD = 1000) for the intercept and fixed effects, Inverse Gamma (alpha = 0.001, beta = 0.001) for random effect variations, and Gamma (alpha = 0.01, beta = 0.01) for the Gamma shape parameter. We ran models with four chains with 5000 iterations per chain, discarding the first 1000 as burn-in (Hobbs and Hooten, 2015). After we ran the models, we extracted posterior sample draws to estimate differences in sneak-spawning delays between satellite and

sneaker males in the different sneaking categories. For all model parameters, we then computed the evidence ratio—the ratio of the posterior probability that shared the same sign as the estimate to the posterior probability with the opposite sign (Bürkner, 2018). For example, suppose a model parameter estimated effect was positive. In that case, an evidence ratio of ten indicates that the parameter is ten times more likely to have a positive effect than a negative effect. To avoid null-hypothesis significance testing with arbitrary cut-offs (Muff et al., 2022), we interpret our results considering the evidence ratio, estimated effect sizes, and 95% credible intervals (CIs).

We next wanted to test how social interactions between males and the overall nest activity influenced the sneak-spawning delay of sneaker and satellite males. Because many of the behaviors and measurements of nest activity were highly correlated, we performed a principal component analysis (PCA) on all variables collected during live observations described in the first section (n = 34 nests). We then performed randomization tests to (1) make sure that the data was structured in a way that a PCA would be meaningful; and (2) select principal components (PC) that were biologically meaningful (e.g., variance explained was not due to random chance; Björklund, 2019). See the supplemental methods for more details (Figures S1.2-4).

We used two PCs in our analysis. PC1 was representative of variables related to nest activity (Figure S1.3). Specifically, it was positively loaded by the total number of spawns (17.258% contribution), sneaks (16.987% contribution), sneaks performed by sneakers (16.120% contribution), female spawns (14.866% contribution), female visits (13.346% contribution), and sneaks performed by satellite males (12.903% contribution). PC2 was representative of variables that explained male-male interactions (Figure S1.3). Specifically, it was positively loaded by the number of satellite male

to sneaker aggressions (31.142% contribution), satellite to nesting male submissions (21.899% contribution), and average sneaker presence (23.883% contribution). We then performed a hierarchical Bayesian generalized linear model with a Gamma distribution predicting sneak-spawning delay with fixed effects of male type, nest activity (PC1), male interactions (PC2), and the interaction of these effects with male type (n = 111 satellite sneaks; n = 317 sneaker sneaks). We included random effects of spawning events nested within fish nest of an observation nested within year to account for the non-independence of observations. We used the same priors and model methodology (e.g., number of chains and iterations) described in the first analysis.

All statistical models reached convergence without error, and all parameters had $\hat{r} \approx 1$, indicating that the separate chains converged. Additionally, posterior predictive checks indicated that all statistical models reasonably fit the data. To ensure the choice of priors did not substantially influence our results, we also ran all models using weakly informative regularizing priors for parameter effects, Normal (mean = 0, SD = 3). Using these weakly regularizing priors yielded qualitatively and quantitatively similar results (Tables S1.1,2). We also included a full model with both nest-level effects and spawning situations to ensure that they did not explain each other (e.g., the effect of sneaking situations might be explained by differences in nest activity). This generally resulted in qualitatively and quantitatively similar results (unless noted otherwise) as when the effects were analyzed separately (Table S1.3). We chose to analyze them separately so we would not have to exclude the situations when there were spawning events with multiple sneakers and no satellite male to test nest-level effects. We conducted all analyses and made all figures in R V.4.1.2 (R Core Team, 2018) using the "tidyverse" suite of packages (Wickham et al., 2019). We give an in-depth breakdown of sample

sizes across years and male types in Table S1.5.

§ 1.4 Results

1.4.1 How much do satellite and sneaker males differ in sneak-spawning delay?

We found strong support (evidence ratio was large and CIs did not overlap with zero) that satellite males had, on average, shorter sneak-spawning delays than sneaker males across all sneaking situations (satellite sneak-spawning delay – sneaker sneak-spawning delay < 0, Figure 1.2B; Table 1.1*). This was true when a "single sneaker or satellite" sneak-spawned alone (estimated median difference: -0.0959 seconds; 95% CI [-0.1627, -0.0286]; evidence ratio: 89.3955), when a "single sneaker and satellite" sneak-spawned at the same event (estimated median difference: -0.1801 seconds [-0.2549, -0.1103]; evidence ratio: Inf**), and when "multiple sneakers and satellite" sneak-spawned at the same event (estimated median difference: -0.0998 seconds [-0.1554, -0.0439]; evidence ratio: 389.2439). We found that satellite males and sneakers in the "multiple sneakers and satellite" situation generally had shorter sneak-spawning delays than other sneaking situations (Table 1.1; Figure 1.2A). We also found that there was likely an interaction between male type and when a "single sneaker and satellite" sneak-spawned (Table 1.1). Specifically, sneaker males were slower with a satellite male present ("single sneaker and a satellite") compared to alone ("single sneaker or

*Table 1.1 gives estimations of specific model effects, while estimates and credible intervals in the text are from contrasts extracted from the model.

**All posterior samples indicated satellite males had shorter sneak-spawning delays than sneaker males.

satellite"; median difference 0.0434 seconds [-0.0312, 0.122]; evidence ratio: 4.6919) or with many sneakers and a satellite male ("multiple sneakers and satellite"; median difference 0.1014 seconds [0.0291, 0.1779]; evidence ratio: 99.6289). The general pattern for sneaker-only spawns with the same number of total males was similar but effect sizes and evidence ratios were much smaller (Table S1.4; Figure S1.5). Moreover, the credible intervals always overlapped for zero indicating that the difference between "single sneaker and satellite" and "multiple sneakers and satellite" was in part driven by the presence of the satellite male and not just number of males present (Table S1.4; Figure S1.5). In summary, satellite males typically have shorter sneak-spawning delays than sneaker males, and this difference is largest when a single sneaker male and satellite male compete in the same sneaking event. Further, sneakers had shorter sneak-spawning delays when multiple sneakers and a satellite sneak in the same sneaking event compared to when a single sneaker and a satellite sneak.

Table 1.1: Results of fixed effects from hierarchical Bayesian generalized linear model with a Gamma distribution predicting sneak-spawning delay as a function of male type, sneaking situation, and their interaction. The random effects for this model were spawning event nested within the fish nest of an observation nested within year. The shape parameter of this model was estimated to be 4.2030 with an error of 0.3808; Bayesian R^2 was estimated to be 0.2883 with an error of 0.0539. Bolded rows indicate estimates with 95% credible intervals that did not overlap with zero; underlined rows indicate evidence ratio >10 (ten times more likely effect shares the same sign as estimate than not). Model effects are compared to the sneak-spawning delay of a sneaker male when he is sneaking alone. Abbreviations in the figures are as follows: satellite male (sat) and sneaker male (sn).[1] means all posterior samples were negative.

Model Effect	Estimate	Evidence Ratio	Error	Lower 95 % CI	Upper 95% CI	Tail Effective Sample Size
Intercept	***-0.8221***	***Inf*** [1]	***0.0894***	***-0.9953***	***-0.6470***	***6552***
Sat	***-0.2472***	***89.3955***	***0.1064***	***-0.4522***	***-0.0359***	***9577***
sn + sat	0.0927	4.6919	0.0995	-0.1020	0.2883	11458
Multiple sn + sat	*-0.1411*	*17.5400*	*0.0879*	*-0.3131*	*0.0323*	*11504*
Sat: sn + sat	*-0.2174*	*12.8528*	*0.1506*	*-0.5145*	*0.0780*	*10325*
Sat: multiple sn + sat	-0.0568	1.8556	0.1501	-0.3515	0.2381	10506

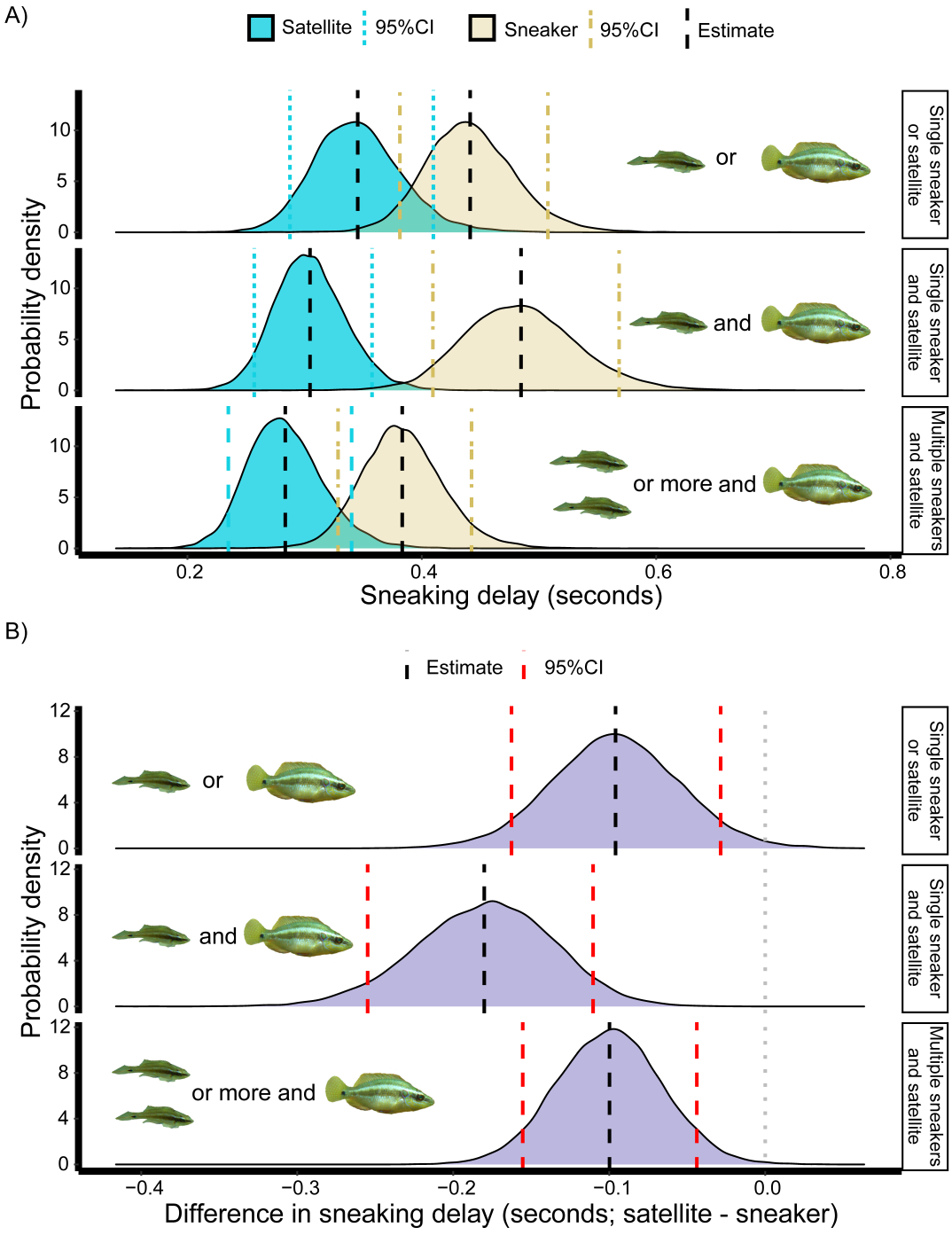
A)
Satellite
95%CI
Sneaker
95%CI
Estimate
Probability density
10
5
0
or
and
or more and
Single sneaker or satellite
Single sneaker and satellite
Multiple sneakers and satellite
0.2
0.4
0.6
0.8
Sneaking delay (seconds)
B)
Estimate
95%CI
12
8
4
0
−0.4
−0.3
−0.2
−0.1
0.0
Difference in sneaking delay (seconds; satellite - sneaker)

Figure 1.2: Male tactic and spawning scenario interact to influence the temporal dynamics of mating. Satellite males, on average, have a shorter sneak-spawning delay than sneaker males, with this effect being strongest during paired spawns. (A) Plots are posterior probability distributions of estimated average sneak-spawning delay for satellite (blue) and sneaker males (gold) when sneaks were performed with "single sneaker or satellite," "single sneaker and a satellite," or "multiple sneakers and satellite." Black dashed lines indicate medians, and colored dashed lines indicate 95% CI for different male types. (B) Plots are the posterior probability distributions of the hypotheses testing the difference in sneak-spawning delay between satellite males and sneaker males when sneaks were performed at different mating scenarios. Black dashed lines indicate medians, red dashed lines indicate 95% credible intervals, and the dotted grey line is at zero (i.e., no difference). Left of the dotted grey line (negative numbers) indicate satellite males had shorter sneak-spawning delays; right of the dotted grey line, sneakers had faster sneak-spawning delays (positive numbers). Posterior distributions are all from the same model described in Table 1.1. Images of sneaker and satellite male are from the photo in Figure 1.1 taken by Susan Marsh-Rollo.

1.4.2 How do nest activity and male interactions affect sneak-spawning delays?

We found strong support (the evidence ratio was large, and CIs did not overlap with zero) for an interaction between nest activity (PC1) and male type on sneak-spawning delay (Figure 1.3A; Table 1.2). Specifically, sneaker male delays decreased with increasing nest activity, but satellite male delays were not influenced by nest activity (Figure 1.3A; Table 1.2). We found weak support that sneak-spawning delays for both male types decreased with more male interactions (PC2). Although the evidence ratio was > 10, this effect was relatively small, and the 95% CIs overlapped with zero (Figure 1.3B; Table 1.2). Further, including spawning situations in the same model almost halved the evidence ratio (Table S1.3). In summary, sneakers had quicker sneak-spawns at busier nests, but male interactions had little influence on sneak-spawning times for

both satellites and sneakers.

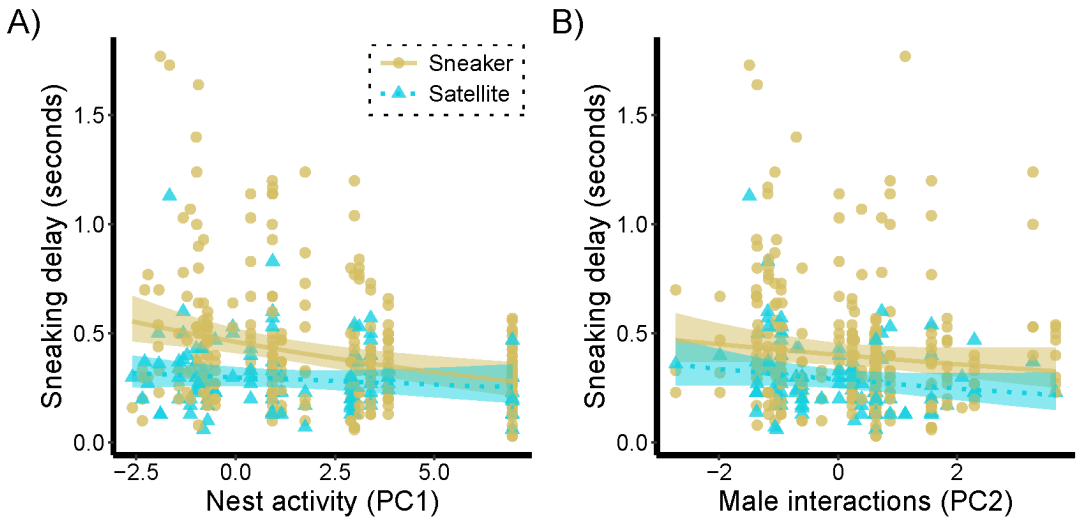

Figure 1.3: Social environment influences the temporal dynamics of mating. (A) Sneak-spawning delay decreases with increasing nest activity (PC1) for sneakers but not satellite males; (B) sneak-spawning delay for both males decreases slightly with more male interactions (PC2). Lines are the median, and shading is the 95% credible intervals of the predicted sneaker (gold) or satellite (blue) sneak-spawning delay; points are raw data for sneakers (gold and circle) and satellites (blue and triangle). (A) High values of nest activity indicate nests with a high number of spawns, sneaks, and female visits. (B) High values of male interactions indicate a high average number of sneakers, a high number of satellite male to sneaker male aggressions, and a high number of satellite male to nesting male submissions. Predictions come from posterior samples of the model presented in Table 1.2 conditioned on the mean of other model effects.

§ 1.5 Discussion

In the context of postmating intra-sexual selection, reproductive success depends on (1) traits and behaviors that decrease the time delay between multiple male mating

Table 1.2: Results of fixed effects from hierarchical Bayesian generalized linear model with a Gamma distribution predicting sneak-spawning delay as a function of male type, nest activity (PC 1), male interactions (PC2) and their interaction with male type. The random effects for this model were spawning event nested within the fish nest of an observation nested within year. The shape parameter of this model was estimated to be 4.1043 with an error of 0.3904; Bayesian R^2 was estimated to be 0.2709 with an error of 0.0581. Bolded rows indicate estimates with 95% credible intervals did not overlap with zero; italicized rows indicate evidence ratio > 10 (ten times more likely effect shares the same sign as estimate than not).[1] means all posterior samples were negative.

Model Effect	Estimate	Estimate Ratio	Error	Lower 95 % CI	Upper 95% CI	Tail Effective Sample Size
Intercept	***-0.7610***	***Inf*** [1]	***0.0620***	***-0.8790***	***-0.6377***	***9925***
Satellite male	***-0.4290***	***Inf*** [1]	***0.0711***	***-0.5664***	***-0.2893***	***11954***
Nest activity	***-0.0714***	***2665.667***	***0.0202***	***-0.1128***	***-0.0314***	***8550***
Male interactions	*-0.0560*	*17.244*	*0.0350*	*-0.1246*	*0.0146*	*10890*
Satellite: nest activity	***0.0476***	***53.4218***	***0.0231***	***0.0030***	***0.0933***	***12107***
Satellite: male interactions	-0.0201	2.0075	0.0478	-0.1123	0.0746	11925

events and (2) ejaculate traits that favor fertilization. Although there has been extensive research on ejaculate traits, few studies have quantified variation in mating time delays or determined how the social environment might explain this variation. Differences in the timing of mating will affect selection on ejaculate traits and therefore are likely critical to understanding the evolution and maintenance of ARTs. Here, we analyzed detailed behavioral observations from multiple years to better understand: (1) how alternative reproductive tactics differ in sneak-spawning delays, (2) how nest activity and (3) social interactions influence the relative timing of male gamete release. We found that satellite males had shorter sneak-spawning delays than sneaker males, which was greatest when both a satellite and a single sneaker snuck at the same sneaking event. We also found that sneak-spawning delay decreased with increasing nest activity for sneaker males but not for satellite males. Given that small differences in sperm velocity have already been shown to affect paternity in this species (Alonzo et al., 2016) and that time delays in other fish species with external fertilization affect paternity (Egeland et al., 2015; Stoltz

and Neff, 2006b; Yeates et al., 2007), it is reasonable to assume that these differences in the timing of sperm release will likely affect paternity as well.

Regardless of the sneaking scenario, satellite males had shorter delays than sneaker males. This result is consistent with previous observations that satellite males are allowed closer to the nest than sneaker males (Stiver and Alonzo, 2013; Taborsky et al., 1987). This difference was most prominent in sneaking situations with a "single sneaker and satellite," primarily due to sneakers experiencing long delays when competing directly with a satellite male (Figure 1.2A). This could be because larger satellite males physically interfere with sneakers when sneaking together. Larger males also have an advantage when sneak-spawning in Masu salmon (*Oncorhynchus masou*; Koseki and Maekawa, 2000) and dusky frillgoby (*Bathygobius fuscus*; Takegaki et al., 2012). Combined with these results, our finding suggests a need to re-evaluate the general assumption that smaller size is favored when sneak-spawning (Ota et al., 2010). This result also indicates that faster sneak spawning (and likely higher fertilization success) is one advantage of adopting a partially cooperative ART and may have enabled this third alternative male tactic to evolve.

We found that sneaker delay times were shortest when the spawning situation was busiest ("multiple sneakers and satellite") and nest activity was higher (more number of spawns, sneaks, and female visits). These results imply that sneaker males, on average, benefit from sneaking when sperm competition is intense (more than two competitors). In these situations, sneaker males may be able to get closer to the nest or get to the nest faster, as nesting males may be preoccupied with courting females and fending off other sneakers. This dynamic is analogous to predator dilution effects. An additional and non-mutually exclusive possibility is that sneaker males may adjust

effort (swim faster or get closer to the nest) to get a competitive edge over other parasitic males in these highly competitive scenarios. Spawning faster likely increases the probability of fertilization and thus could counteract some of the negative impacts of higher sperm competition intensity (i.e., paternity being shared with more males). However, current theoretical models have not incorporated how the social environment influences the dynamics of sperm competition. Incorporating these effects could alter current theoretical predictions that males should invest less when the intensity of sperm competition is high (Parker et al., 1996; Parker and Pizzari, 2010) and could explain why these predictions are not generally well supported (Kelly and Jennions, 2011).

Ignoring aspects of the relative timing and positioning of gamete release may also explain why sperm competition theory has failed to predict differences in ejaculate allocation among alternative reproductive tactics reliably. Sneakers are almost always in sperm competition. Postmating sexual selection is therefore important in systems with ARTs, and existing theory predicts that sneakers should invest more in ejaculate production than dominant (territory-holding) males (Dougherty et al., 2022; Kustra and Alonzo, 2020; Montgomerie and Fitzpatrick, 2009; Parker, 1990). However, a recent review (Kustra and Alonzo, 2020) and meta-analysis (Dougherty et al., 2022) found this predicted pattern did not hold generally across species. We suggest that this could, in part, be due to selection on the timing of sperm release overriding selection on ejaculate traits, or the spatiotemporal dynamics of mating in these systems may explain how ejaculate investment varies between tactics. To improve our understanding of post-mating sexual selection, we need more empirical work quantifying the temporal mating dynamics of ARTs. We also need to develop theory on ejaculate allocation in ARTs that (1) incorporate investment into non-ejaculate components that influence the

competitive weighting of ejaculates (e.g., the energy devoted to quicker sneaking), and (2) allow the number of sneakers in a mating to influence the competitive weighting of sneaker ejaculates. Incorporating these aspects may better align theoretical expectations with empirical realities.

Instead of overriding selection on ejaculate traits, the timing of fertilization could also change the relative importance of ejaculate components (Egeland et al., 2015). For example, spawning at time-delays decreased the relative importance of sperm number in the Arctic charr (*Salvelinus alpinus*), which may explain why subordinate males produce faster sperm (Egeland et al., 2015). Similarly, in the ocellated wrasse, sneaker males may compensate for sneak-spawning delays as they release 4x more sperm than satellite or nesting males (Alonzo and Warner, 2000a). However, nesting males produce higher-quality ejaculates than sneaker males (Alonzo et al., 2016), and sperm production does not differ between male types (Alonzo et al., 2021). Sperm do not compete in a vacuum, and in many species, females can have considerable influence on sperm competition dynamics (Gasparini et al., 2020; Myers et al., 2020; Zadmajid et al., 2019). For example, in this system, ovarian fluid influences sperm competition dynamics by increasing the relative importance of sperm velocity and the speed at which sperm may fertilize the egg, further favoring nesting males (Alonzo et al., 2016). The total selective pressure acting on ejaculate traits is likely a combination of sperm competition, the female environment, and the temporal dynamics of mating. Developing new theory that considers all three aspects can help guide future work and provide testable predictions. Further, future empirical work should explicitly test these interactions to help improve our understanding of the evolution of ejaculate traits and behavior.

There are a few key limitations to this study that may affect our interpretation of

our results. First, in this system we do not currently know the extent at which these time-delays may influence paternity. However, we do know that fertilization occurs rapidly in species with external fertilization and small time delays can have huge influences in paternity in other systems (Egeland et al., 2015; Stoltz and Neff, 2006b; Yeates et al., 2007). Further, small differences in sperm velocity in this system (Alonzo et al., 2016) and others influence paternity. Second, our measure of male interactions (PC2) was from the entire 10-minute observation period which could have driven our finding of weak evidence for an effect of male interactions. It is quite plausible that the interactions between males that happen immediately before spawning events may have impacts on time-delays and are more important than average rates of interactions. To improve our understanding of temporal dynamics of mating and fertilization success, future empirical work in this system and others should analyze how sequences of behaviors/interactions influence time-delays that in-turn influence fertilization outcomes.

Most research on postmating sexual selection, both theoretical and empirical, have ignored how the social environment may influence the absolute timing of mating. Our study shows that the social environment can affect the fine-scale temporal dynamics of mating and highlights the potential for important feedbacks between the social environment, sperm competition, and the evolution of ARTs. Future work in other systems should explore how the social environment and interactions between competitors influence the temporal dynamics of mating and how time differences in mating interact with sperm competition and cryptic female choice. Further, our results show that the social makeup of mating situations can influence the timing of mating. Incorporating such dynamics into future theory may better align theoretical predictions and empirical results.

§ 1.6 Acknowledgments

We thank A. Chinn, D. Waller, K. Jobes, E. Wilson, and R. Williams for helping collect sneak-spawning delays from the videos. We thank the staff at STARESO for assistance during fieldwork, especially C. Steibel. We thank S. Munch for help with statistics. We thank D. Weiler, L. Alissa, M. Molinari, J. Fitzpatrick, B. Lyon, B. Sinervo, and S. Beyer for helpful feedback that greatly improved this manuscript. M.C.K. was supported by the US National Science Foundation via a GRFP (Award number: DGE-1842400) and an Achievement Rewards for College Scientists Foundation fellowship. This research, S.H.A., and S.E.M were supported by the National Science Foundation (Award numbers IOS-0950472 and IOS-1655297) and funds from Yale University and the University of California Santa Cruz. K.A.S. was supported by the National Science Foundation (IOS-1655217) and from Southern Connecticut State University and CSU-AAUP creative activity grants.

§ 1.7 Statement of Authorship

M.C.K. and S.H.A conceived of the study and S.H.A designed the protocols. All authors collected the data used in this paper. M.C.K performed the statistical analyses and drafted the manuscript. All authors critically revised the manuscript and approve the publication of this article.

§ 1.8 Data and Code Accessibility

Code and data needed to run the model, make figures, and perform statistical analyses are deposited in the Dryad Digital Repository: https://doi.org/10.7291/D17698

Chapter 2

Warm waters may undermine cryptic female choice for preferred males

§ 2.1 Abstract

Female reproductive fluids play an essential role in fertilization by enhancing male sperm performance, guiding sperm to eggs, and biasing fertility to preferred males. However, we know little about how temperature influences interactions between female reproductive fluids and sperm. We studied the effect of temperature on female reproductive fluid and sperm interactions by measuring sperm velocity of a Mediterranean fish species, the ocellated wrasse (*Symphodus ocellatus*), at three temperatures relevant to both seasonal variation they experience and future conditions due to climate change with and without female reproductive fluid. This species has alternative male phenotypes, including a small sneaker male and a large nesting male. Females have a strong mating preference for nesting males. Sneakers override this preference by releasing more sperm than nesting males. Female reproductive fluid in this species decreases the importance of sperm number but increases the importance of sperm velocity in determining fertilization success, effectively favoring nesting males. We performed an experiment on these two male types to see if warming temperatures could influence the

relative fertilization success of these males. We find that nesting males had better or similar sperm velocity as sneakers with ovarian fluid at 16℃. However, at 22℃ and 28℃ sneaker males had higher sperm velocity than nesting males with ovarian fluid. Our results show that cryptic female preferences are temperature-dependent. Thus, the relative fertilization between preferred and non-preferred males may vary both within a season and in the future, given current climate change predictions. Our results imply that temperature may change patterns of selection and relative fitness in ways with long-term evolutionary consequences and more immediate population dynamic consequences.

§ 2.2 Introduction

Climate change poses a significant threat to marine species by changing ocean chemistry (e.g., ocean acidification, less dissolved oxygen), increasing the frequency of extreme climatic events, and rising average temperatures (Buckley and Huey, 2016; Deutsch et al., 2015; IPCC, 2023). Rising average temperatures and extreme heatwaves can harm organisms, especially ectotherms, that depend on the environment for temperature regulation (Buckley and Huey, 2016; Kingsolver et al., 2013). Thus, identifying the thermal sensitivity of important performance traits is crucial to identifying organisms' acute and chronic thermal limits (Williams et al., 2008). Although there has been a significant amount of work in this space, research has focused primarily on traits related to survival and viability (e.g., movement Walsh et al., 2019). Such traits may be overly optimistic as critical thermal limits for viability often exceed those for fertility limits (Dougherty et al., 2024). In other words, even if individuals survive at certain temperatures, they may not be fertile, putting the species at risk (Parratt et al., 2021; Walsh et al., 2019).

Marine animals with external fertilization are likely susceptible to warming waters and extreme climatic events because they directly expose their gametes to the ambient water temperature (Leuchtenberger et al., 2022). Indeed, extreme temperatures can have negative impacts on the performance and fertilizing capability of sperm (Fenkes et al., 2017; Vasudeva et al., 2021; Wang and Gunderson, 2022). However, like most performance traits, sperm performance can have a parabolic relationship with temperature, where intermediate warm temperatures can enhance sperm performance (Dadras et al., 2017; Purchase et al., 2010). Moreover, sperm do not function in a vacuum,

and female physiology can greatly influence sperm function regardless of fertilization mode (Gasparini et al., 2020; Pitnick et al., 2020; Zadmajid et al., 2019). For species with internal fertilization, the female reproductive tract influences sperm (Lüpold and Pitnick, 2018; Pitnick et al., 2020). For species with external fertilization, eggs and the fluid surrounding eggs can positively impact sperm performance (reviewed in Gasparini et al., 2020; Zadmajid et al., 2019) and influence fertilization dynamics. For example, female reproductive fluids can influence the relative importance of different sperm characteristics (e.g., sperm velocity over sperm number) in determining fertilization success (Alonzo et al., 2016; Hadlow et al., 2020). Female reproductive fluids can also select for higher quality sperm or sperm that are more genetically compatible, i.e., cryptic female choice, resulting in the production of fitter offspring (Cattelan et al., 2023; Evans and Marshall, 2005; Hadlow et al., 2023; Lehnert et al., 2017; Lymbery et al., 2017; Oliver and Evans, 2014). Such impacts of female physiology on sperm could help buffer male thermal fertility limits. We are only aware of one study to look at this, which found evidence of such thermal buffering in the spiny lava lizard (*Tropidurus spinulosus*), a species with internal fertilization (Rossi et al., 2021). It remains unknown how taxonomically widespread such thermal buffering could be and if it is even possible in marine species with external fertilization.

Although increasing temperatures may reduce fertility, warming temperatures could also act more subtly and may influence the outcomes of reproductive interactions (reviewed in Leith et al., 2022). Temperature could have this effect by influencing the interactions between sperm and female reproductive fluids. Female reproductive fluids consist of a mixture of proteins, chemoattractants, hormones, carbohydrates, ions, and other organic compounds (reviewed in Gasparini et al., 2020; Zadmajid et al.,

2019), all of which interact with and influence the performance of sperm. Increases in temperature could denature proteins, change pH and osmolality, potentially influencing female reproductive fluid and sperm interactions. Sperm motility is also likely altered by the physical properties of reproductive fluids, such as viscosity, which is also temperature dependent (Graziano et al., 2023; Rosengrave et al., 2009). However, we know little about how temperature can modulate the influence of female reproductive fluid on sperm characteristics, limiting our ability to understand how climate change could alter reproductive interactions.

Here, we investigate how temperature influences sperm motility and how temperature influences the effect of female reproductive fluid on sperm motility in the Mediterranean fish species, the ocellated wrasse (*Symphodus ocellatus*). The ocellated wrasse has three distinct male phenotypes: a large dominant nesting male, an intermediate-sized satellite male, and a small sneaker male (Alonzo et al., 2000; Lejeune, 1984; Taborsky et al., 1987). These tactics are likely condition-dependent based on juvenile growth rates (Alonzo et al., 2000). Males with fast juvenile growth will be satellite males in their first reproductive season and nesting males in their second and final reproductive season. Males with slow growth will be sneaker males in their first year and satellite males in their second and final reproductive year (Alonzo et al., 2000). Females have strong mating preferences for nesting males who build nests and provide all parental care (Alonzo and Pizzari, 2010). To override female preferences, sneaker males perform sneak spawns after the nesting male spawns (Kustra et al., 2023), invest more in ejaculate production (Alonzo et al., 2021), and release ~3x as much sperm as nesting males (Alonzo and Warner, 2000a). Female reproductive fluid (ovarian fluid) counteracts this numerical advantage by decreasing the importance of sperm number

but increasing the importance of sperm velocity in determining fertilization success, effectively favoring the preferred nesting males (Alonzo et al., 2016).

The ocellated wrasse is an ideal study species for studying how temperature influences postmating female-ejaculate interactions for a few reasons. First, ovarian fluid improves sperm velocity and changes fertilization dynamics in ways that favor preferred nesting males (Alonzo et al., 2016). Second, the ocellated wrasse is an ectotherm with external fertilization—their gametes/fluids are directly exposed to the ambient temperature during reproduction and could be vulnerable to heatwaves. Third, there is a large change in temperature within the reproductive season (Figure 2.1A;Fullgrabe et al., 2020, 2023. Fourth, the Mediterranean Sea, where this research will take place, is a climate change hotspot (Giorgi, 2006), with both average temperatures as well as the intensity and frequency of marine heatwaves steadily increasing (Figure 2.1B; Dayan et al., 2023; Garrabou et al., 2022). Thus, understanding how temperature influences interactions between ovarian fluid and sperm performance will provide insight into how seasonal temperature changes and heatwaves can affect postmating male-female interactions.

We tested how the temperature of the fluid within which sperm were swimming (seawater or ovarian fluid) influences sperm velocity (Figure 2.2). This allowed us to tease apart the effects of temperature and ovarian fluid on sperm velocity. We chose three temperatures that represent temperatures the fish currently experience and realistic future heatwave conditions (16℃, 22℃, and 28℃; Figure 2.1; Dayan et al., 2023; Garrabou et al., 2022). We ran these experiments for both nesting males and sneaker males but ignored satellite males because depending on their age (not possible to easily determine in the field) they could be either one year-old males with fast growth

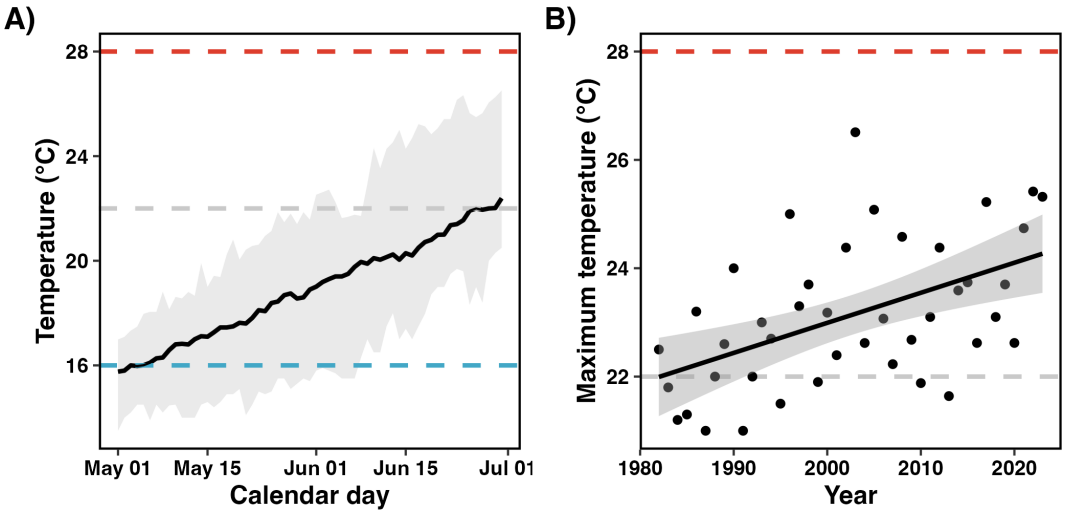

Figure 2.1: (A) Water temperature increases during the reproductive season, and (B) maximum temperature during the reproductive season has increased since 1982. (A) The black line shows the median temperature across all years from 1982 to 2023 at different days of the reproductive season. Grey shading represents the maximum and minimum temperatures on a given calendar day across all years from 1982 to 2023. (B) Scatter plot with a line of best fit of maximum temperature during the reproductive season (β_{year} 0.09, t = 5.393, p <0.001). Grey shading represents the 95% confidence interval. Colored dashed lines indicate the test temperatures of the experiment with blue being the coldest test temperature (16℃), grey being the intermediate temperature (22℃), and red being the hottest test temperature (28℃). Thus, the temperatures used in our experiment are both relevant currently within reproductive seasons and in the future given climate change projections of average seawater temperature increases of 1.8℃ to 3.5℃ by 2100 (UNEP 2020). Data from Fullgrabe et al., 2020, 2023.

or two-year-old males with slow growth. We predicted that increased temperature would have a negative effect on sperm velocity, but that ovarian fluid would help buffer this. We, however, found that sperm velocity increased with temperature, especially for non-preferred sneaker males, and that increasing temperature limited the positive influence that ovarian fluid has on sperm velocity. Our results imply that temperature can profoundly influence postmating interactions between males and females and, therefore,

sexual selection.

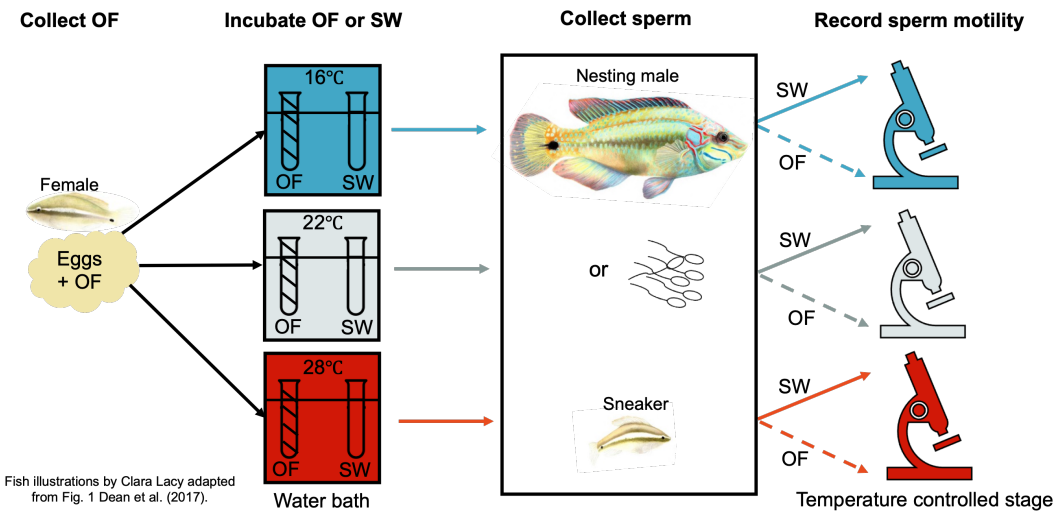

Figure 2.2: Diagram of the experimental design. A single replicate consisted of a single nesting male or single sneaker male. We first collected ovarian fluid (OF) from a female and diluted it with filtered seawater into three samples. We incubated diluted ovarian fluid and pure seawater (SW) at three different temperatures (16℃, 22℃, and 28℃). We then collected sperm from a male and placed the sperm on a temperature-controlled stage, matching the same temperature as the incubation treatments. We then activated sperm with each fluid treatment at each test temperature and recorded sperm motility. This resulted in six separate sperm motility measurements per male and female combination.

§ 2.3 Methods

2.3.1 Animal collection

We collected all fish from the wild at the University of Liege Marine Station (STARESO) near Calvi, Corsica, France (42.5806° N, 8.7243° E) from mid-May to mid-June in 2023. This period is at the peak of their breeding season, which typically runs from May to July (Lejeune, 1984). Individuals were kept in a holding tank with

flow-through water from the ocean until used in the experiments. Fish were used in the experiment within a day of collection. We did not repeat the use of fish across experimental replicates.

2.3.2 Experimental design

To test the effect of temperature on sperm velocity, we used three different temperature treatments: 16℃, 22℃, and 28℃. We chose these temperatures because they cover both the low, average, and extreme heat waves experienced by fish in this area of the Mediterranean during the reproductive season (Figure 2.1). To test how temperature interacts with ovarian fluid, we tested each male's sperm velocity with and without ovarian fluid at each temperature. This resulted in six different treatment combinations per individual male. All six treatment combinations were performed within two hours of one another (Figure 2.2).

We first stripped a female of her eggs into a petri dish, pipetted 2 μL of the ovarian fluid surrounding the eggs, and mixed it into a microcentrifuge tube with 4 μL of filtered seawater (similar concentrations to previous work; Alonzo et al., 2016). This was done three times for each female (one per temperature treatment). We also prepared three microcentrifuge tubes (one per temperature treatment) with 6 μL of filtered seawater. We incubated these tubes in three separate water baths each set at one of the three different water temperature treatments. The temperature of each water bath was kept within 0.1℃ of the target temperature using INKBIRD ITC-306T thermostats and DaToo flat thermostatic heaters. We incubated these microcentrifuge tubes (containing either seawater or seawater plus ovarian fluid) for at least five minutes prior to use in the experiment.

We next collected 0.2 μL of milt (semen) from a male fish and added this milt directly to a 2 μL four-chamber LeJa slide. We then set a microscope with a temperature-controlled stage (LinkamWCP) to the desired test temperature to prevent temperature shock. Next, we flushed the milt into the chamber slide with either 1.8 μL diluted ovarian fluid or seawater incubated at the same test temperature. We then recorded sperm motility at 50 frames per second with a Nikon Ci-L microscope at 100x with phase contrast. Sperm characteristics were analyzed with Microptic CASA software immediately after collection. We took continuous frame captures for two minutes, moving the field of view every two captures to record the velocity of many unique sperm. We then haphazardly moved the field of view around and took two captures at three, four, and five minutes to capture sperm longevity. We purposely excluded sperm that were clumped (preventing sperm movement) or sperm that failed to activate. This process was done manually with the person excluding these data blinded to the experimental treatments and male identity. We additionally excluded any sperm that had not been recorded for at least 20 frames to improve the accuracy of the velocity measure. For each male, we repeated the sperm velocity analysis for every temperature with both diluted ovarian fluid/seawater treatment (six treatments/male). We used freshly collected milt for each treatment. We purposefully changed the order of treatments across replicates (i.e., different males and females). We ended up with 38 full replicates (19 nesting males, 19 sneakers). However, due to exclusion factors (and non-demonic intrusions Hurlbert, 1984), for certain analyses, we did not have all six treatments for each replicate. The exact sample sizes per treatment for each analysis are given in Tables S2.1, S2.2.

2.3.3 Statistical analysis of the experiment

We first examined how ovarian fluid and temperature interact to influence the initial sperm velocity of the different male tactics. We focused on initial sperm velocity because this is a good predictor of fertilization in this species (Alonzo et al., 2016) and other externally fertilizing fish (Fitzpatrick, 2020; Hoysak and Liley, 2001). Specifically, we looked at sperm velocity at 30 seconds following activation, which we refer to as initial sperm velocity. We chose 30 seconds because this was the shortest time in which we could reliably measure a reasonable number of sperm across all six treatments per male: 225± 15.2 (mean ± s.e.) sperm. We had measurements across all six treatments for 17 nesting male replicates and 17 sneaker replicates. We had measurements for four or five treatments for the other replicates. We included the incomplete replicates because mixed effects models account for incomplete blocks, and the data can be used to estimate the treatment effects they contained (see Table S2.1 for a summary of sample size by treatment). For all sperm velocity analyses, we used the curvilinear sperm velocity (VCL; μm/s) because most sperm did not have linear paths.

To test how activating fluid, male type, and temperature influence initial sperm velocity we performed a linear mixed effects model in R V.4.1.2 (R Core Team, 2018) using the package *lme4* (Bates et al., 2015). The fixed effects were the three-way interaction between male type (nesting male or sneaker), activating fluid (ovarian fluid or seawater), and temperature (16℃, 22℃, and 28℃), as well as all lower-level effects. We included a random intercept of the field of view nested within the trial. This allowed us to account for the non-independence of sperm measurements in the same field of view and belonging to the same male. We also included random slopes for activating fluid treatment and temperature treatment effects. The p-values of fixed effects were

calculated with a Type II ANOVA χ^2 test using the *car* package (Fox and Weisberg, 2019). Finally, if there was a significant interaction, we performed post hoc comparisons using the *emmeans* package where *p* values were adjusted with the Tukey method (Lenth, 2021). Model fit was assessed using the *DHARMa* package (Hartig, 2022). All models reasonably fit the data when looking at residuals and Q-Q plots. Although there were sometimes slight deviations from model assumptions, transformations did not improve fit, and mixed effects models are generally robust to slight deviations (Schielzeth et al., 2020).

Next, we wanted to test how ovarian fluid and temperature interact to influence sperm longevity using different male tactics. Longevity could also be important in terms of fertilization success, not necessarily for the initial clutch of eggs, but future clutches of eggs due to rapid sequential matings of the same or different females in this system. To assess longevity, we looked at sperm velocity at 5 minutes. We chose this time to be consistent with previous work in this system (Alonzo et al., 2016). We also did not do a time series of sperm velocity to avoid the complexity of interpreting and analyzing a potential 4-way interaction among time, activating fluid, experimental temperature, and male tactic. For this analysis, we had 12 complete nesting male replicates and 14 complete sneaker male replicates. We had measurements for four or five out of the six treatments for the incomplete replicates. We included the incomplete replicates because mixed effects models account for incomplete blocks, and the data can be used to estimate the treatment effects they contained (see Table S2.2 for a summary of sample size by treatment). The average number of sperm used was 91.5± 6.24 per measurement. We ran the same statistical analyses as the initial sperm velocity analysis.

Understanding individual differences in responses is important to understand the

population's evolutionary potential. So we tested if there was significant variation among males in the response of their sperm to either ovarian fluid or temperature. To do this, we used a log-likelihood ratio test comparing models with only random intercepts to models with a random slope for activating fluid treatment and water test temperature. We performed this analysis for both sperm velocity at 30 seconds and at 5 minutes.

Because temperature changes throughout the reproductive season (Figure 2.1A), fish may be acclimated to different temperatures when used in the experiment. We thus ran supplementary analyses that included a fixed effect of the difference in ocean water temperature on the day of the experiment and trial. This effect did not influence any of the results (Table S2.3), did not improve model fit (Table S2.4), and was highly correlated with temperature treatment (r = 0.9775). Therefore, we did not include this effect in any of the results discussed in the main text. We conducted all analyses and made all figures in R V.4.1.2 (R Core Team, 2018) using the "tidyverse" suite of packages (Wickham et al., 2019).

§ 2.4 Results

2.4.1 Increasing temperature reduces the positive effect of ovarian fluid and favors low-quality males

Initial sperm velocity (i.e., sperm velocity in the first 30 seconds after activation) was significantly influenced by the three-way interaction between activating fluid, experimental temperature, and male tactic (Table 2.1A; Figure 2.3). Initial sperm velocity increased with temperature for both males in seawater, but velocity only increased with

temperature for sneaker males in ovarian fluid (Figure 2.3; Table 2.1A; Table S2.5). At 16℃, ovarian fluid positively affected nesting male swimming velocity (estimate =37.548 μm/s, z = 4.688, p < 0.001). At 22 and 28℃, there was no significant difference in velocity between sperm activated in seawater and ovarian fluid for nesting males (22℃: estimate = -6.645 μm/s, z = -0.839, p= 0.401, 28℃: estimate = -3.823 μm/s, z = -0.489, p = 0.625). At 16℃ and 22℃, ovarian fluid had a significant positive effect on sneaker male velocity (16℃: estimate = 15.719 μm/s, z = 2.013, p = 0.044; 22℃: estimate = 22.347 μm/s, z = 2.950, p = 0.003). However, at 28℃ ovarian fluid had no significant influence on sperm velocity of sneaker males (estimate = -4.846 μm/s, z = -0.623, p=0.533). When directly comparing the velocity of nesting males and sneakers in ovarian fluid, there was no significant difference between nesting males and sneakers at 16℃ (estimate = 10.423 μm/s, z = 1.262, p =0.207; Figure 3B). However, at 22℃, sneakers had significantly faster sperm (estimate = 21.701 μm/s, z= 2.393, p =0.017; Figure 2.3B). At 28℃ the estimated difference between sneakers and nesting males was similar to 22℃. However, there was more variation in that estimate (estimate = -18.915 μm/s, z = -1.921, p = 0.055; Figure 2.3B). All possible pairwise comparisons are reported in tables (S2.5-7).

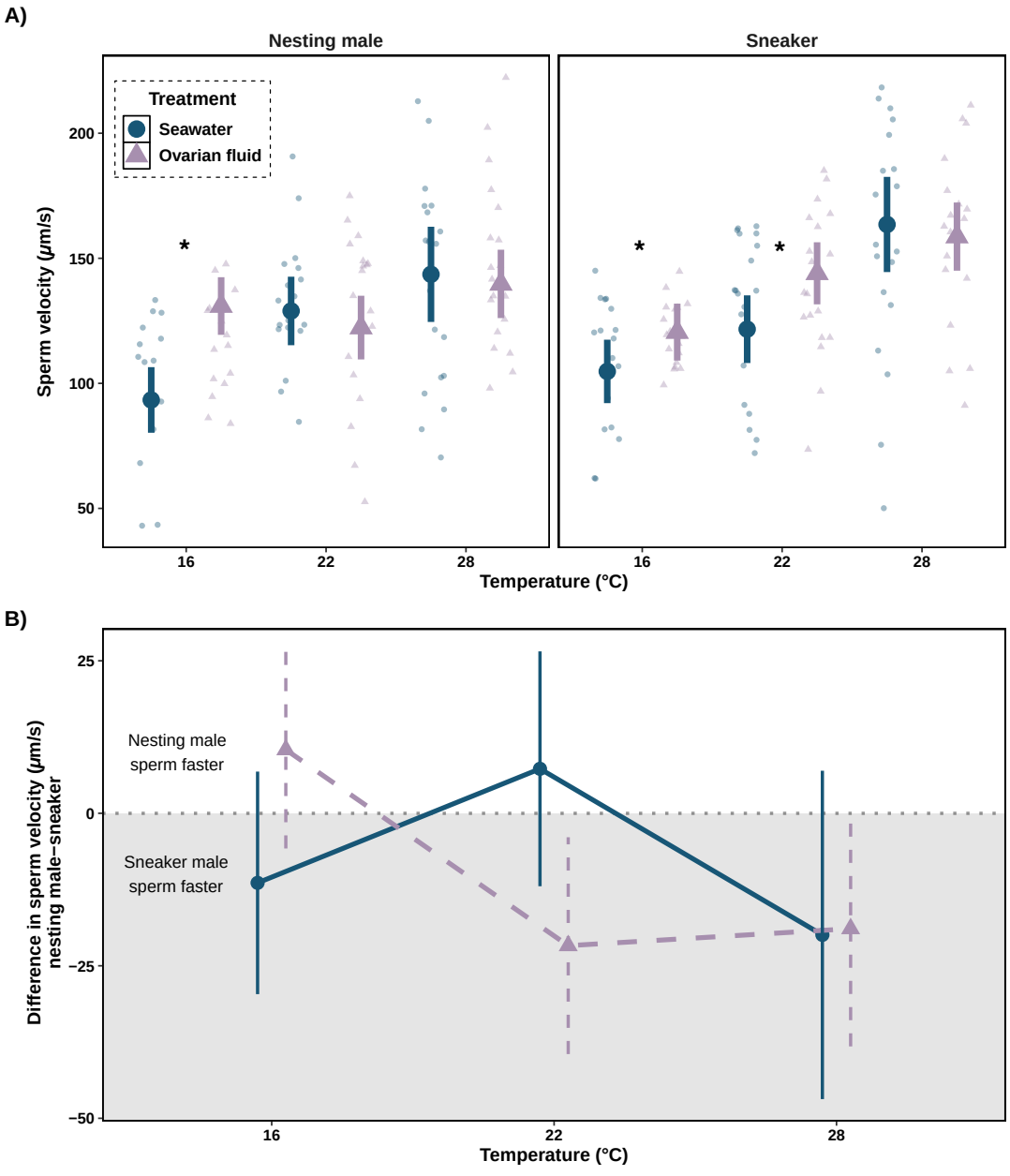
A)
Nesting male
Sneaker
Treatment
Seawater
Ovarian fluid
Sperm velocity (μm/s)
200
150
100
50
16
22
28
Temperature (°C)
B)
Difference in sperm velocity (μm/s)
nesting male−sneaker
25
0
−25
−50
Nesting male
sperm faster
Sneaker male
sperm faster
16
22
28
Temperature (°C)

Figure 2.3: Increasing temperature increases initial sperm velocity but limits the positive effect of ovarian fluid, resulting in sneakers having higher initial sperm velocity than nesting males. (A) Solid shapes indicate the predicted value of each treatment based on the model described in Table 2.1A, and bars represent the 95% confidence intervals. The smaller points in the background are averages of sperm velocity of all motile sperm at 30 seconds of activation. Asterisks indicate significant differences between seawater and ovarian fluid. The statistical significance of other pairwise comparisons is given in tables (S2.5-S2.7). (B) Solid shapes indicate the contrast between sneaker males and nesting males at each treatment based on the model described in Table 2.1A, and bars represent the 95% confidence intervals of those contrasts. The dotted grey line at zero means no difference between nesting males and sneakers. Positive values (white background) indicate nesting males have faster sperm than sneaker males, negative values (grey background) indicate sneakers have faster sperm than nesting males. Contrasts are reported in Table S2.7.

Initial sperm velocity may tradeoff with sperm longevity, so we also looked at sperm velocity at 5 minutes. We found that sperm velocity at 5 minutes was also significantly influenced by the three-way interaction between activating fluid, experimental temperature, and male tactic (Table 2.1B; Figure 2.4A). Nesting male sperm velocity decreased with increasing temperature in ovarian fluid (Figure 2.4A) but not seawater. This was likely due to sperm velocity being low in seawater across all temperature treatments at 5 minutes. Temperature did not influence sperm velocity of sneaker males in both seawater and ovarian fluid. Ovarian fluid increased sperm velocity for nesting males at 5 minutes compared to seawater across all temperatures, although the magnitude of this effect was strongest at 16℃ (16℃: estimate = 41.767 μm/s, z = 6.508, p < 0.001; 22℃: 15.491 μm/s, z = 2.429, p = 0.015; 28℃: 19.18 μm/s, z = 2.926, p = 0.003; Figure 4A). Ovarian fluid only increased sperm velocity at 5 minutes for sneaker males at 16℃: and 22℃: (16℃: 18.099 μm/s, z = 2.926, p = 0.003; 22℃: 24.558 μm/s, z = 3.955, p = <0.001; 28℃: 8.386 μm/s, z = 1.338, p = 0.181). When directly

comparing the sperm velocity at 5 minutes of nesting males and sneakers in ovarian fluid at 16℃, sneaker males and nesting males had similar sperm swimming velocity (estimate = 0.694μm/s, z = 0.105, p = 0.916; Figure 2.4B). However, at 22℃ and 28℃, sneakers had significantly faster sperm at 5 minutes (22℃: 20.896 μm/s, z = 2.959, p = 0.003; 28℃: 21.625 μm/s, z = 2.142, p = 0.032; Figure 2.4B). All possible pairwise comparisons are reported in tables (S2.8-S2.10).

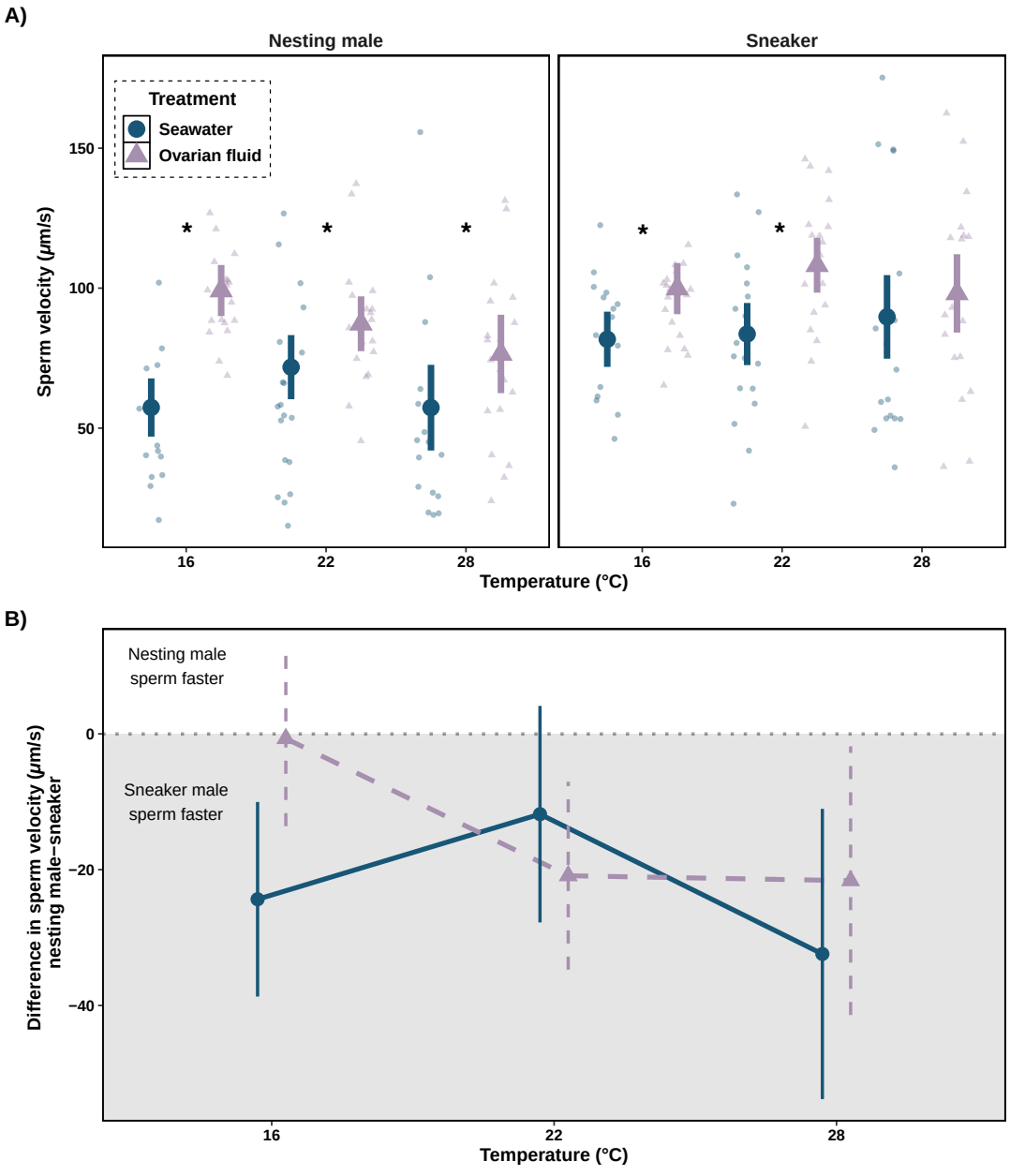
A)
Nesting male
Sneaker
Treatment
Seawater
Ovarian fluid
Sperm velocity (μm/s)
150
100
50
16
22
28
Temperature (°C)
B)
Nesting male sperm faster
Sneaker male sperm faster
Difference in sperm velocity (μm/s) nesting male−sneaker
0
−20
−40
16
22
28
Temperature (°C)

Figure 2.4: Increasing temperature decreases sperm longevity for nesting males and limits the positive effect of ovarian fluid, resulting in sneakers having higher sperm velocity after 5 minutes at warmer temperatures. (A) Solid shapes indicate the predicted value of each treatment based on the model described in Table 2.1B, and bars represent the 95% confidence intervals. The smaller points in the background are averages of sperm velocity of all motile sperm at 30 seconds of activation. Asterisks indicate significant differences between seawater and ovarian fluid. The statistical significance of other pairwise comparisons is given in tables (S2.8-10). (B) Solid shapes indicate the contrast between sneaker males and nesting males at each treatment based on the model described in Table 2.1B, and bars represent the 95% confidence intervals of those contrasts. The dotted grey line at zero means no difference between nesting males and sneakers. Positive values (white background) indicate nesting males have faster sperm than sneaker males, negative values (grey background) indicate sneakers have faster sperm than nesting males. Contrasts are reported in Table S2.10.

Table 2.1: Results of the (A) linear mixed effects model explaining sperm velocity at 30 seconds (VCL) and (B) sperm velocity at 5 minutes. The significance of fixed effects was determined with a Type II ANOVA Wald χ^2 test. Random effects for both models were a random intercept for the field of view nested within the trial and random slopes for both activating fluid treatment and temperature treatment of each trial. Significant effects are bolded.

Trait	Model Effect	χ^2	$p(>\chi^2)$
(A) Sperm velocity at 30 seconds	**Temperature**	**40.711**	**<0.0001**
	Activating fluid	3.712	0.0540
	Male tactic	1.477	0.224
	Activating fluid: temperature	**60.949**	**<0.0001**
	Activating fluid: male tactic	0.0977	0.7546
	Temperature: male tactic	3.599	0.1654
	Activating fluid: temperature: male tactic	**43.181**	**<0.0001**
(B) Sperm velocity at 5 minutes	Temperature	3.358	0.1866
	Activating fluid	**24.646**	**<0.0001**
	Male tactic	**7.481**	**0.0062**
	Activating fluid: temperature	**53.100**	**<0.0001**
	Activating fluid: male tactic	0.8455	0.3578
	Temperature: male tactic	4.868	0.0877
	Activating fluid: temperature: male tactic	**74.412**	**<0.0001**

2.4.2 Significant variation exists in individual responses to temperature and ovarian fluid

We tested the significance of random slopes to determine whether there was significant variation in individual responses to either temperature or ovarian fluid. Understanding individual differences is important to understand the evolutionary potential of the population. We found that including a random slope for both the activating fluid treatment and temperature treatment significantly improved the model fit for both sperm velocity at 30 seconds (χ^2 = 1304.9 , p <0.0001; Table S2.11A) and sperm velocity at 5 minutes (χ^2 = 3064.1, p <0.0001; Table S2.11B). This indicates that there was significant among-individual variation in reaction norms in both treatments for sperm velocity at 30 seconds (Figure S2.1) and sperm velocity at 5 minutes (Figure S2.2).

§ 2.5 Discussion

Female reproductive fluids play an essential role in fertilization by enhancing male sperm performance, guiding sperm to eggs, and sometimes even biasing fertility to produce higher-quality offspring (reviewed in Gasparini et al., 2020). However, we know little about how temperature may influence interactions between female reproductive fluids and sperm. Here, we tested how the presence or absence of female ovarian fluid interacts with temperature to affect sperm performance in a marine fish species with distinct male types: the heavily preferred nesting male and non-preferred sneakers. We find that temperature generally increases initial sperm velocity in both male types, but this effect is stronger in sneakers. Further, increasing temperature decreased sperm longevity for nesting males but did not influence sneaker male sperm longevity. This

resulted in nesting males having better or similar sperm velocity as sneakers in the presence of ovarian fluid at 16℃. However, at 22 and 28℃, sneaker males had higher sperm performance than nesting males in ovarian fluid. Because small differences in sperm velocity influence fertilization success in this species (Alonzo et al., 2016), this suggests that non-preferred sneakers will have higher fertilization success than the preferred nesting male at higher temperatures (e.g., the end of the reproductive season and in future climate projections).

The initial sperm velocity of both male tactics increased with increasing temperature in both seawater and ovarian fluid. However, this effect was greatest in sneaker males. This result is consistent with other studies demonstrating a positive impact of temperature on initial sperm velocity in fish (reviewed in Dadras et al., 2017). This could be due to increased metabolism, resulting in a reduction in longevity. Indeed, we found this to be the case for nesting males whose sperm velocity at five minutes decreased with increasing temperature in ovarian fluid. Interestingly, sperm velocity at 5 minutes did not decrease with increasing temperature for sneaker males. One potential reason for this difference could be physiological differences in the sperm. Sneakers in this system typically have longer-lived sperm than nesting males (Alonzo et al., 2016). Although not explicitly tested in this species, this could indicate higher energy stores in sneaker male sperm cells (i.e., ATP). A recent meta-analysis (Dougherty et al., 2022) and review (Kustra and Alonzo, 2020) found that sneaker males in fish generally have sperm with higher ATP content. Future work should look at sperm physiology in this species and test the effect of temperature on metabolism. Our results imply that in warmer temperatures, sneaker males, which are generally not preferred by the females, will likely have higher fertilization success than the preferred nesting males, independent of the effects

of ovarian fluid.

Ovarian fluid positively influences sperm velocity and longevity for fish generally (Zadmajid et al., 2019) and in this species specifically (Alonzo et al., 2016). Further, ovarian fluid in this species increases the relative importance of sperm velocity in fertilization. Here, we found that ovarian fluid only increased initial sperm velocity at cooler temperatures for both males. Ovarian fluid improved sperm longevity for nesting males across all temperatures, but this effect weakened as temperature increased to the point that sperm longevity was significantly lower than that of sneakers at 22 and 28℃. Future work in this system should explore the mechanism of this negative temperature dependence. One possibility is that temperature could be influencing viscosity or osmolality, characteristics known to influence sperm motility in other taxa (Graziano et al., 2023; Rosengrave et al., 2009; Zadmajid et al., 2019). Other species should be examined to see if temperature has similar effects on female reproductive fluid. To our knowledge, the only other study to explore temperature effects on female reproductive fluid was in an internally fertilizing lizard (Rossi et al., 2021), which found that increasing temperature did not influence the impact that female reproductive fluid had on sperm motility.

The temperature dependence of female-male postmating interactions has exciting implications for improving our understanding of sexual selection. Overall, our results, in combination with previous work in this system, suggest that mechanisms of cryptic female choice that bias paternity to nesting males may only function early on in the reproductive season. Because temperature typically increases from 16℃ to 22℃ over the reproductive season (Figure 2.1A), this could mean that nesting males have higher fertilization success in competitive matings at the beginning of the season than at the

end. This could influence how nesting males alter behavior to improve mating success and prevent sperm competition (e.g., mate guarding) across the reproductive season (Alonzo and Warner, 2000a). This adds a novel way for temperature to change the dynamics of sexual selection across the reproductive season (Kvarnemo, 1997). In the context of climate change, temperature shifts could also shape male tactics' relative reproductive success. This could result in a shift in the frequency of these male phenotypes and/or population size, given that sneaker males may be of lower quality due to strong female preferences against them and their slower juvenile growth rates (Alonzo et al., 2000). Alternatively, selection could act on existing variation in thermal tolerance. We found that there was significant variation in individuals' response to temperature and activating fluid. If this variation is heritable, it could mean selection could favor females who produce ovarian fluid that is more temperature resistant or males whose sperm are more temperature resistant. Future work should be conducted in other systems to see if temperature has similar influences on postmating female-male interactions.

One limitation of this study is that we use sperm velocity as a proxy for fertility, although this is a good proxy in this species (Alonzo et al., 2016) and in other fish with external fertilization (reviewed in Fitzpatrick, 2020). None of the test temperatures resulted in a massive reduction in sperm velocity; if anything, sperm velocity increased at higher temperatures. Heatwaves with higher temperatures than what we explored in this study could have a negative effect on sperm performance. However, anecdotally, sperm did not completely lose motility until 38℃, an unrealistic temperature. Past work in other systems has shown that at higher than normal temperatures, sperm can still fertilize eggs, but there may be adverse paternal effects resulting in reduced offspring

viability (Kekäläinen et al., 2018; Lymbery et al., 2020). This could occur at our highest test temperature, but due to constraints, we only focused on sperm velocity. We also only looked at the immediate temperatures that sperm and female reproductive fluids faced. Although this is still relevant in the context of marine heatwaves (Garrabou et al., 2022), exploring chronic exposure to warming temperatures and the possibility of plasticity in gamete/fluid production would be interesting.

We know surprisingly little about how temperature can influence male-female interactions after mating. Our results show that increasing temperatures could undermine the positive impact of female reproductive fluid and bias paternity toward lower-quality males. Future research into the consequences of climate change on female-male post-mating interactions is essential in understanding how climate change will influence fertility, population persistence, and sexual selection.

§ 2.6 Acknowledgments

We thank M. Rogers, L. Alissa, K. Stiver, S. Marsh-Rollo, J. Hellmann, and M. Molinari for collecting fish and helping run the experiment. We thank the staff at STARESO for assistance during fieldwork, especially C. Steibel. We thank D. Weiler, L. Alissa, M. Molinari, J. Fitzpatrick, M. Servedio, B. Lyon, and M. Gamble for helpful feedback that greatly improved this manuscript. M.C.K. was supported by the US National Science Foundation via a GRFP (Award number: DGE-1842400), an Achievement Rewards for College Scientists Foundation fellowship, and an American Society of Naturalists student research award. Long-term acquisition of temperature data was supported by the STARESO marine station, the Rhone-Mediterranean and

Corsica Water Agency, and the Collectivity of Corsica under the STARECAPMED project.

Chapter 3

The coevolutionary dynamics of cryptic female choice

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Kustra, M. C., & Alonzo, S. H. (2023). The coevolutionary dynamics of cryptic female choice. *Evolution Letters*, *7*(4), 191–202

§ 3.1 Abstract

In contrast to sexual selection on traits that affect interactions between the sexes before mating, little theoretical research has focused on the coevolution of post-mating traits via cryptic female choice (when females bias fertilization toward specific males). We used simulation models to ask (1) whether and if so how non-directional cryptic female choice (female-by-male interactions in fertilization success) causes deviations from models that focus exclusively on male-mediated post-mating processes and (2)

how the risk of sperm competition, the strength of cryptic female choice, and tradeoffs between sperm number and sperm traits interact to influence the coevolutionary dynamics between cryptic female choice and sperm traits. We found that incorporating cryptic female choice can result in males investing much less in their ejaculates than predicted by models with sperm competition only. We also found that cryptic female choice resulted in the evolution of genetic correlations between cryptic female choice and sperm traits, even when the strength of cryptic female choice was weak, and the risk of sperm competition was low. This suggests that cryptic female choice may be important even in systems with low multiple mating. These genetic correlations increased with the risk of sperm competition and as the strength of cryptic female choice increased. When the strength of cryptic female choice and risk of sperm competition was high, extreme co-divergence of sperm traits and cryptic female choice preference occurred even when the sperm trait traded off with sperm number. We also found that male traits lagged behind the evolution of female traits; this lag decreased with increasing strength of cryptic female choice and risk of sperm competition. Overall, our results suggest that cryptic female choice deserves more attention theoretically and may be driving trait evolution in ways just beginning to be explored.

§ 3.2 Introduction

Sexual selection can drive the evolution of complex traits and result in trait divergence between populations and species (Coyne and Orr, 2004; Irwin, 2020; Lande, 1981; Mead and Arnold, 2004; Mendelson et al., 2014; Servedio and Boughman, 2017). Changes in the preferences of one sex can impose selection on traits in the other sex, leading to co-divergence of these traits among isolated populations (Coyne and Orr, 2004; Lande, 1981; Mead and Arnold, 2004; Servedio and Boughman, 2017). Despite numerous theoretical models on intersexual selection (Mead and Arnold, 2004; Servedio and Boughman, 2017; Turelli et al., 2001), almost all theory has focused on pre-mating sexual selection (but see, Lorch and Servedio, 2007; Rushworth et al., 2022). In contrast, little is known about the coevolution between female and male traits shaped by post-mating sexual selection (i.e., selection on traits affecting fertilization after mating; Howard et al., 2009; Parker, 1970; Shuker and Kvarnemo, 2021). Thus, theory that explicitly considers post-mating intersexual selection is needed to understand these coevolutionary dynamics.

Post-mating sexual selection occurs in two non-mutually exclusive forms: sperm competition, when sperm from two or more individuals compete for the fertilization of the same ova (Parker, 1970), and cryptic female choice, when females bias fertilization towards a specific male or sperm trait (e.g., sperm size; Firman et al., 2017; Thornhill, 1983. Cryptic female choice is often mediated by female reproductive physiology (Beirão et al., 2015; Firman et al., 2017; Gasparini et al., 2020; Higginson et al., 2012; Lupold et al., 2016; Lüpold and Pitnick, 2018; Miller and Pitnick, 2002; Pitnick et al., 2003, 2020; Poli et al., 2019; Rosengrave et al., 2008). Cryptic female choice can

either be directional, when all females share the same preference, or non-directional, when preferences differ among females (Birkhead and Pizzari, 2002; Firman et al., 2017). Non-directional cryptic female choice results in female-by-male interactions in fertilization success and has been demonstrated in a wide variety of taxa(Bjork et al., 2007; Clark et al., 1999; Devigili et al., 2018; Firman et al., 2017; Fitzpatrick and Lüpold, 2014; Fitzpatrick et al., 2020; Lüpold, Boer, et al., 2020; Oliver and Evans, 2014; Pitnick et al., 2020; Poli et al., 2019; Rosengrave et al., 2008; Urbach et al., 2005). These female-by-male interactions may be a mechanism for the rapid evolution of sperm, which are the most diverse cell type across taxa, and also show rapid divergence between populations (Hogner et al., 2013; Lüpold and Pitnick, 2018; Manier and Palumbi, 2008; Pitnick, Hosken, and Birkhead, 2009; Pitnick et al., 2003). Further, there is evidence for the codivergence of female reproductive tract morphology and sperm morphology in various taxa (Briskie et al., 1997; Higginson et al., 2012; Pitnick, Wolfner, and Suarez, 2009; Pitnick et al., 2020), suggesting non-directional cryptic female choice may play a major role in generating such diversity.

One hypothesized mechanism for the coevolution of pre-mating female preferences and male traits is the Fisher Process (Fisher, 1930; Henshaw and Jones, 2020; Kirkpatrick, 1982; Lande, 1981). In the Fisher Process, male traits evolve due to higher mating success. Female preferences evolve from indirect selection arising from genetic correlations between preferences and traits because females mate with males that carry traits associated with their preference. Although a similar process could occur in post-mating sexual selection (e.g., sexy sperm hypothesis; Keller and Reeve, 1995; Yasui, 1997), there exists no model that explicitly explores this. Because most pre-mating sexual selection models assume that females are monogamous, the factors that post-

mating sexual selection empiricists study (e.g., degree of polyandry) are not explored. Additionally, genetic correlations may be harder to establish due to greater stochasticity in the post-mating coevolutionary process resulting from (1) selection among males only occurring when multiple mating happens and (2) fertilization not being a "winner-take-all situation" as there will often be mixed paternity in a brood (Bocedi and Reid, 2015; Cramer et al., 2023). Thus, we need theory that explicitly considers post-mating sexual selection.

The focus of most post-mating sexual selection theory is on intrasexual selection (exclusive sperm competition), not intersexual selection (cryptic female choice; Ah-King, 2022; Parker and Pizzari, 2010; but see Ball and Parker, 2003; Cramer et al., 2023; Lorch and Servedio, 2007). Moreover, most post-mating sexual selection theory takes a game theory approach focused on strategic ejaculate allocation (Parker and Pizzari, 2010). While powerful for predicting some evolutionary outcomes, game theory does not address the underlying genetic correlations needed for coevolution (Kirkpatrick, 1982; Kuijper et al., 2012; Lande, 1981). The evolution of genetic correlations between post-mating sexually-selected traits and preferences (e.g., sperm length and female reproductive tract length;Miller and Pitnick, 2002) could be hindered by selection simultaneously acting on sperm number, especially if tradeoffs exist. Previous models have explored this tradeoff (Immler et al., 2011; Parker, 1993; Parker and Pizzari, 2010), but did not explicitly consider cryptic female choice.

We first tested if non-directional cryptic female choice causes deviations in predicted ejaculate investment from models that exclusively focus on male-mediated processes. We did this by contrasting a "traditional" game theory model of sperm competition to a genetically explicit individual-based model that either did or did not incorporate

cryptic female choice with sperm competition. We then asked how the risk of sperm competition, preference strength, and a tradeoff between sperm number and sperm trait interact to influence post-mating coevolutionary dynamics. Specifically, we looked at the magnitude of these genetic correlations between the cryptic female choice trait and the sperm trait, and whether these genetic correlations resulted in trait codivergence across a suite of scenarios.

§ 3.3 Methods

Below, we first describe our analytical model of exclusive sperm competition. We then describe the basic structure of our genetically explicit individual-based model (IBM). Next, we present equations representing how selection acts on male ejaculate traits and explain the details of our simulations and the analyses we performed (see Table 3.1 for a summary).

3.3.1 Analytical model of sperm competition

We developed an analytically tractable game theory model based on Parker, 1993 to compare to our IBMs. We assumed a tradeoff between post-mating and pre-mating traits. Specifically, we assumed that the mating success probability (n) of a male with ejaculate allocation (x_i) would decrease as a sigmoidal function with increasing x_i such that α is a shape parameter and β is the inflection point of the sigmoidal shape,

$$n_{(x_i)} = 1 - \frac{1}{1 + e^{-\alpha(x_i - \beta)}} \tag{3.3.1}$$

We chose a sigmoidal relationship rather than the inverse relationship used in Parker, 1993 because we believe that it is more realistic to assume that there should be diminishing returns in n when decreasing x_i after a certain point.

The relative expected mating success (n_r) for a mutant with ejaculate allocation (x_m) to a male with equilibrium ejaculate allocation (x_e) is,

$$n_r(x_m, x_e) = \frac{1 - \frac{1}{1+e^{-\alpha(x_m-\beta)}}}{1 - \frac{1}{1+e^{-\alpha(x_e-\beta)}}} \tag{3.3.2}$$

The expected fertilization success (υ) of mutant with ejaculate allocation (x_m) relative to the male with equilibrium ejaculate allocation (x_e) is,

$$\upsilon(x_m, x_e) = (1 - q) + 2q\frac{x_m}{x_e + x_m} \tag{3.3.3}$$

where q is the risk of sperm competition (probability that a female mates with more than one male). Assuming that females do not differ in fecundity, the fitness of a mutant relative to a male at equilibrium ($W(x_m, x_e)$) is the product of n_r and υ,

$$W(x_m, x_e) = n_r(x_m, x_e)\upsilon(x_m, x_e) \tag{3.3.4}$$

To find the evolutionary stable strategy (ESS) ejaculate allocation equation, we found when the derivative of the fitness equation was equal to 0 evaluated at $x_m = x_e$,

$$\frac{-\alpha x + \frac{q(e^{\alpha(\beta-x)}+1)}{2}}{x(e^{(\alpha(\beta-x))} + 1} = 0 \tag{3.3.5}$$

The solution requires the use of the Lambert function, which yields two possible solutions (Lehtonen, 2016):

$$\frac{\frac{q}{2} + LambertW(\frac{qe^{\alpha\beta-\frac{q}{2}}}{2})}{\alpha}, \frac{\frac{q}{2} + LambertW(\frac{-qe^{\alpha\beta-\frac{q}{2}}}{2})}{\alpha} \tag{3.3.6}$$

We only use the first solution because the second does not yield positive values for valid values of q (i.e., between 0 and 1). Ejaculate allocation increases monotonically with risk of sperm competition with the shape of this curve depending on α and β (Fig. S3.1; SI web app).

3.3.2 General IBM description

Individuals in this model were diploid and had three positive, continuous traits with sex-limited expression: sperm number (s), sperm trait (m), and cryptic female choice trait (f; Fig. S3.2). We performed simulations for both cryptic female choice and sperm competition only (see Fig. S3.3 for a flow chart of the model). We ran two sperm competition only models: (1) fixed stabilizing selection on m (e.g., optimum sperm velocity given a tradeoff with sperm longevity; Levitan, 2000) and (2) fair raffle where only sperm number (s) mattered. Like most models of sperm competition (Parker and Pizzari, 2010), we always assumed that pre-mating and post-mating success traded off with one another. In addition to this pre- and post-mating tradeoff, we varied whether there was a tradeoff between s and m because not all sperm traits will tradeoff with sperm number. For example, we would not necessarily expect a tradeoff between chemical signals/receptors for sperm function and the number of sperm produced by a male (Fitzpatrick et al., 2020). We refer to these different scenarios as "tradeoff" or "no

tradeoff."

We assumed that females mate with at most two males, with the probability of a second mating occurring being the risk of sperm competition (q). If sperm competition (i.e., multiple mating) occurred, a male's fertilization success depended on how high his sperm number (s) was relative to a competitor and how closely his sperm trait (m) matched an "optimum" relative to the sperm trait of his competitor. For cryptic female choice simulations, the "optimum" was the cryptic female choice trait (f); for simulations of stabilizing selection with sperm competition only, this was a set constant. The strength of preference (ω) on m determined the selective advantage of differences in the sperm trait value between the two competing males when there was sperm competition. For simplicity we ignore mate order effects. We assumed non-overlapping generations to keep population size and sex-ratio constant.

3.3.3 Pre-mating sexual selection

We modeled pre-mating sexual selection like Eq.3.3.1. In model runs with a tradeoff between m and s, the probability that a female mated with male z_i ($P(z_i)$) where n_m is the number of males in the population is,

$$P(z_i) = \frac{1 - \frac{1}{1+e^{-\alpha((s_i m_i)-\beta)}}}{\sum_{j=1}^{n_m} 1 - \frac{1}{1+e^{-\alpha((s_j m_j)-\beta)}}} \tag{3.3.7}$$

In model runs without a tradeoff between s and m, the probability that a female mated with male $z_i(P(z_i))$ was only dependent on s:

$$P(z_i) = \frac{1 - \frac{1}{1+e^{-\alpha((s_i)-\beta)}}}{\sum_{j=1}^{n_m} 1 - \frac{1}{1+e^{-\alpha((s_j)-\beta)}}} \tag{3.3.8}$$

For simulations without a tradeoff, we set α = 1/20 to make the predicted investment moderately distinct across q (Fig.S3.1), and β = 50 to make the inflection point around starting trait averages. To keep the shape of the tradeoff the same when multiplying m and s, we set α = 1/1000 and β= 2500 for simulations with a tradeoff. Preliminary analyses varying these parameters did not influence qualitative results.

3.3.4 Post-mating sexual selection

During a competitive mating with male z_i and male z_j, male z_i's probability of fertilization $\psi(z_i, z_j)$, was determined by his sperm number (s_i) and sperm trait (m_i), the competitor male's sperm number (s_j) and sperm trait (m_j), the cryptic female choice trait (f_i) of the female involved in the mating event, and the strength of preference acting on sperm trait (ω; Fig. S3.4; SI web app),

$$\psi(z_i, z_j) = \frac{s_i e^{\frac{-(m_i - f_i)^2}{2\omega}}}{s_i e^{\frac{-(m_i - f_i)^2}{2\omega}} + s_j e^{\frac{-(m_j - f_i)^2}{2\omega}}} \tag{3.3.9}$$

For simulations of stabilizing selection with sperm competition only, ω can be thought of as the strength of selection. For simulations of fair raffle, only s factored in to determine ψ. We assume that females are not under direct selection as fertilization is guaranteed.

To explore the balance between selection on m and s, we varied the value of ω for both cryptic female choice and stabilizing selection simulations. Specifically, we ran simulations when ω was 50, which we refer to as weak selection; 12.5, which we refer to as moderate selection; and 1, which we refer to as strong selection (Fig. S3.4).

To model stabilizing selection with sperm competition only as a comparison to cryptic female choice simulations, we fixed f to 50. Preliminary analyses showed that varying this value did not qualitatively change results.

3.3.5 Model process

We first generated populations with equal sex ratio of size N. Each run (population) was randomly initialized with trait values drawn from a normal distribution with mean = 50 and standard deviation (SD) = 5. We did this by assuming a "continuum-of-alleles" (alleles have continuous effects on trait values) and randomly generated two alleles per locus per trait for each individual by drawing from a normal distribution with mean = 1.25 and SD =$\sqrt{0.625}$. We converted any negative numbers to zero, as negative trait values are not biologically meaningful. Each trait was determined by adding each copy of all twenty unlinked loci (Fig.S3.2). During each generation, we recorded the mean and standard deviation of traits, the Pearson correlation between the f and m genetic values of individuals, and the multivariate selection coefficients of each trait (Lande and Arnold, 1983; Stinchcombe et al., 2008; see supplemental methods). We calculated ejaculate investment (x in the analytical model) as s for simulations without a tradeoff and ms for simulations with a tradeoff.

Each female mated with at least one male and, with probability q a second male. Males, since their potential number of matings were unrestricted, were assumed to experience ejaculation depletion with no recovery at a constant rate of c after mating:

$$s^{'} = se^{-c} \tag{3.3.10}$$

We assumed $c = 0.2$ for all runs, preliminary analyses showed that this did not influence results. This ejaculate depletion affected only post-mating outcomes (i.e., ψ) and not pre-mating success (i.e., P). Each female then produced a female and male offspring, with each offspring's paternity determined by randomly selecting one of the two males with probability ψ (Eq.3.3.9). In a single mating, that single male sired both offspring. We determined the genotype of each offspring by randomly sampling one allele per locus from each parent. During this process, each allele mutated with probability μ = 0.005 and with a mutational effect drawn from a normal distribution with mean = 0 and SD =$\sqrt{0.00625}$. We limited our evolutionary simulations to 30,000 generations because genetic correlations stabilized by generation 20,000 for all parameters. We ran the model for 50 replicates per parameter combination.

3.3.6 Analysis of deviation and lags

To test how deviated sperm traits were from their optima, we calculated the deviation of average m from the value at which fertilization would be highest (assuming equal s). We refer to this as the "optimal" sperm trait value in the results. For cryptic female choice, this was calculated using the average f of a population, and for stabilizing selection simulations with sperm competition only, this was 50 (pre-set optimum).

To test for the possibility of evolutionary lags between f and m, we looked at the deviation between f and m in the final 2000 generations of our simulations. We standardized both traits using the mean of a trait across the final 2000 generations to keep the scale consistent. We then calculated the mean absolute error (MAE) between standardized f and m across the final 2000 generations at different generational lag times (from 0 to 300 generations).

Table 3.1: Parameter, variable, and function definitions and corresponding values used in the models. * The first value is for no tradeoff between s and m; the second value is with a tradeoff between s and m. Values were changed to keep the same scale for total investment. IBM = from individual-based model; AM = from analytical model; Both = used in both models.

	Symbol	Definition	Values/Equations
Parameters			
	N	Population size (IBM)	10,000
	m	Mutation rate (IBM)	0.005
	q	Probability that a female will mate with more than one male (Both)	0.25; 0.5; 0.75; 1
	a*	Shape parameter for post- and pre- mating tradeoff (Both)	1/20; 1/1000
	b*	Scale parameter for post- and pre- mating tradeoff (Both)	50; 2,500
	w	Width of optimality function which determines strength of selection; lower values result in stronger selection (IBM)	50; 12.5; 1
	c	Ejaculate depletion rate (IBM)	-0.2
Variables			
	x	Ejaculate investment (AM)	Evolves
	m	Sperm trait value (IBM)	Evolves
	f	Cryptic female choice trait value (IBM)	Evolves
	s	Sperm number (IBM)	Evolves
Function			
	$n_r(x_m, x_e)$	Expected mating success of mutant relative to a male at equilibrium (AM)	Eq.3.3.2
	$v(x_m, x_e)$	Expected fertilization success of mutant relative to a male at equilibrium (AM)	Eq.3.3.3
	$W(x_m, x_e)$	Fitness of a mutant relative to a male at equilibrium (AM)	Eq.3.3.4
	$P(z_i)$	Probability of male z_i being selected for mating (IBM)	Eq.3.3.7 and 3.3.8
	$\psi(z_i, z_j)$	Probability that male z_i fertilizes an egg given male competitor z_j (IBM)	Eq.3.3.9

We ran all models using Julia v1.6.4 (Bezanson et al., 2017) and performed analyses/made figures in R (R Core Team, 2018) using the "tidyverse" suite of packages (Wickham et al., 2019). We report a summary of parameters, variables, and values used in this model in Table 3.1. We conducted several sensitivity analyses on the number of loci that determined each trait, the population size, starting averages, and the starting variation (see supplemental methods). All results discussed below are from the IBM and were robust to these sensitivity analyses (Figs. S3.5-11; SI web app). Unless stated otherwise, results reported are from the final generation.

§ 3.4 Results

3.4.1 What factors influence the evolution of cryptic choice trait, sperm trait and sperm number?

We first summarize how mean trait values coevolve and diverge across populations, which is essential to understand the potential for reproductive isolation. After 30,000 generations, the presence of a tradeoff between sperm number (s) and sperm trait (m) did not influence the average trait values of m or cryptic choice trait (f; Fig.3.1A). The overall range of average trait values for f and m increased both with preference strength and risk of sperm competition. There was an interaction such that risk of sperm competition had a much larger influence when preferences were strong (Fig.3.1A). We found that s increased with the risk of sperm competition as predicted by the analytical model. Unlike m and f, the range of s increased with the risk of sperm competition only when there was a tradeoff.

We then looked at variation maintained within populations after extended coevolution to understand the ability for continual coevolution. We found that the coefficient of variation (CV) was generally highest in s followed by f and then m, with f being over double m across all scenarios (Fig.3.1B). The CV of all three traits decreased with the risk of sperm competition (Fig.3.1B). As the strength of preference increased, the CV of f and m decreased while s increased. This effect was most notable when preferences were strong (Fig. 3.1B). We also found that the range of s CV among populations was largest when there was a tradeoff and preferences were strong.

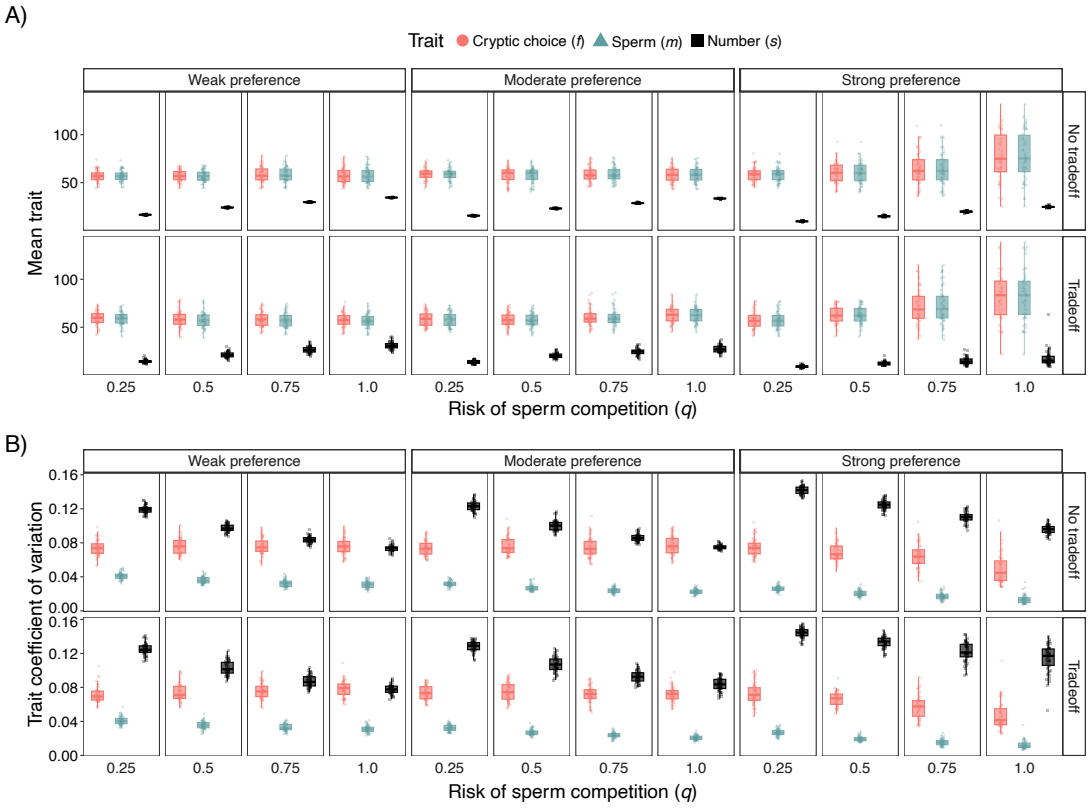

Figure 3.1: Strong selection and high risk of sperm competition results in higher trait divergence but lower trait variation within populations. (A) Box plots and jittered points of population average of cryptic choice (f; left red circles), sperm trait (m; center blue triangles), and sperm number (s; right black squares) at generation 30,000. (B) Box plots and jittered points of within-population coefficient of variation of all traits at generation 30,000. Similar graphs at other parameter combinations can be made on the SI web app

3.4.2 Strong cryptic female choice results in less overall ejaculate investment.

To understand whether cryptic female choice causes deviations from previous theory, we compared the evolutionary stable ejaculate investment from simulations of cryptic female choice to the analytical model with sperm competition only. Our

analytical model predicts that sperm number (s) should increase with risk of sperm competition. We found that with cryptic female choice when there was a tradeoff between sperm trait (m) and s, s only increased with the risk of sperm competition when comparing populations with similar m (Fig.3.2A). This was unique to cryptic female choice as the simulations with sperm competition only matched the analytical model both qualitatively and quantitively (Fig.3.2A).

We found that when preference strength was weak, overall investment was larger than predicted by analytical models regardless of the risk of sperm competition and whether a tradeoff was included (Fig.3.2B). This pattern was also true for simulations with sperm competition only both with and without selection on m (Fig.3.2B; S3.5), indicating that this is a feature of differences in assumptions between the simulations and the analytical model. When preferences were strong, however, it uniquely resulted in much lower ejaculate investment than models with sperm competition only (Fig.3.2B; S3.5). As the risk of sperm competition increased, overall ejaculate investment became closer to the analytical predictions, but was still ~25% lower.

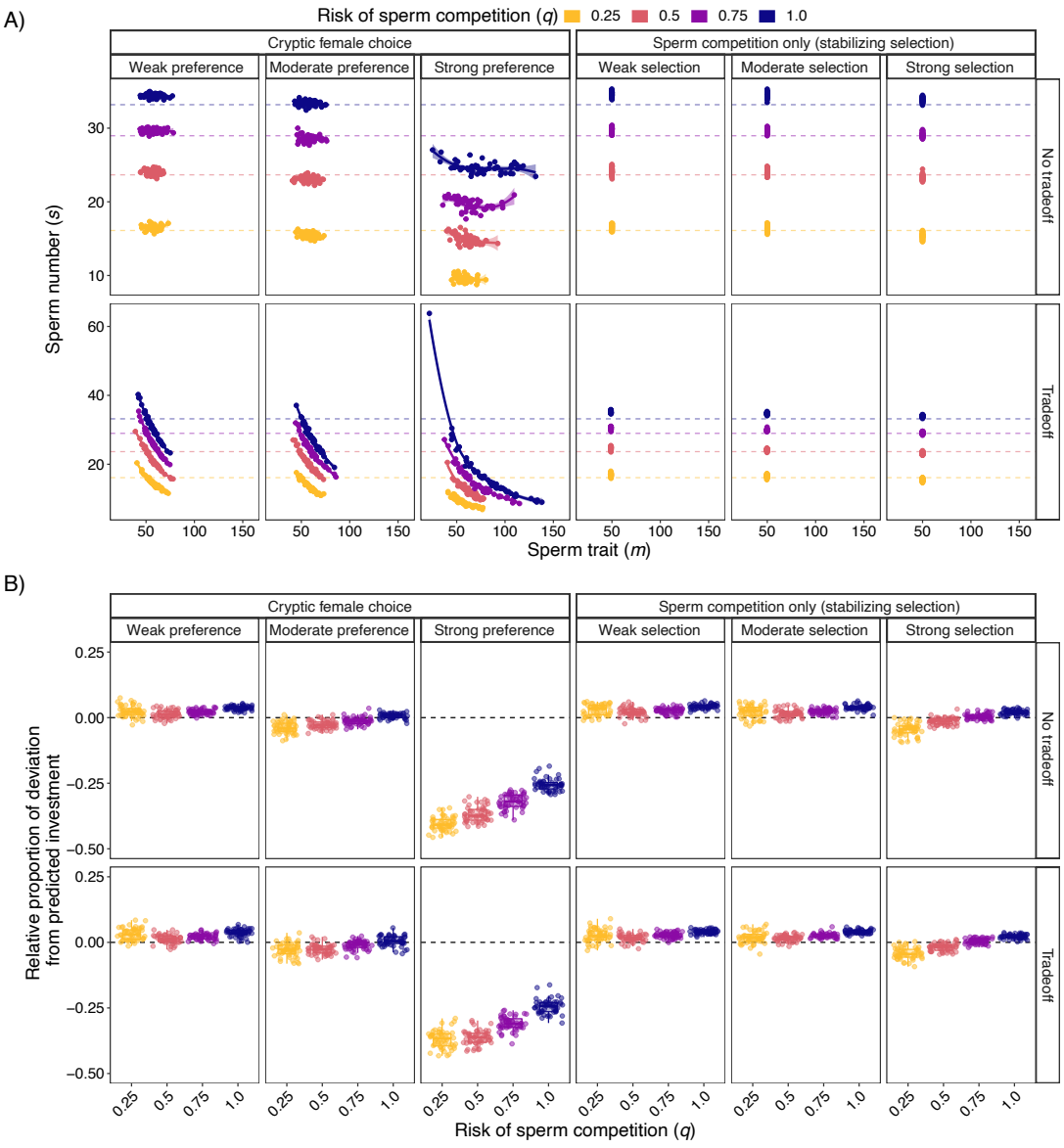
A)
Risk of sperm competition (q) 0.25 0.5 0.75 1.0
Cryptic female choice
Sperm competition only (stabilizing selection)
Weak preference
Moderate preference
Strong preference
Weak selection
Moderate selection
Strong selection
No tradeoff
Tradeoff
Sperm number (s)
Sperm trait (m)
B)
Relative proportion of deviation from predicted investment
Risk of sperm competition (q)

Figure 3.2: Cryptic female choice can result in less ejaculate investment than models with sperm competition only. (A) Scatter plots of average population sperm trait (m) and sperm number (s) at generation 30,000 with dashed lines indicating predicted sperm number from the game theory model (x_e). Line shown is the best fit local polynomial regression ("LOESS" function) between m and s. Similar graphs at other parameter combinations can be made on the SI web app. (B) Box plots and jittered points of the population average relative deviation of simulations at generation 30,000 compared to the analytical model's predicted investment ($\frac{simulationinvestment-predictedinvestment}{predictedinvestment}$). Values at the black dashed line indicate that a simulation exactly matched the game theory model prediction; values above the line indicate more investment than predicted; values below the line represent lower investment than predicted. Simulations of sperm competition only are from simulations with stabilizing selection, fair raffle results are shown in Fig. S3.5.

3.4.3 Cryptic female choice results in correlated trait evolution even when female preference is weak and risk of sperm competition is low.

We found a positive genetic correlation arose and was maintained between the cryptic female choice trait (f) and sperm trait (m) across all model scenarios with cryptic female choice (Fig.3.3). Genetic correlations did not develop for models with sperm competition only (SI web app). Within a few generations, genetic correlations became positive, indicating f and m loci entered linkage disequilibrium (Fig.3.3A). This genetic correlation peaked within the first 200 generations, then later declined and stabilized by 10,000 generations (Fig.3.3A; SI web app). The genetic correlation between f and m increased with preference strength and the risk of sperm competition. There was an interaction such that the effect that the risk of sperm competition had on genetic correlations increased with preference strength (Fig.3.3A). These patterns still

held when starting f was much larger than m and there was net directional selection on m (Fig. S3.6,7). The genetic correlations resulted in the codivergence of f and m (Fig.3.3B). Populations with a tradeoff that were not under strong preferences drifted along a line slightly below a line of perfect correlation (Fig.3.3B).

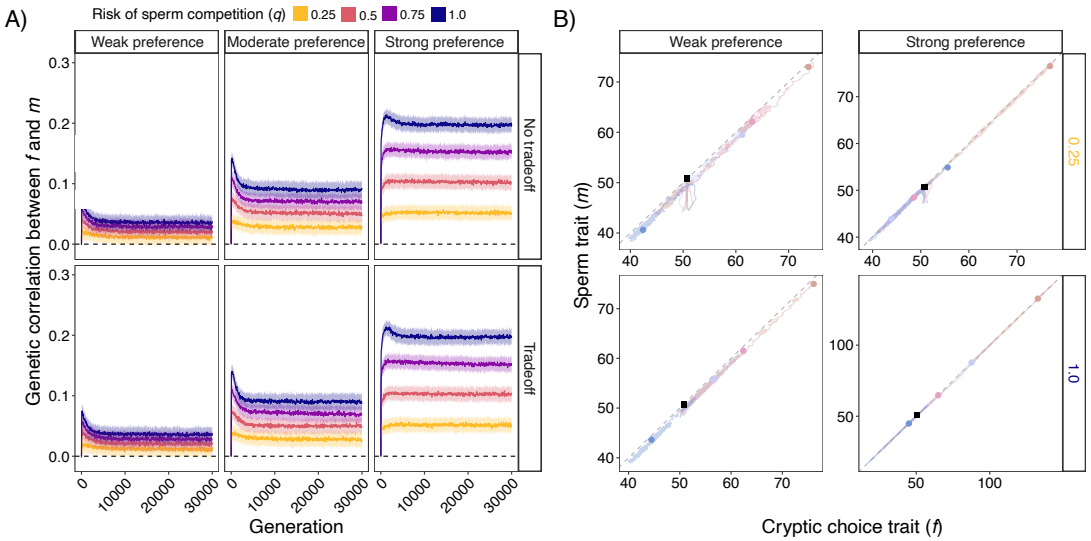

Figure 3.3: Cryptic female choice results in coevolution even with weak selection and low risks of sperm competition. (A) Genetic correlations between cryptic female choice trait (f) and sperm trait (m) evolve within the first 200 generations and are maintained due to linkage disequilibrium. Black dashed line is at zero representing no correlation. Lines represent mean and bands represent standard deviation of 50 populations (separate runs) at each parameter combination. (B) When looking across populations, average f and m are highly correlated. Shown are the highest, lowest, and two random population trajectories of average f and m when there was a tradeoff and for different preference strengths (weak, strong) and risks of sperm competition (1.0, 0.25). Black dashed line represents a perfect correlation; black square represents starting values; circle dots represent the population ending point after 30,000 generations with different colors representing different populations. Note that the axes differ for the different subpanels. Only every 50 generations are shown due to computer memory constraints when plotting. Similar graphs at other parameter combinations can be made on the SI web app.

3.4.4 Cryptic female choice results in a greater deviation from the "optimal" sperm trait predicted by sperm competition only

.

To understand the degree of trait matching we might see empirically, we tested for deviations between sperm traits and their "optimal" value—where fertilization would be highest when not considering sperm number or mating success. For both cryptic female choice and sperm competition only stabilizing selection simulations, when a tradeoff with sperm number (s) was present, the deviation of the sperm trait (m) from its optimum was lowest (~1 lower) with weak selection/preference and only slightly lower (~0.25 lower) for moderate selection/preference (Fig. 4A). When selection/preference on m was strong, m did not deviate much from this optimum, indicating selection for m overpowered selection on s. For simulations with sperm competition only, the range of deviations from the realized optimum (i.e., range of m) decreased with increasing selection strength but were not affected by the risk of sperm competition. For cryptic female choice simulations, however, there were sizable deviations from the optimum even with strong preferences (Fig. 3.4A). These deviations were not influenced by the risk of sperm competition.

We tested if differences in deviations were due to differences in the realized strength of selection (gamma quadratic selection coefficient; Lande and Arnold, 1983). We found that gamma became more negative (stronger realized selection) with increasing risk of sperm competition and preference strength ($\frac{1}{\omega}$). There was an interaction such that the effect the risk of sperm competition had on gamma increased with the strength of preference (Fig.3.4B). When preference was weak or moderate, the sperm competition

only and cryptic female choice simulations had similar gamma estimates despite cryptic female choice simulations having larger deviations in these scenarios (Fig.3.4B). However, when selection was strong, simulations with only sperm competition had larger absolute gamma estimates than cryptic female choice simulations.

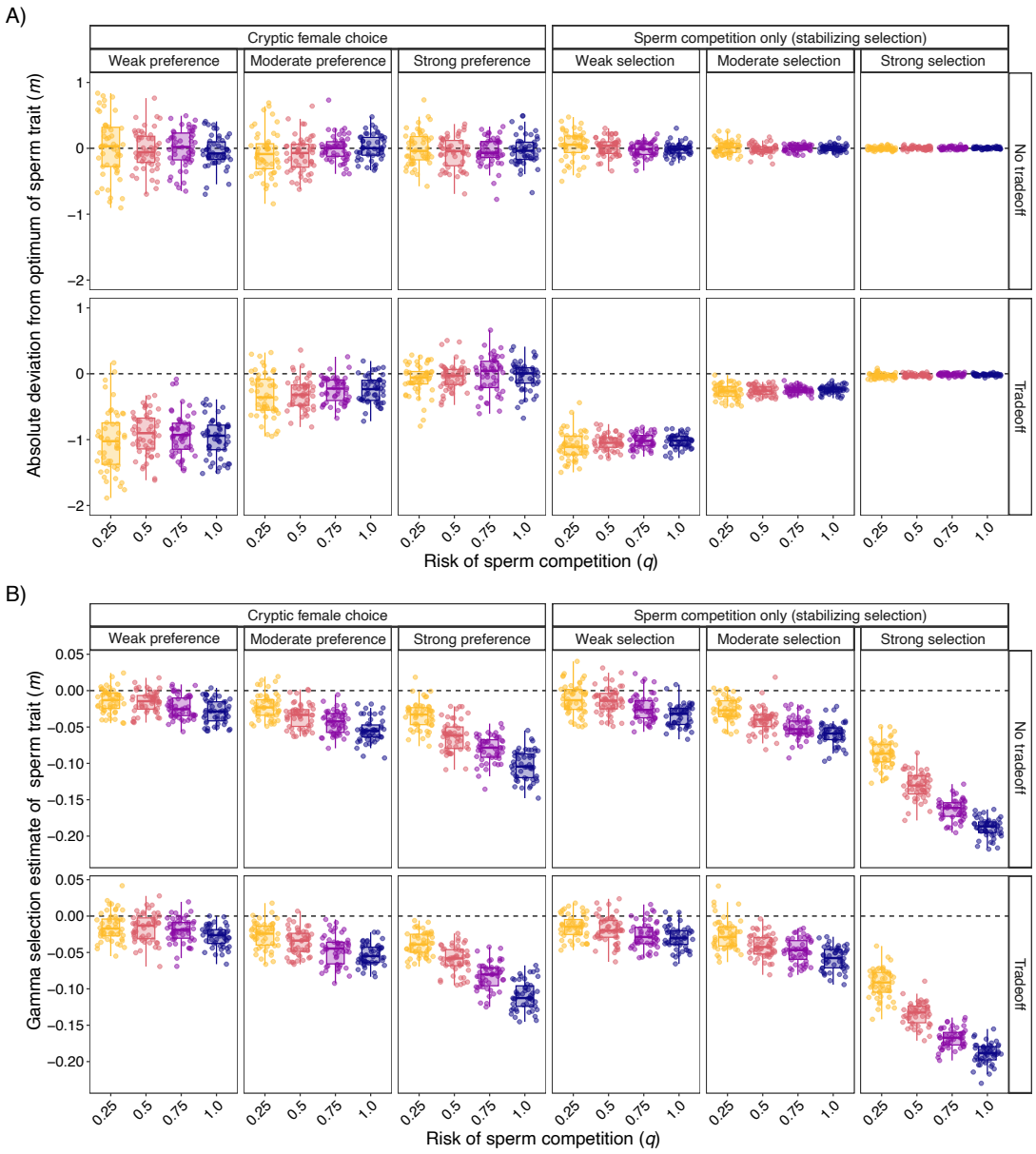

Figure 3.4: Cryptic female choice results in more deviation from sperm trait optimum than sperm competition only. (A) Box plots and jittered points of simulation deviation from "optimal" sperm trait value (m; the value where fertilization is maximized when only considering m) at generation 30,000. For cryptic female choice, deviation was calculated by subtracting mean m from mean cryptic female choice trait (f). For sperm competition only, deviation was calculated by subtracting mean m from 50, the optimum set during those runs. Black dashed line indicates zero or no deviation from the optimum. (B) Box plots and jittered points of gamma quadratic selection estimates of m after 30,000 generations (Lande and Arnold, 1983). Zero means no quadratic selection (black dashed line), negative values represent stabilizing selection, positive values represent disruptive selection. Coefficient estimates remained stable after 10,000 generations. Similar graphs at other parameter combinations can be made on the SI web app.

3.4.5 Sperm trait evolution lags cryptic choice trait evolution.

We next tested if deviations between cryptic choice (f) and sperm trait (m) could be explained by lags in evolution. We found that regardless of scenario, incorporating an evolutionary lag of m improved fit (Fig.3.5). The length of these generational lags decreased with increasing risk of sperm competition and preference strength (Fig.3.5A). Further, the relative improvement in deviation between traits increased with risk of sperm competition and preference strength (Fig.3.5B).

§ 3.5 Discussion

Despite extensive research on sperm competition and the evolution of sperm traits, little is known about the coevolutionary dynamics between sperm traits and female preferences that exert selection on sperm (Lindsay et al., 2019; Parker and Pizzari, 2010). We found that the correlated evolution of cryptic female choice and sperm traits

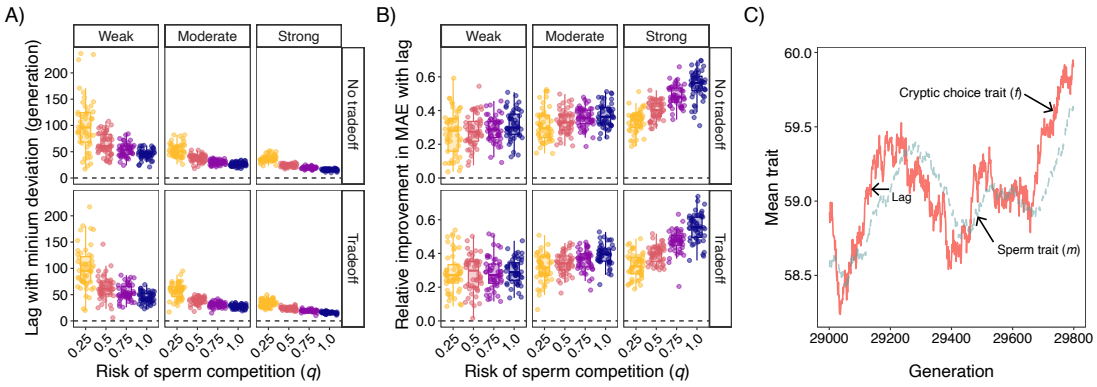

Figure 3.5: Evolution of sperm trait lags cryptic female choice trait and the length of this lag decreases with strength of cryptic female choice and risk of sperm competition..(A) Box plots and jittered points of generational lag that resulted in minimum mean absolute error (MAE) for different replicates during the last 2000 generations of the simulation. Black dashed line is at zero indicating no lag best fit the data. (B) Box plots and jittered points of the relative improvement in MAE of incorporating the best fit generational lag versus no lag. Black dashed line is at zero indicating lag did not improve fit. (C) Line plot of an example population showing how the evolution of sperm trait (m; dashed light blue) lags the evolution of cryptic choice trait (f; solid red). The optimal lag in this example population was 49 generations and is when there was no tradeoff, risk of sperm competition was 0.25, and selection was strong.

could occur even when there was a tradeoff between sperm trait and number, selection on sperm traits was weak, and the risk of sperm competition was low. We also found that strong cryptic female choice results in males investing less in their ejaculate than predicted by models without cryptic female choice.

Most post-mating theory focuses exclusively on male-mediated processes. Thus, it is essential to understand how incorporating cryptic female choice can result in deviations from existing expectations. When selection via non-directional cryptic female choice was strong, we find males evolved to invest less in their ejaculates than when there was sperm competition only. This counterintuitive result may arise because there is no "best" male trait when the outcome is dependent on female-by-male interactions.

Since the sperm trait that maximizes fertilization success depends on female traits, it is likely advantageous to invest more resources toward gaining mating opportunities even when the risk of sperm competition is high. Less investment came at the cost of sperm number since tradeoffs did not limit the extent to which the sperm trait could exaggerate, as the largest sperm trait values evolved under strong selection (Fig.3.1A). This fits well with empirical work on the Drosophila genus, where cryptic female choice is known to occur, with some species producing extremely large but very few sperm (Immler et al., 2011; Lupold et al., 2016). Although the qualitative prediction that ejaculate investment increases with the risk of sperm competition remained true for a given preference strength, species likely differ in preference strength. Thus, failing to account for preference strength may cause qualitative predictions to no longer hold. For example, our model predicts that overall investment for a species with strong cryptic female choice and a risk of sperm competition = 1 would have as high of a sperm number as a species with moderate cryptic female choice and risk of sperm competition = 0.5. These results highlight the importance of better characterizing the actual strength of cryptic preferences in different species. Without taking this into account, many previous theoretical predictions (i.e., sperm number increases with risk of sperm competition) may be inaccurate.

Establishing and maintaining a genetic correlation between female preferences and male traits is essential for the pre-mating Fisher Process (Fisher, 1930; Henshaw and Jones, 2020; Kirkpatrick, 1982; Lande, 1981), but under what scenarios can these develop with post-mating traits and what factors influence their magnitude? We found that within-population genetic correlations between m and f increased with preference strength and risk of sperm competition. There was an interaction such that the effect the

risk of sperm competition had on genetic correlations increased with preference strength (Fig. 3A). Although the magnitude of genetic correlations was generally small (< 0.05) when the risk of sperm competition was low with weak preferences, it still resulted in large phenotypic divergence between replicates (Figs. 3.1A; 3.3B). The resulting phenotypic coevolution of the cryptic female choice trait and the sperm trait predicted by our model is consistent with comparative evolutionary studies documenting the co-diversification of the female reproductive tract and sperm morphology (Higginson et al., 2012; Pitnick, Wolfner, and Suarez, 2009; Pitnick et al., 2020) and female and male genitalia (Evans et al., 2013; Simmons and Fitzpatrick, 2019). Divergence between populations could be aided by female variation being more than twice as large as male variation regardless of modeling scenario. Our results imply that post-mating intersexual selection could be an under-appreciated evolutionary force driving reproductive divergence and isolation, even in systems with rare multiple mating. We think it is important for future empirical efforts to look for evidence of cryptic female choice in systems with low to moderate rates of multiple mating.

When designing empirical studies, it is important to know what deviations between coevolving traits we might expect and the biological reasons for these deviations. Logically, we might predict that the amount of deviation between male and female traits important in cryptic female choice will be negatively correlated with the strength of preference and the risk of sperm competition. Our results (Figs.3.4;3.5), however, predict that deviations between male and female post-mating traits are likely to exist and not correlate with preference strength or the risk of sperm competition (Fig.3.4A). The deviations observed are in part a result of time-lags in male sperm trait tracking a co-evolving female trait and not just a lower realized selection strength than sperm

competition only models (Figs.3.4;3.5). The lower realized selection could be due to variation in female traits (Cramer et al., 2023). Incorporating a generational time lag improved correspondence between male and female traits, especially when preferences were strong and risk of sperm competition was high. To increase the likelihood of detecting cryptic female choice, we suggest using individuals from a wide range of populations for both experimental and comparative work to have sufficient trait variation. Further, the importance of time lags in understanding the coevolution of these traits means that analyzing long-term data sets of population traits will also help with the detection of cryptic female choice.

Our modeling framework provides a strong starting point for future cryptic female choice models and future work should relax some of our assumptions. For simplicity, we only considered a single pair of coevolving traits and sperm number, however, the actual number of traits that may be interacting with one another is much higher (Lüpold, Boer, et al., 2020; Pitnick et al., 2020; Snook, 2005). Future models could incorporate multiple traits that may be simultaneously under both intra- and inter- sexual selection. We also assumed that preferences and traits were unrelated to fecundity or survival, which impacts pre-mating sexual selection models (Kirkpatrick and Ryan, 1991; Kuijper et al., 2012). Relaxing these assumptions with a good-genes approach could allow positive covariation between pre-mating and post-mating success (see Mautz et al., 2013) and potentially allow for the evolution of costly preferences. In our model, we focused on non-directional cryptic female choice and chose a stabilizing closed preference function to model these female-by-male interactions. Modeling directional cryptic female choice with an open preference function could alter some of the predictions like it does in pre-mating sexual selection models (Jennions and Petrie, 1997; Millan et al., 2020). It

would also be interesting to explore sexual conflict (Brennan and Prum, 2015), evolution of negative correlations (Simmons and Kotiaho, 2007), mate order effects (Parker and Pizzari, 2010), the evolution of polyandry (e.g., sexy sperm hypothesis Bocedi and Reid, 2015; Keller and Reeve, 1995; Yasui, 1997), and preference strength. We also assumed that population size and sex ratio were constant; incorporating eco-evo dynamics to relax these assumptions would be an interesting extension. Finally, future work should explicitly explore the degree to which codivergences generated by cryptic female choice can cause reproductive isolation given previous theory showing that pre-mating sexual selection alone can often be ineffective (Irwin, 2020; Servedio and Bürger, 2014). Such a model could allow fitness costs associated with divergence in male and female traits similar to Lorch and Servedio (2007) and Rushworth et al. (2022), which modeled the evolution of conspecific gamete precedence, post-mating-prezygotic incompatibilities and reinforcement.

We demonstrate that incorporating cryptic female choice results in strong deviations from predictions based on models that focused exclusively on male-mediated processes. We also find that the strength of selection/preference and the risk of sperm competition often have interactive effects, something that most previous theory and comparative work do not consider. Further, we find that coevolution between female and male traits occurs even with weak cryptic preferences and low rates of multiple mating. Our results highlight the importance of considering cryptic female choice in understanding the evolution of male traits, the need to develop further theory on cryptic female choice, and the importance of conducting more empirical research, especially on the strength of selection arising from cryptic female choice.

§ 3.6 Acknowledgements:

We thank B. Lyon, B. Sinervo, D. Weiler, J. Fitzpatrick, L. Alissa, M. Gamble, M. Molinari, M. Glasenapp, M. Servedio, S. Beyer, and anonymous reviewers for helpful feedback that greatly improved this manuscript. This work was supported by the US National Science Foundation via a GRFP award to M.C.K (Award number: DGE-1842400), an ARCS fellowship award to M.C.K., and a National Science Foundation grant awarded to S.H.A (Award number: IOS-1655297). The authors declare no conflicts of interest.

§ 3.7 Author Contributions

MCK and SHA designed the modeling framework. MCK wrote the model script and ran analyses on the resulting data. MCK drafted the manuscript and SHA critically revised the manuscript. Both authors approve of the publication of this article.

§ 3.8 Data Accessibility

Data and code to run the model and make figures are deposited in the Dryad Digital Repository: https://doi.org/10.7291/D1310W.

Chapter 4

Conspecific sperm precedence can maintain reproductive isolation

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§ 4.1 Abstract

Sexual selection has long been considered an important mechanism of speciation. Despite growing empirical evidence that postmating sexual selection—selection on traits that affect fertilization—is common, most speciation theory has focused on premating sexual selection. Postmating sexual selection can result in assortative fertilization, i.e., conspecific sperm precedence—a process where fertilization is biased towards conspecific males during sperm competition. Although there is empirical evidence of conspecific sperm precedence in a wide range of taxa (plants, fish, birds, insects, mollusks, and echinoderms), there is little theory on conspecific sperm precedence, limiting our understanding of how it contributes to speciation. We use simulation models

of secondary contact to ask under what circumstances conspecific sperm precedence is effective at maintaining reproductive isolation (and therefore contributing to species divergence and maintenance). We found that conspecific sperm precedence alone can maintain reproductive isolation under limited but realistic conditions, specifically when the migration rate is low, conspecific sperm precedence is strong, and multiple mating is intermediate. When found in combination with ecological divergence, conspecific sperm precedence was able to maintain reproductive isolation even at high rates of migration. We also found that divergence in cryptic preferences could evolve through reinforcement (selection on divergent preferences due to less fit hybrids). Our results demonstrate that conspecific sperm precedence could maintain reproductive isolation and, therefore, can contribute to species divergence and maintenance. We also suggest that further research on how post-mating sexual selection affects speciation is needed.

§ 4.2 Introduction

Sexual selection is often considered a potent force driving speciation by maintaining reproductive isolation between populations (Coyne and Orr, 2004; Lande, 1981; Ritchie, 2007; Servedio and Boughman, 2017; Shaw et al., 2024). Isolated populations may diverge in traits and preferences, resulting in conspecific assortative mating upon secondary contact. Assortative mating within divergent populations can act as a premating prezygotic barrier, resulting in reproductive isolation, and ultimately speciation (Servedio and Boughman, 2017; Shaw et al., 2024). There is also growing empirical evidence that postmating sexual selection—selection on traits affecting fertilization after mating—may play an important role in speciation (reviewed in Garlovsky et al., 2023; Howard et al., 2009). Yet, almost all theory on speciation has ignored the role of postmating sexual selection (but see; van Doorn et al., 2001; Lorch and Servedio, 2007).

Postmating sexual selection likely drives the rapid evolution of specific reproductive proteins (McDonough et al., 2016; Palumbi, 2009; Swanson and Vacquier, 2002; Wilburn and Swanson, 2016) and quantitative traits (e.g., female reproductive tracts and sperm morphology; Briskie et al., 1997; Kahrl, Kustra, et al., 2021; Lupold et al., 2016; Lüpold and Pitnick, 2018; Pitnick, Wolfner, and Suarez, 2009; Pitnick et al., 2020; Simmons and Fitzpatrick, 2019). Sperm are one of the most diverse cell types across taxa and show rapid divergence between populations of the same species (Hogner et al., 2013; Lifjeld et al., 2023; Lüpold and Pitnick, 2018; Manier and Palumbi, 2008; Pitnick et al., 2003). Moreover, comparative evolutionary studies have documented the co-diversification of the female reproductive tract and sperm morphology (Briskie et al.,

1997; Higginson et al., 2012; Pitnick, Wolfner, and Suarez, 2009; Pitnick et al., 2020) and male and female genitalia (Evans and Sherman, 2013; Simmons and Fitzpatrick, 2019). This co-divergence of reproductive proteins and traits can result in postmating prezygotic reproductive isolation barriers—i.e., reproductive barriers that occur after mating but before fertilization (Coyne and Orr, 2004; Garlovsky et al., 2023).

Postmating prezygotic reproductive isolation barriers can occur as a reduction in either fertilization success or female fecundity during a heterospecific mating (competitive or non-competitive reviewed in Garlovsky et al., 2023). Postmating prezygotic reproductive isolation can also occur in a competitive setting without any intrinsic reduction in fertility or fecundity via conspecific sperm precedence. Conspecific sperm precedence (also referred to as conspecific gamete precedence) is when conspecific males have higher fertilization success than heterospecific males when the sperm of these males compete to fertilize the eggs of one female. This is likely driven by cryptic female choice, i.e., females biasing fertilization towards conspecific males either through direct actions or due to conspecific sperm being better competitors in the conspecific female's environment (e.g., reproductive tract or reproductive fluid). Conspecific sperm precedence is widespread across taxa in both animals and plants (reviewed in Garlovsky et al., 2023). In *Drosophila*, conspecific sperm precedence evolves at a similar rate to premating isolation, both of which are faster than postzygotic isolation (Turissini et al., 2018). In some species, conspecific sperm precedence is present when premating reproductive isolation is not (*Poecilia fish* Devigili et al., 2018; Ludlow and Magurran, 2006; *Allonemobius* crickets Howard et al., 1998; or not possible, e.g., broadcast spawners *Echinometra* urchins Geyer and Palumbi, 2005, and plants *Solanum* tomatoes Baek et al., 2016). Further, even in species with existing premating isolating barriers, there

is evidence for conspecific gamete precedence, such as in birds (*Ficedula* flycatchers Cramer et al., 2016 and fish with external fertilization *Salmo* salmonids Yeates et al., 2013). The existence of conspecific sperm precedence is generally interpreted in the literature as evidence for cryptic female choice playing an important role in speciation. However, previous theory on premating sexual selection has demonstrated that the relationship between female choice and speciation is complex (Irwin, 2020; Servedio and Boughman, 2017; Servedio and Bürger, 2014; Weissing et al., 2011).

For example, previous theoretical work on premating sexual selection suggests that female choice in the face of gene flow can be ineffective or even impede reproductive isolation between incipient species (Irwin, 2020; Servedio and Boughman, 2017; Servedio and Bürger, 2014; Weissing et al., 2011). Theoretical predictions about the effectiveness of cryptic female choice at maintaining reproductive isolation may deviate from premating sexual selection models for a few reasons. First, most sexual selection models assume that females sample the entire population when making a mating choice (albeit sometimes at a cost). For conspecific sperm precedence, selection only occurs among males mating with the same female. Second, traits involved in postmating sexual selection (e.g., sperm morphology) are less likely to be under viability selection than premating traits (e.g., coloration). Despite these fundamental differences, only a few theoretical models on conspecific gamete precedence have been developed (e.g., van Doorn et al., 2001; Lorch and Servedio, 2007). van Doorn et al. (2001) focused on protein recognition in broadcast spawners and assumed that all sperm in the population competed with one another. Lorch and Servedio (2007) focused on how conspecific gamete precedence coevolved with premating isolation with a deterministic population genetics model. They assumed the females always mated with exactly two males.

However, multiple mating rate varies empirically and is a parameter often explored in existing postmating sexual selection models (reviewed in Parker and Pizzari, 2010). Thus, we have a limited understanding of the importance of multiple mating rate in maintaining reproductive isolation. The development of new theory is needed to help our understanding of if and when conspecific sperm precedence can promote speciation by maintaining reproductive isolation between incipient species upon secondary contact.

A previous postmating sexual selection model focused on cryptic female choice demonstrated that isolated populations can rapidly codiverge in cryptic preferences (e.g., sperm storage tubule) and male sperm traits (e.g., sperm size Kustra and Alonzo, 2023). These results imply that cryptic female choice could generate reproductive isolation via conspecific sperm precedence. However, in the face of migration, these divergences could collapse for a few reasons: (1) there is no direct selection acting on migrating females, which means preferences could homogenize between the two populations, and (2) conspecific sperm precedence is only effective if a female mates with both a heterospecific male and conspecific male. Thus, it is unclear if conspecific sperm precedence via divergence in cryptic preferences will enable speciation by restricting gene flow upon secondary contact between two incipient species.

Here, we use a similar individual-based model as Kustra and Alonzo (2023) to test how effective conspecific sperm precedence is at maintaining reproductive isolation between two populations that diverged in allopatry and are now in secondary contact. We modelled three scenarios. First, when there was only initial divergence in cryptic preferences and sperm traits but no divergent ecological selection. Second, when there was only divergent ecological selection and initial divergence in the associated

ecological trait (e.g., beak size). Third, when there were both divergences in ecological optima (and associated trait) and cryptic preferences and sperm traits. We then tested how migration rate, the probability females mate with more than one male (i.e., the risk of sperm competition), the number of males with which females mate (i.e., the intensity of sperm competition), and the strength of conspecific sperm precedence influenced the degree of reproductive isolation maintained during secondary contact. We looked at the amount of gene flow that occurred in neutral loci (i.e., admixture) and the evolution (or maintenance) of divergence in cryptic preferences and sperm traits. Together these analyses allow us to ask how conspecific sperm precedence contributes to speciation both in isolation and when combined with ecological divergence.

§ 4.3 Methods

4.3.1 Model overview

We use an individual-based model to test the effectiveness of conspecific sperm precedence at maintaining reproductive isolation upon secondary contact. Individuals in our model are sexually reproducing and diploid. We keep track of four traits: a sperm trait (t; e.g., sperm size), a cryptic female preference trait (f; e.g., sperm storage tubule length), sperm number (s), and an ecological trait (ϵ) that influences the survival of both sexes (e.g., beak size for foraging). Each trait was positive, continuous and was determined additively by 20 physically unlinked loci. To keep track of the amount of admixture that occurs during secondary contact, individuals also had 20 physically unlinked neutral population markers coded as zero or one, representing the ancestral population of origin. This allowed us to track the proportion of their neutral genome

that originated from the different populations. We assumed there were no mutations, the standard deviation (SD) of all traits was initialized at 2.5 (similar to stabilized variation in Kustra and Alonzo, 2023), and the initial average trait values depended on the secondary contact scenario (described below).

To simulate secondary contact, we assumed a two-island model in which two populations (A and B) have evolved in complete allopatry. During secondary contact, there is limited symmetrical migration between the two populations. We modeled three possible starting scenarios of secondary contact: (1) there is no ecological divergent selection, but cryptic preferences and associated sperm traits have diverged; (2) there is ecological divergent selection, but cryptic preferences and associated sperm traits have not diverged; (3) there is ecological divergent selection, and both cryptic preferences and associated sperm traits have diverged. Viability and sexual selection still occur in each scenario, even if there is no initial divergence. When there was cryptic preference (f) and sperm trait (t) divergence, we assumed that the average starting preference and sperm trait was ~40 arbitrary units for Population A and ~80 for Population B. Trait values could take on any positive value bounded by initial variation in starting loci because we assumed no mutation. These values were chosen as this level of divergence between isolated populations was commonly observed in Kustra and Alonzo (2023). When there was no divergence in cryptic preferences, both populations and traits/preferences were initialized at ~40 arbitrary units. We modeled two scenarios, one where sperm number (s) and sperm trait (t) trade off with one another ("tradeoff") and where they did not trade off with one another ("no tradeoff"). We did this because not every sperm trait might trade off with sperm number, for example if conspecific sperm precedence was arising from chemical interactions not differences in morphology (Devigili et al., 2018; Yeates

et al., 2013), this likely would not directly impact the number of sperm a male could produce. Initial values of sperm number (s) were set based on evolutionary stable sperm number from single population scenarios given the risk/intensity of sperm competition, strength of cryptic preferences, and presence/absence of a tradeoff with sperm trait (t). For simplicity, when there was ecological divergent selection, we assumed populations were already adapted to their environment (e.g., near each population's respective optima for the ecological trait). To match the level of divergence in sperm traits and cryptic preferences, when there was ecological divergent selection, we set the ecological trait optima (and starting trait value) at 40 for population A and 80 for Population B. When there was no ecological divergent selection, the ecological trait (ϵ) optima and the starting average value of the ecological trait was set at 60.

We assumed non-overlapping generations with the following lifecycle. First, symmetrical migration of both sexes occurs at rate m, followed by stabilizing viability selection on the ecological trait where only half the population size survives (see *Viability selection*). Surviving individuals then enter the mating pool where premating sexual selection occurs (see *Premating sexual selection*). If a female mates with more than one male, then postmating sexual selection occurs (see *Postmating sexual selection*). Since the potential number of matings per male was unrestricted, males were assumed after a mating to experience ejaculation depletion with no recovery at a constant rate of c: $s' = se^{-c}$. We assumed no fecundity selection and that females produce two male and two female offspring to keep the population size stable for the next generation. We then repeat this process for 3,000 generations as preliminary analyses showed that populations stabilized by 2,000 generations. See Figure 4.1 for a summary of the model and significant assumptions of the model. For each parameter combination, we ran

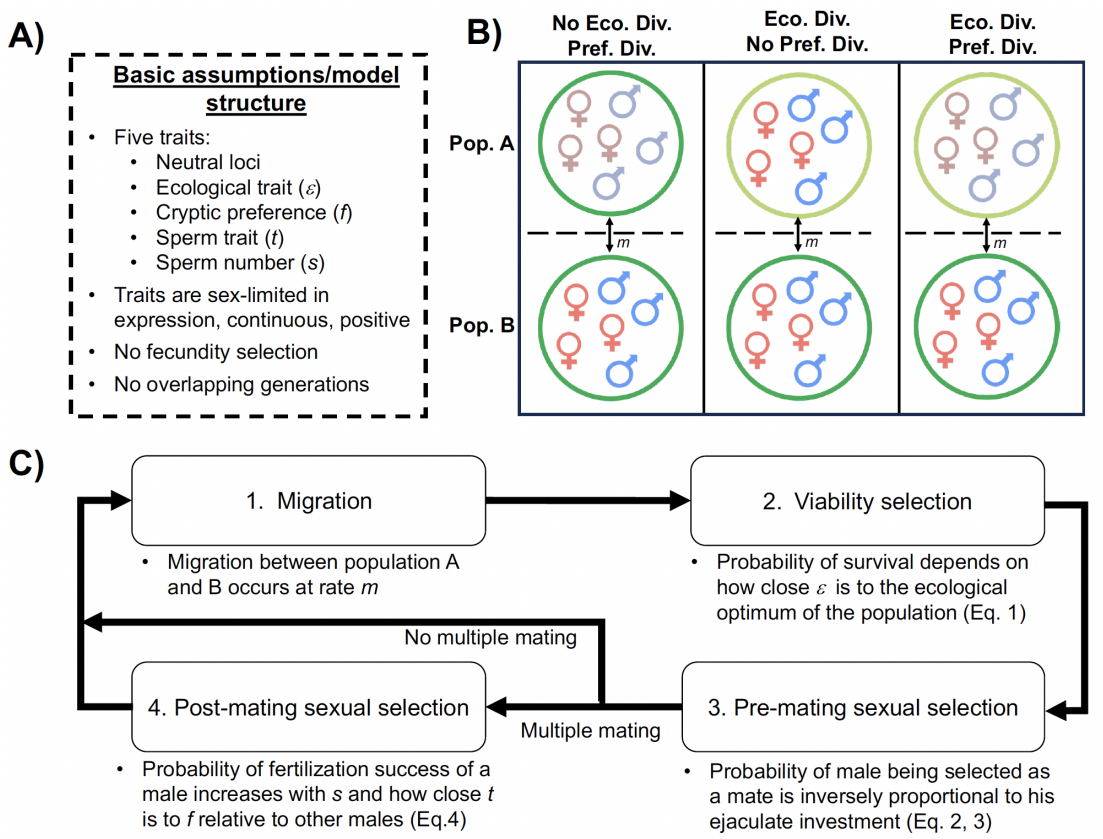

Figure 4.1: Summary of model. (A) Basic assumptions and description of traits in the model. (B) Visualization of the different secondary contact scenarios explored in this model. Specifically, (left) there is no ecological divergent selection (same colored green circles), but cryptic preferences and male traits have diverged between populations A and B (different shades of blue and red symbols); (middle) there is ecological divergent selection (different shaded green circles), but cryptic preferences and male traits have not diverged between populations A and B (same shade of blue and red symbols); and (right) there is both ecological divergent selection (different shaded green circles), and cryptic preference divergence between populations A and B (different shades of blue and red symbols). Cryptic female choice/sexual selection was present even when there was no initial divergence in cryptic preferences. (C) Main stages of the model.

simulations 50 times.

We ran all models using Julia v1.9.2 (Bezanson et al., 2017) and performed analyses/made figures in R 4.1.2 (R Core Team, 2018) using the "tidyverse" suite of

packages (Wickham et al., 2019). We report a summary of parameters, variables, and values used in this model in Table 4.1.

4.3.2 Viability selection

We modeled viability selection as stabilizing selection on the ecological trait ϵ, such that exactly half of the population survived. The probability $P_s(z_i)$ that an individual z_i survived depended on how well their ecological trait matched the ecological optimum E relative to other individuals in the population such that ω is the strength of selection:

$$P_s(z_i) = \frac{\frac{e^{-(\epsilon_i - E)^2}}{2\omega}}{\sum_{j=1}^{n} \frac{e^{-(\epsilon_j - E)^2}}{2\omega}} \tag{4.3.1}$$

For scenarios with ecological divergent selection, the ecological optimum for populations A and B was set at 40 and 80 arbitrary trait units (e.g., optimal beak size for foraging), respectively. These numbers matched the divergence of cryptic preferences and sperm traits. When there was no ecological divergent selection, the ecological optimum was set to 60. After viability selection, only half of the population survived, with the probability of survival based on equation 4.3.1.

4.3.3 Premating sexual selection

We assumed that premating sexual selection was determined only by male-male competition, and there was no premating female choice. Because there was no premating choice, females did not distinguish between migrants and non-migrants when mating.

Like most sperm competition models, we assume that male premating competitive ability trades off with ejaculation allocation (Parker and Pizzari, 2010). The probability that a female mates with male z_i ($P_m(z_i)$) where n_m is the number of males in a population, α is a shape parameter, and β is the inflection point of the sigmoidal shape for the tradeoff scenario is as follows:

$$P(z_i) = \frac{1 - \frac{1}{1+e^{-\alpha((s_i t_i)-\beta)}}}{\sum_{j=1}^{n_m} 1 - \frac{1}{1+e^{-\alpha((s_j t_j)-\beta)}}} \tag{4.3.2}$$

For the no tradeoff scenario, ejaculate investment is only determined by sperm number (s):

$$P(z_i) = \frac{1 - \frac{1}{1+e^{-\alpha((s_i)-\beta)}}}{\sum_{j=1}^{n_m} 1 - \frac{1}{1+e^{-\alpha((s_j)-\beta)}}} \tag{4.3.3}$$

See the supplemental web app for graphical interpretations of these equations.

4.3.4 Postmating sexual selection

Postmating sexual selection can occur if a female mates with more than one male. We varied either the risk (q_r) or the intensity of sperm competition (q_i). The risk of sperm competition is the probability a female mates with more than one male (between 0 and 1). The intensity of sperm competition is the number of males with which a female mates. A risk of sperm competition of one (i.e., females always mate with more than one male) is equivalent to an intensity of sperm competition of two (i.e., females mate with two males). We varied either the risk or the intensity of sperm competition in our simulations. Although the probability of multiple mating and the degree of multiple

mating when it happened could be varied simultaneously, we treated these as distinct scenarios to align our work with existing sperm competition models (Parker and Pizzari, 2010).

We modeled cryptic female preferences as a closed preference function, meaning that preferences were stabilizing. During a competitive mating with n_c number of males, male z_i and male(s) z_j, male z_i's probability of fertilization $\psi(z_i, z_j)$, is determined by his sperm number (s_i) and sperm trait (t_i), the competitor(s) male's sperm number (s_j) and sperm trait (t_j), the cryptic female choice trait (f) of the female involved in the mating event, and the strength of preference acting on sperm trait (υ):

$$\psi(z_i, z_j) = \frac{s_i e^{\frac{-(t_i - f)^2}{2\upsilon}}}{\sum_{j=1}^{n_c} s_j e^{\frac{-(t_j - f)^2}{2\upsilon}}} \tag{4.3.4}$$

In words, a male's probability of fertilization increases with sperm number s and with how close his sperm trait (t) is to the cryptic preference (f) of the female he mated with relative to that of other male competitors. Smaller values of υ indicate stronger selection. We examined three parameter values, which we refer to as strong conspecific sperm precedence when $\upsilon = 1$, moderate when $\upsilon = 12.5$, and weak conspecific sperm precedence when $\upsilon = 50$. See the supplemental web app for graphical interpretations of this equation when there are only two males in competition.

4.3.5 Selection analysis

To help understand the dynamics driving the results of our model, we kept track of the survival and reproductive success of migrants and non-migrants at each generation.

We also performed multivariate selection analyses to estimate how selection acted on male and female reproductive traits separately by sex. This allowed us to assess the degree to which any divergence in preference was due to drift or emergent selection acting on traits. This information also allows us to determine the source of selection causing patterns of divergence. For all selection analyses, we standardized each trait within a sex to a mean of zero and a standard deviation of one in order to calculate selection coefficients (Lande and Arnold, 1983; Stinchcombe et al., 2008). We ran selection analyses using a few different measurements of relative fitness (W_r), where fitness was standardized by the mean fitness. For female selection analyses, we used the number of offspring produced by their sons as our proxy for fitness. We did this because, in the situation when there was no divergent ecological selection, we would not expect differences in the survival of migrant offspring or differential female reproductive success. Using the proxy allowed us to make similar comparisons across model scenarios. We acknowledge that this estimation of selection could potentially confound sources of selection across generations (see Fitzpatrick and Wade, 2022; Wolf and Wade, 2001). For situations with divergent ecological selection, using number of surviving offspring gave qualitatively similar results. Additionally, we performed a regression analysis to estimate directional selection coefficients (β) and quadratic selection coefficients (γ ; Lande and Arnold, 1983; Stinchcombe et al., 2008):

$$W_r = \beta_f f + \frac{1}{2}\gamma_f f^2 \tag{4.3.5}$$

For male selection analyses, we used the number of offspring before viability selection (e.g., mating and fertilization success). We performed a regression analysis

to estimate directional selection coefficients (β) and quadratic selection coefficients (γ; Lande and Arnold, 1983; Stinchcombe et al., 2008):

$$W_r = \beta_t t + \beta_s s + \frac{1}{2}\gamma_{tt}t^2 + \frac{1}{2}\gamma_{ss}s^2 + \gamma_{ts}ts \tag{4.3.6}$$

Table 4.1: Parameter, variable, and function definitions and corresponding values used in the models. [a]The risk of sperm competition of 1 is equivalent to the intensity of sperm competition of 2. *The first value is for no tradeoff between s and t; the second value is with a tradeoff between s and t. Values were changed to keep the same scale for total investment.

	Symbol	Definition	Values/Equations
Parameters			
	N	Population size	2,000; 10,000
	m	Migration rate (%)	1; 2; 3; 4; 5
	q_r	Probability that a female will mate with more than one male	0.25; 0.5; 0.75; 1[a]
	q_i	the number of males a female mates with.	2[a] -10
	$a*$	Shape parameter for post- and pre- mating tradeoff	1/20; 1/1000
	$b*$	Scale parameter for post- and pre- mating tradeoff	50; 2,500
	E	Optimal ecological trait value	40; 60; 80
	w	Width of optimality function which determines strength of selection on e; lower values result in stronger selection	1
	v	Width of optimality function which determines strength of selection on t; lower values result in stronger selection	50; 12.5, 1.0
	c	Ejaculate depletion rate	-0.2
Variables			
	ϵ	Ecological trait (expressed in both sexes)	Evolves
	t	Sperm trait value (male limited expression)	Evolves
	f	Cryptic preference (female limited expression)	Evolves
	s	Sperm number (male limited expression)	Evolves
Function			
	$P_s(z_i)$	Probability of individual z_i surviving viability selection	Eq. 4.3.1
	$P_m(z_i)*$	Probability of male z_i successfully mating	Eqs. 4.3.2, 4.3.3
	$\psi(z_i, z_j)$	Probability that male $z_i$fertilizes an egg given male competitor z_j	Eq. 4.3.4

§ 4.4 Results

4.4.1 Conspecific sperm precedence can help prevent admixture upon secondary contact

To test how effective conspecific sperm precedence is at preventing gene flow (admixture) upon secondary contact, for each individual, we tracked the proportion of

the neutral genome that originated from either Population A or Population B. We first tested whether conspecific sperm precedence alone (i.e. in the absence of divergent ecological selection) could prevent admixture. We found that at population sizes of 2000, partial reproductive isolation was maintained through 3000 generations only when there was a combination of moderate or strong conspecific sperm precedence, intermediate levels of sperm competition intensity, and weak migration rates (top row of Figure 4.2). However, at population sizes of 10,000 conspecific sperm precedence alone was usually insufficient to prevent admixture in large populations (Figure S4.1; see subsection conspecific sperm precedence alone is more effective at maintaining reproductive isolation in smaller populations). Simulations with partial admixture were stable over the final 2000 generations of the simulations when looking at individual runs (Figure S4.2), indicating gene flow had stopped. We next tested if ecological divergent selection (and initial divergence) with no initial cryptic preference divergence could maintain reproductive isolation. We found that complete admixture occurred at 5% migration (middle panels Figure 4.2). At 4% migration, partial reproductive isolation was maintained when the strength of conspecific sperm precedence was strong and the intensity of sperm competition was high (Figure 4.2). This was due to the rapid evolution of cryptic preferences (f) and correlated evolution of sperm traits (t) (see Cryptic preferences can evolve through reinforcement and prevent gene flow). Simulations with partial admixture were stable over the final 2000 generations of the simulations when looking at individual runs (Figure S4.3), indicating that gene flow was stopped after divergent preferences evolved. At lower migration rates reproductive isolation was maintained (Figure 4.2; S4.1). Finally, we looked at how effective ecological divergent selection in combination with initial cryptic preference divergence was at maintaining

reproductive isolation. We found that reproductive isolation was maintained across most parameter combinations and even with high migration rates (5%; bottom panels Figure 4.2). Interestingly, the presence or absence of a tradeoff between sperm trait (t) and sperm number (s) did not influence results (Figure S4.1). In summary, conspecific sperm precedence can help prevent admixture upon secondary contact, especially in combination with ecologically divergent selection.

4.4.2 Conspecific sperm precedence can maintain initial cryptic preference divergence

Despite admixture in most of the genome, incipient or recently diverged species can still maintain regions of genomic divergence that underly traits under divergent selection (Martin and Wainwright, 2013). We therefore examined when patterns of divergence in neutral loci differ from divergence in cryptic female preference (f) and sperm traits (t). Because the patterns of sperm trait divergence (t) were similar to divergence in cryptic female preferences (Figure S4.4, S4.5), we focus our description below on the predicted patterns of divergence in cryptic preferences. We first tested if divergence in preferences could be maintained without any divergent ecological selection. We found that initial divergence in female preferences was lost when migration was greater than 1% (Figure 4.3). However, at 1% migration, divergence in cryptic female preference traits were maintained when conspecific sperm precedence was strong and at intermediate levels of sperm competition intensity regardless of population size (Figures 4.3; S4.4,S4.5). In other words, although admixture in neutral loci still occurred (Figures 4.2; S4.1), divergence in cryptic preferences (f) and sperm traits (t) was

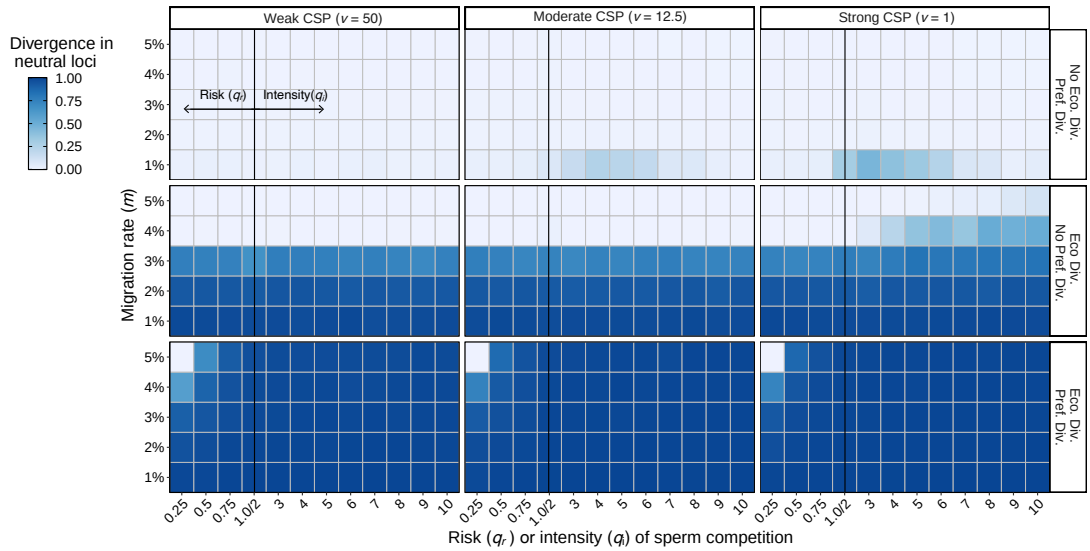

Figure 4.2: Conspecific sperm precedence (CSP) with ecological divergent selection can prevent admixture under most conditions. Heatmap of average divergence in neutral loci after 3000 generations of secondary contact across different scenarios. Divergence in neutral loci is measured as the absolute difference in the proportion of ancestry between two populations experiencing secondary contact. 1.0 would mean no admixture, and 0 would mean complete admixture between the two populations. The black solid line marks the shift between the risk of sperm competition (probability of a single multiple mating; 0.25 to 1.0) and the intensity of sperm competition (number of multiple matings 2 to 10) scenarios. The three panels in the top row are the predicted patterns of divergence when there is no ecological divergence and a starting divergence in cryptic female preference traits. The middle row shows the predicted patterns for no initial divergence in female cryptic preference traits, when there is initial ecological divergence and divergent selection. The bottom row gives the results for the situation where there is divergence in cryptic female preference traits and ecological divergent selection. The three panels at the far left show the results for simulations assuming weak CSP, the middle column moderate, and the far right strong CSP. The simulations shown here assume there is no tradeoff between sperm number and sperm trait; the population size is 2,000. See Figure S4.1 for similar graphs across a broader range of parameter values and biological scenarios.

maintained (Figures 4.3, S4.1, S4.2). We then tested if divergence in cryptic preferences would also be maintained when there was also ecological divergent selection. We found that in this situation, cryptic preferences stayed diverged across most parameter

combinations and even with high migration rates (5%; bottom panels Figure 4.3). Interestingly, the presence or absence of a tradeoff between sperm trait (t) and sperm number (s) did not influence results (Figures S4.4, S4.5). In summary, conspecific sperm precedence can maintain initial divergence of cryptic preferences in more situations than that of divergence in neutral loci.

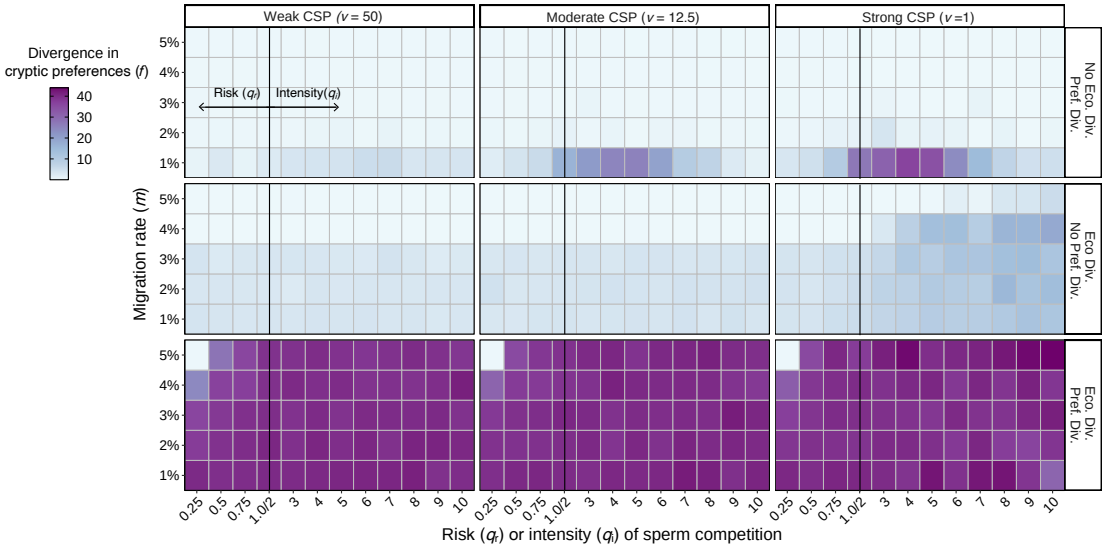

Figure 4.3: Strong conspecific sperm precedence (CSP) can lead to the evolution of divergence in cryptic preferences. Heatmap of average divergence in cryptic female preference traits after 3000 generations of secondary contact across different biological scenarios. The black solid line marks the shift between the risk of sperm competition (probability of a single multiple mating; 0.25 to 1.0) and the intensity of sperm competition (number of multiple matings 2 to 10) scenarios. The three panels in the top row assume no ecological divergence (or divergent selection) and a starting divergence in cryptic female preference traits of 40. The middle row assumes no initial divergence in female cryptic preference traits, but there is initial ecological divergence and divergent selection. The bottom row assumes both divergences in cryptic female preference traits (40) and ecological divergent selection. The three panels at the far left show the results for simulations assuming weak CSP, the middle panels show the results for moderate CSP, and the far-right panels show the results for strong CSP. The simulations shown here assume no tradeoff between sperm number and sperm trait; the population size is 2,000. See Figure S4.2 for similar graphs across a broader range of parameter values and biological scenarios.

4.4.3 Cryptic preferences can evolve through reinforcement and prevent gene flow

Although cryptic preferences can stay diverged in the presence of gene flow (top and bottom rows Figure 4.3), we wanted to know if divergences in preferences can evolve due to selection acting on females to avoid costly heterospecific fertilizations—i.e., reinforcement. We found that when there was ecological divergent selection but no initial cryptic preference divergence, divergence in cryptic preferences still evolved as long as migration rates were lower than 5% (middle panels Figure 4.3; S4.3, S4.4). The divergence in cryptic female preference increased with the strength of conspecific sperm precedence and the intensity of sperm competition (Figures 4.3, 4.4, S4.4). Divergence was also greater at larger population sizes, likely due to selection being more effective relative to drift at larger populations (Figure S4.4). The rate of trait divergence evolution increased with the migration rate (Figure 4.4). This was due to stronger divergent selection acting on cryptic preferences at higher migration rates due to ecological selection against hybrids (Figure S4.6). As correlations (i.e., linkage disequilibrium) between ecological trait (ϵ) and sperm trait (t) increased (Figure S4.7A), so did selection on cryptic preferences (Figure S4.7B). This resulted in greater divergence in cryptic traits (Figure S4.7C), limiting gene flow between the two populations (Figure S4.7D). This resulted in both measures of divergence increasing with the intensity of sperm competition when the strength of conspecific sperm precedence was strong at migration rates of 4% (Figure 4.2, 4.3). When looking at variation between simulation runs, populations that evolved high preference divergence early on prevented gene flow earlier and maintained a higher divergence in neutral loci (Figure 4.5; S4.7). Thus, early

preference divergence was a better predictor of final neutral loci divergence than final preference divergence (Figure 4.5). In summary, cryptic preferences quickly evolved from no initial divergence in the face of high migration rates when there were high rates of multiple mating. Populations that evolved cryptic preferences faster were able to stop gene flow earlier and limit the final amount of admixture in neutral loci.

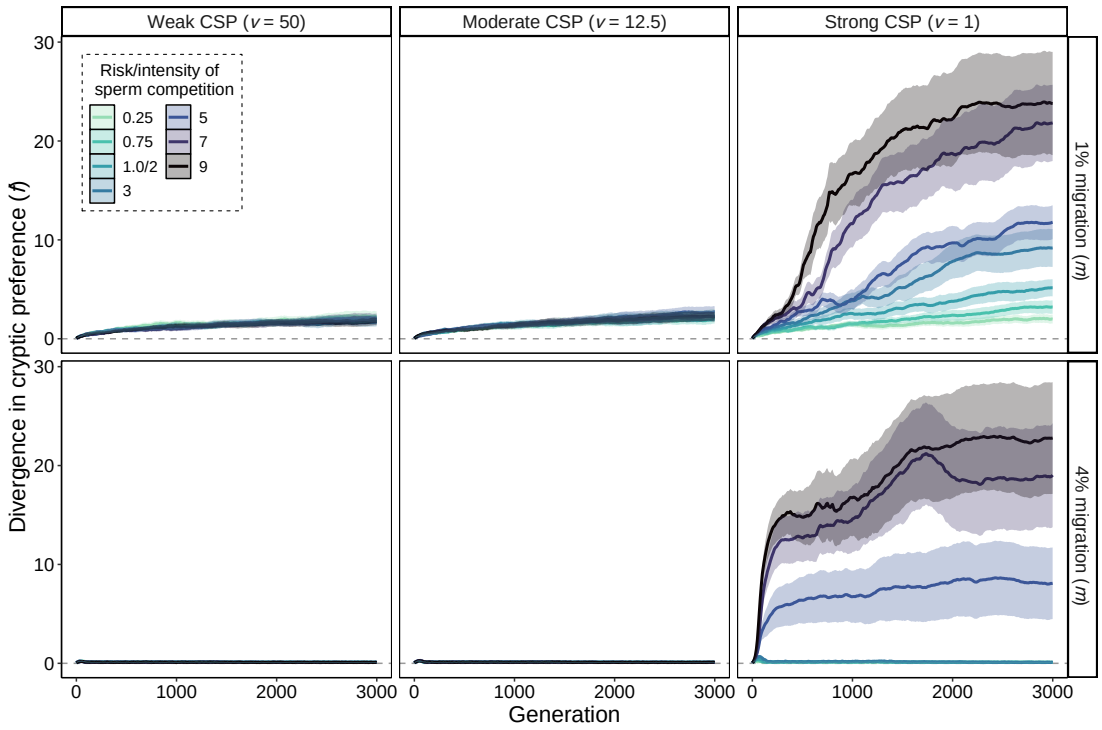

Figure 4.4: Strong conspecific sperm precedence (CSP) and high sperm competition intensity can cause the evolution of divergent cryptic preferences . Lines are the average divergence in cryptic preference over time for when there is ecological divergence but no initial cryptic preference divergence. Shading represents two standard errors in the mean across 50 replicates. The dashed horizontal line indicates an initial divergence of zero. Simulations shown here are when there is no tradeoff between sperm number and sperm trait and at when population size = 10,000.

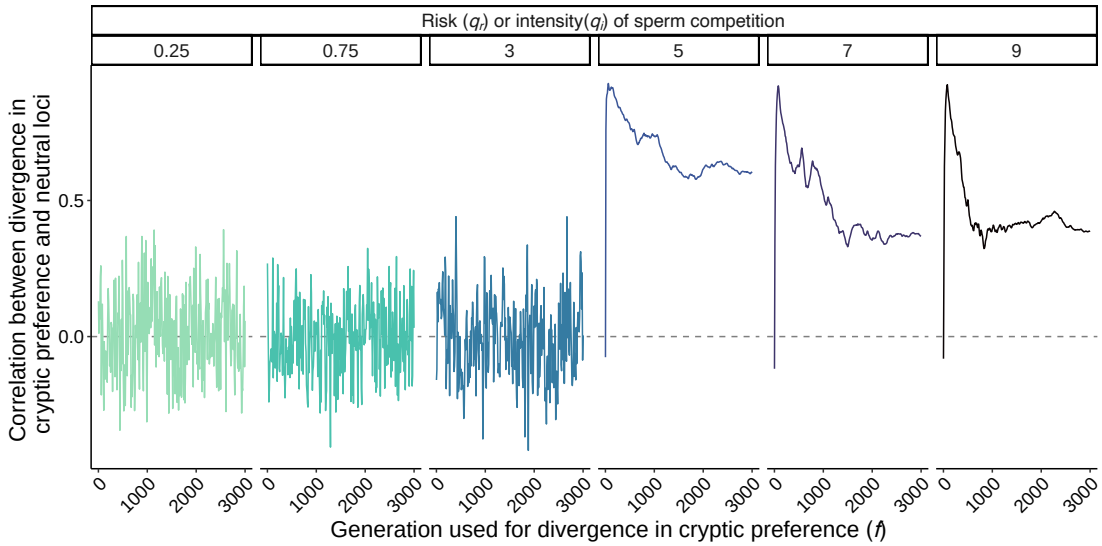

Figure 4.5: Divergence in cryptic preferences early on in secondary contact better predicts stable neutral loci divergence when complete admixture is prevented (intensity of sperm competition ≥ five). No divergence was maintained for risk/intensity of sperm competition of three or less. The line shown is the correlation between divergence in the cryptic female preference at generation x and the divergence of neutral loci at generation 3000. For example, the line at generation 1000 shows the correlation of cryptic divergence at generation 1000 and divergence of neutral loci at the end of the simulation at generation 3000. Results are for no initial divergence in cryptic female preference but with ecological divergence, population size = 10,000, migration rate of 4%, strong conspecific sperm precedence, and no tradeoff between sperm number and sperm trait.

4.4.4 Conspecific sperm precedence alone is more effective at maintaining reproductive isolation in smaller populations

Interestingly, population size only influenced the patterns of reproductive isolation when there was no ecological divergent selection (Figure 4.6; S4.1, S4.4, S4.5). At smaller population sizes (N = 2,000) with moderate and strong strengths of conspecific sperm precedence, conspecific sperm precedence alone prevented complete admixture at sperm competition intensities from 2 to 7, peaking at 3-4 (Figure 4.6A). Divergence

in neutral loci was maintained by cryptic preference divergence (Figure 4.6B). When looking at variation between replicate simulations, the amount of divergence in cryptic preferences maintained upon initial secondary contact correlated with the amount of divergence in neutral loci maintained (Figures S4.8, S4.9). At the highest levels of neutral loci divergence (intensity of sperm competition 3-5), populations that maintained higher preference divergence early on due to stochastic processes prevented gene flow earlier and maintained a higher divergence in neutral loci (Figure S4.8,S4.9). This resulted in early preference divergence better predicting final neutral loci divergence than final preference divergence (Figure S4.9). At other risk/intensity of sperm competition, final preference divergence was a better predictor of neutral loci divergence (Figure S4.9). However, the amount of neutral divergence was much lower (Figure 4.6). In these cases, the evolution of cryptic preference divergence after initial loss allowed some divergence in neutral loci to be regained (Figure S4.2,S4.8). This was due to existing starting linkage disequilibrium between neutral loci and traits/preferences. Gene flow was stopped after cryptic preferences evolved/stabilized due to low male migrant reproductive success (Figure 4.6C). Relative female reproductive success did not differ across these parameters and was not dependent on population size (Figure S10). This resulted in differential selection between the two populations on cryptic preferences (Figure 4.6D). In other words, females with cryptic preferences that were more divergent from the preferences in the other population had sons with higher reproductive success (Figure 4.6C, D).

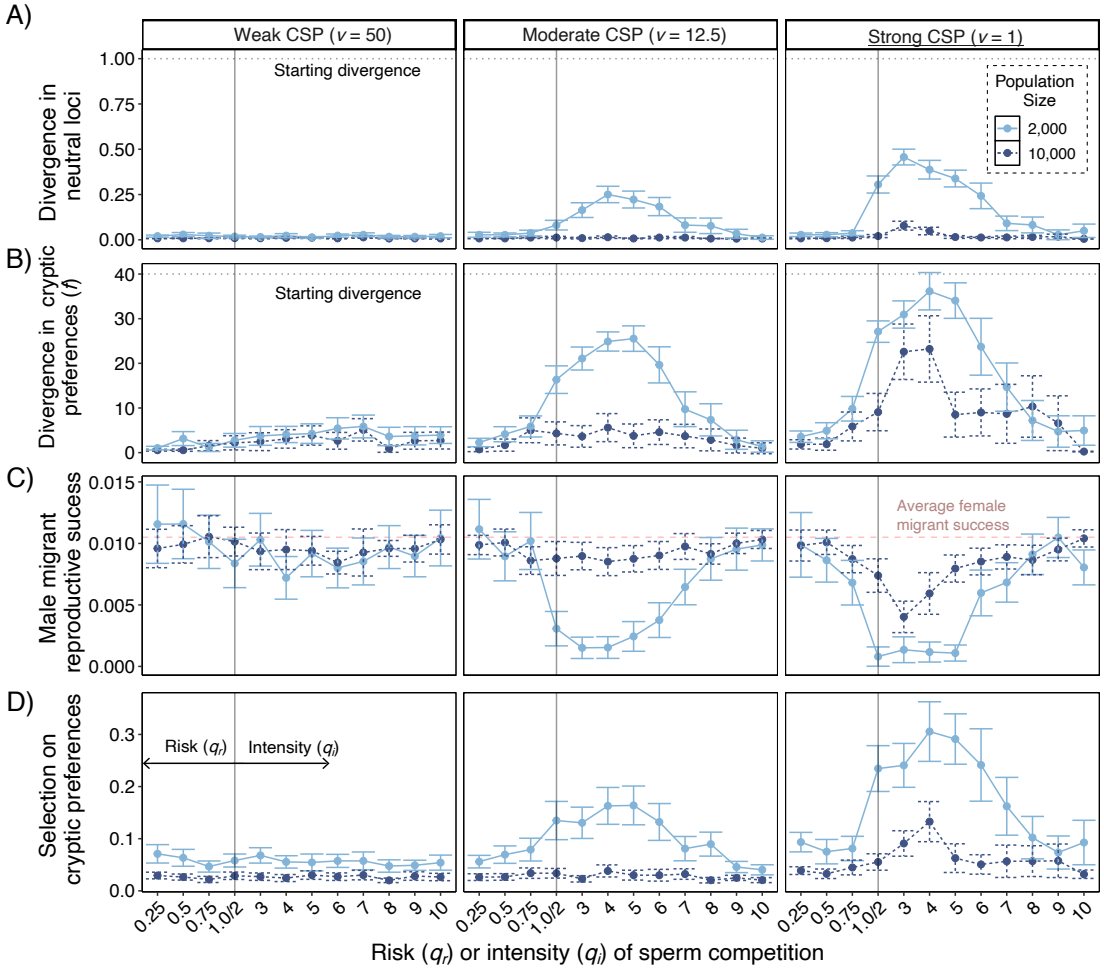
A)
Weak CSP (v = 50)
Moderate CSP (v = 12.5)
Strong CSP (v = 1)
Starting divergence
Population Size
2,000
10,000
Divergence in neutral loci
1.00
0.75
0.50
0.25
0.00
B)
Divergence in cryptic preferences (t)
40
30
20
10
0
C)
Male migrant reproductive sucess
0.015
0.010
0.005
0.000
Average female migrant success
D)
Selection on cryptic preferences
0.3
0.2
0.1
0.0
Risk (q_r)
Intensity (q_i)
0.25 0.5 0.75 1.0/2 3 4 5 6 7 8 9 10
Risk (q_r) or intensity (q_i) of sperm competition

Figure 4.6: Conspecific sperm precedence (CSP) alone can maintain partial reproductive isolation and trait divergence in smaller populations. Patterns and drivers of divergence across different risks and intensities of sperm competition and population size after 3000 generations for when there is initial divergence in cryptic preferences but no ecological divergence. (A) Proportion of neutral loci that are not admixed. The dotted line indicates the starting divergence or if there was no admixture. (B) Divergence in cryptic preferences. The starting divergence in these simulations was 40. (C) The relative number of surviving offspring for male migrants compared to non-migrant males. The dashed line indicates the average relative number of surviving offspring for female migrants, which was not affected by these parameters (Figure. S4.10). (D) The absolute difference in beta coefficient from selection analysis on cryptic preferences where fitness was measured as the mating success of sons. Solid vertical lines divide the risk of sperm competition and the intensity of sperm competition scenarios. Simulations shown here are when there is no tradeoff between sperm number and sperm trait, and the migration rate is at 1%. Error bars represent two standard errors in the mean across 50 replicates.

4.4.5 Secondary contact did not have long term evolutionary consequences on sperm number

Secondary contact could potentially shape the relative importance of sperm number (s) compared to sperm trait (t) because a higher sperm number will always help with fertilization, even in a competitive mating with a heterospecific male and female. We focus this analysis on the no tradeoff scenario because the sperm number in the tradeoff scenario depended on the final sperm trait value (t). We found that the initial sperm number was often very similar to the ending sperm number across all scenarios, meaning there was little predicted evolutionary change in sperm number in our model (Figure S4.11, S4.12). Because initial sperm number was chosen based on average stable sperm number from single population simulations, our results suggest that secondary contact did not have a notable influence on the long-term evolution of sperm

number (s). However, when conspecific sperm precedence was strong there were transient evolutionary dynamics, where sperm number initially decreased upon secondary contact (Figure S4.11 and S4.12). The magnitude of this initial decrease increased with the intensity of sperm competition. This initial decrease was likely due to the tradeoff between pre- and postmating success. Males that invested less in sperm number had higher mating success in our model, so investing less in sperm number increased the likelihood of mating with a female with cryptic preferences (f) similar to the sperm trait (t). Afterward, sperm number increased back to approximately the same starting conditions, likely after preferences stabilized. This pattern occurred regardless of migration rate (1% Figure S4.11; 5% Figure S4.12) and independent of the degree of admixture (see Figure S4.1).

§ 4.5 Discussion

Despite extensive research on the role that premating sexual selection can play in speciation (reviewed in Servedio and Boughman, 2017; Shaw et al., 2024), the role postmating sexual selection plays in speciation has received little theoretical attention (Garlovsky et al., 2023; Howard et al., 2009; Lorch and Servedio, 2007). We, therefore, examined whether and when conspecific sperm precedence maintains reproductive isolation between incipient species that are now in secondary contact. We found that postmating sexual selection via conspecific sperm precedence can help maintain reproductive isolation across a wide range of scenarios, including when there is no other mechanism of reproductive isolation. Further, divergence in cryptic preferences can quickly evolve through reinforcement via ecological divergent selection when there is

no initial divergence.

4.5.1 The effectiveness of conspecific sperm precedence alone at maintaining reproductive isolation depends on population size

Conspecific sperm precedence alone was sufficient to maintain reproductive isolation in smaller populations, but only with the combination of strong conspecific sperm precedence, intermediate levels of multiple mating, and low migration rates (Figure 4.6, S4.1, S4.2). In most cases, initial migration caused a decrease in divergence in both cryptic preferences and neutral loci. However, as cryptic female preferences stabilized, gene flow stopped, resulting in only a partial admixture of the neutral loci. In larger populations, the divergence in neutral loci maintained was smaller. This was likely due to male migrants having much lower reproductive success in smaller populations (Figure 4.6C). In smaller populations, rare migrants are more likely to be lost due to drift. This limited the establishment of a female subpopulation with cryptic preferences that favored migrant males. The success of male migrants was lowest at intermediate levels of multiple mating. This result is likely because multiple mating needs to be high enough for conspecific sperm precedence to happen (i.e., a female mates with both a conspecific and heterospecific male) but also needs to be low enough to prevent rare female migrants from commonly mating with rare male migrants. These results demonstrate the importance of empirical studies to measure the conspecific and heterospecific mating rates in the context of conspecific sperm precedence. To our knowledge, only one study has done this (Larson et al., 2019), so there is much to be done.

4.5.2 Connection with premating sexual selection theory

Most previous models show that sexual selection alone is ineffective at maintaining reproductive isolation (reviewed in Servedio and Boughman, 2017; Weissing et al., 2011). Further, strong sexual selection can degrade ecological divergence and reproductive isolation if the trait is a magic trait (i.e., the male trait is locally adapted and under sexual selection Servedio and Bürger, 2014). However, this main result from Servedio and Bürger (2014) did not hold when females only sampled a small portion of the population (best-of-n with low n) because rare females were less likely to mate with rare males, rendering conspecific sperm precedence irrelevant. As the number of males a female sampled increased (larger n), the model results became more similar to the baseline model assumptions of females sampling the whole population. In many ways, conspecific sperm precedence is most like the best-of-n scenario because females only "sample" the males that they mate with. When there was no ecological divergent selection, we found that as the intensity of sperm competition 4.6). In allopatry, divergence in cryptic preferences and sperm traits may evolve fastest at high intensities of sperm competition (Cramer et al., 2023). Thus, counterintuitively, neither the populations with. The most divergent cryptic preferences nor species with the highest rates of multiple mating may likely maintain divergence upon secondary contact without additional isolating mechanisms. However, low intensities of multiple mating are likely more biologically relevant for non-broadcast-spawning species.

4.5.3 Reinforcement on postmating traits can help maintain reproductive isolation

Although the parameter space in which conspecific sperm precedence alone can maintain reproductive isolation is somewhat limiting, in combination with ecological divergence, it can maintain reproductive isolation and prevent admixture under most scenarios. Even without initial divergence in cryptic preferences, reinforcement can cause the evolution of cryptic preferences and sperm traits upon secondary contact. Although we modeled ecological divergence as the additional factor supporting reproductive isolation, other postzygotic barriers would likely have had a similar reinforcing effect (Lorch and Servedio, 2007; Rushworth et al., 2022). This result of reinforcement acting on postmating sexual selected traits is supported by growing empirical evidence (Garlovsky et al., 2023). For example, sperm length divergence between the common nightingale (*Luscinia megarhynchos*) and the thrush nightingale (*L. luscinia*) is greater in sympatric populations than allopatric populations. Interestingly, although reinforcement can cause the evolution of cryptic preference divergence, we found that it was not needed for reproductive isolation. At low migration rates, the presence of ecological divergent selection alone prevented full admixture. Cryptic preference divergence evolved when conspecific sperm precedence was strong in these scenarios via reinforcement, but it was not needed per se to prevent admixture. At high migration rates, however, reinforcement via the evolution of cryptic preferences was needed to prevent complete admixture and played a vital role in reproductive isolation. Overall, our results imply that when interpreting the importance of traits to initiate speciation caution is required when only looking at the presence of conspecific sperm precedence.

4.5.4 Model extensions and future directions

Although we explored many biological scenarios, further theory could explore how relaxing several assumptions of our model affects these general results to provide further insight into when conspecific sperm precedence is effective at maintaining reproductive isolation. First, although we included premating sexual selection (differential male mating success), we did not incorporate premating preferences and traits. Lorch and Servedio (2007) found that conspecific sperm precedence can slow the evolution of assortative mating and vice versa in line with previous verbal hypotheses (e.g., Marshall et al., 2002) . It would be interesting to see if our modeling framework replicated these results and to test the importance of drift in this process. Second, we only focused on conspecific sperm precedence, although other postmating prezygotic barriers exist in other systems (e.g., reduced fertility; reviewed in Garlovsky et al., 2023). Finally, we held the rate of multiple mating and the strength of conspecific sperm precedence constant. Understanding how these traits evolve during secondary contact would be important. For example, higher rates of multiple mating may evolve if females experience reduced fertility due to postmating prezygotic barriers. This could result in interesting coevolutionary feedbacks with cryptic preferences, sperm number, and sperm traits. Developing theory that relaxes some of our assumptions will provide great insight into the speciation process.

4.5.5 Conclusion

Growing empirical evidence suggests that postmating prezygotic barriers may be more important in speciation than currently appreciated (Garlovsky et al., 2023).

Our theoretical results agree with this empirical finding. Here, we developed theoretical models demonstrating that conspecific sperm precedence alone can be an effective reproductive isolation barrier at intermediate levels of multiple mating and strong selection. With an additional barrier like ecological divergence, conspecific sperm precedence can contribute to maintaining reproductive isolation despite high migration rates. Further, divergence in cryptic preferences can rapidly evolve between populations upon secondary contact. Our results highlight the importance conspecific sperm precedence can play in speciation and the need for future theoretical and empirical investigations into this process.

§ 4.6 Acknowledgements:

We thank B. Lyon, J. Fitzpatrick, L. Alissa, M. Gamble, and M. Molinari for helpful feedback that greatly improved this manuscript. This work was supported by the US National Science Foundation via a GRFP award to M.C.K (Award number: DGE-1842400), an ARCS fellowship award to M.C.K., and a National Science Foundation grant awarded to S.H.A (Award number: IOS-1655297). The authors declare no conflicts of interest.

§ 4.7 Author Contributions

MCK, MRS, and SHA designed the modeling framework. MCK wrote the model script and ran analyses on the resulting data. MCK drafted the manuscript. MRS and SHA critically revised the manuscript.

Synthesis

The complex dynamics of reproductive interactions can drive organismal diversity from variation within species (Gross, 1996) to the creation of new species (Servedio and Boughman, 2017). Although reproductive interactions occur before, during, and after mating, the importance of reproductive interactions occurring after mating (i.e., postmating sexual selection) has only become appreciated in the past 50 years (Ah-King, 2022; Parker, 2020). Despite the rapid growth of this subfield of biology, there remain several gaps in our understanding of this important and taxonomically widespread biological process. Using a combination of empirical and theoretical approaches for my dissertation, I significantly contribute to our understanding of the recent and rapidly growing field of postmating sexual selection.

In Chapter 1, I explored the importance of social roles and the social environment in determining the timing of mating in the ocellated wrasse. Because the timing of mating matters for fertilization success, this chapter helps build connections between various stages of reproductive interactions, which remains a significant gap in our knowledge of postmating sexual selection. I accomplished this by analyzing multiple years of behavioral videos. I found that satellites have shorter sneak-spawning delays than sneakers, a benefit of their cooperation with nesting males. Sneak-spawning delays decreased with increasing nest activity for sneakers but not satellites, suggesting sneakers may benefit from increased sperm competition intensity. My study provides insight into mechanisms that drive variation in the timing of spawning, which could explain mismatches between theoretical and empirical results.

Although there has been a decent amount of work on the effects of temperature on sperm performance (reviewed in Dadras et al., 2017), only one study to date has looked

at the effect of temperature on female reproductive fluids Rossi et al., 2021). Given rising temperatures due to climate change and the importance that female reproductive fluid plays in fertilization by enhancing male sperm performance, guiding sperm to eggs, and biasing fertility to preferred male, this is a critical gap to address. In Chapter 2, I tested how temperature may influence postmating female-male interactions in the ocellated wrasse. Specifically, I tested the sperm performance of different male tactics with and without ovarian fluid at three different temperatures (16℃, 22℃, and 28℃). I find that nesting males had better or similar sperm performance as sneakers with ovarian fluid at 16℃. However, at 28℃, sneaker males had higher sperm performance than nesting males with or without ovarian fluid. My results show that increasing temperatures could undermine cryptic female preferences and highlight that temperature can profoundly impact female-male postmating interactions.

In contrast to sexual selection on traits that affect interactions between the sexes before mating, little theoretical research has focused on the coevolution of post-mating traits via cryptic female choice. Further, most postmating sexual selection theory has focused on male-mediated processes. In Chapter 3, I used simulation models to ask (a) how male ejaculate allocation changes in response to cryptic female choice and (b) when cryptic female choice will result in extreme trait elaboration. I found that when selection from cryptic female choice is strong, investment in ejaculate production is much lower than predicted by models with sperm competition only. I also found that cryptic female choice results in the correlated evolution of male and female traits even when selection is weak, and multiple mating is infrequent, with the potential for extreme trait elaboration. My results suggest that cryptic female choice deserves more attention theoretically and may drive the evolution of male traits in ways that we are only just

beginning to explore.

Sexual selection has long been considered an important mechanism of speciation. Despite growing empirical evidence that postmating sexual selection is common, most speciation theory has focused on premating sexual selection. In Chapter 4, I used simulation models to ask under what circumstances can postmating sexual selection via assortative fertilization (i.e., conspecific sperm precedence) maintains reproductive isolation. I found that conspecific sperm precedence alone can maintain reproductive isolation under limited but realistic conditions. In combination with ecological divergence, conspecific sperm precedence was able to maintain reproductive isolation even at high rates of migration. I also found that divergence in cryptic preferences could evolve through reinforcement (selection on divergent preferences due to less fit hybrids). My results demonstrate that conspecific sperm precedence could maintain reproductive isolation and deserves more attention.

Although my research has filled some of our knowledge gaps, it has also highlighted new gaps and the need for future research. Chapter 1 highlights that the social environment can influence the timing of gamete release and could complicate predictions from current sperm competition theory and may explain why the prediction that males should decrease investment when sperm competition involves more than two males is not well supported (Kelly and Jennions, 2011). Future empirical work can look at how often social environment influences the dynamics of sperm competition, and future theoretical work can incorporate feedbacks between ejaculate investment and social environment. Chapter 2 highlights that warming temperatures can influence postmating female-male interactions. Future work should see how general this result is across other species. Chapters 3 and 4 show that theoretical predictions generated when

focusing on cryptic female choice can deviate from both premating sexual selection theory and postmating sexual selection theory that exclusively focuses on male-mediated processes. Future work incorporating cryptic female choice into the abundant existing theoretical literature on premating sexual selection (reviewed in Kuijper et al., 2012; Servedio and Boughman, 2017) and male-mediated postmating sexual selection (reviewed in Parker and Pizzari, 2010) will significantly improve our understanding of reproductive interactions.

List of supplemental files

- Kustra_Thesis_Supplement.pdf
  - File with all supplemental methods, figures, and tables for the entire thesis.
- SI_Video_1.1.mp4
  - Video of sneaking delay for Chapter 1.

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