

**Post-Copulatory Sexual Conflict and Mate-Guarding Strategies in the Pacific Field Cricket
(*Teleogryllus oceanicus*)**

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Dedication

I dedicate this work to my students, soccer players and the next generation of thinkers and scientists. In a time when education and nature are under threat, it is our duty to build a community rooted in reciprocity and care, ensuring a future where both the planet and its stewards are honored and nurtured.

Abstract

This dissertation explores the role of post-copulatory song as a mate-guarding strategy in the Pacific field cricket, *Teleogryllus oceanicus*. In Chapter 1, I investigate how post-copulatory song influences female behavior and male fertilization success. I provide the first evidence of post-copulatory song in *T. oceanicus* and show that females exposed to this song are more likely to remain in close proximity to the male and retain the spermatophore, increasing the likelihood of fertilization with that male's sperm. In Chapter 2, I examine the role of post-copulatory song in relation to the perceived risk of competition from other males. I find that the likelihood of using post-copulatory song is not influenced by exposure to acoustic cues of rival males. However, males perceiving less competition invest more in the song by decreasing latency to sing and increasing the time spent singing. In Chapter 3, I investigate potential physiological trade-offs between post-copulatory song and sperm viability; however, methodological limitations, particularly delayed sperm staining and buffer-induced degradation, prevent strong conclusions about a biological relationship between song and sperm viability. Overall, this dissertation reveals the role of post-copulatory song as a flexible mate-guarding tactic and highlights its influence on male reproductive success, responses to competition, and the trade-offs involved in reproductive strategy allocation.

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Introduction

Males and females have different evolutionary goals: males aim to maximize the number of offspring they sire, while females typically focus on the quality and investment in each offspring (Trivers, 1972). These opposing objectives create conflicts at various stages of the mating process, from attracting mates to the act of mating itself, and extend beyond mating, as sperm compete to fertilize the egg (Rice, 1998). One key point of sexual conflict emerges after mating, when males attempt to ensure the success of their reproductive efforts by engaging in mate guarding. Sexual conflict continues here, as males strive to control fertilization by limiting female choice and preventing remating with other males, so as to not dilute their chances of reproductive success (Chapman et al., 2003; Arnqvist & Rowe, 2005).

Mate guarding is a widespread behavior observed across many animal species. Males attempt to stay in close proximity to females immediately after mating to control fertilization by preventing the female from prematurely ending sperm transfer and mating with other males (Elias et al., 2014). This behavior is an adaptive strategy for males, as it ensures that their sperm are the ones that fertilizes the female's eggs, increasing their reproductive success (Alcock, 1994; Parker, 1970; Birkhead & Møller, 1992). While this strategy can be highly beneficial for males, it is not without costs, particularly for females (Eberhard, 1996). Multiple mating provides females with direct benefits, such as increased resources or indirect genetic benefits for their offspring (Parker, 2006). Therefore, mate guarding creates a potential for sexual conflict, as females benefit from multiple mating opportunities, while males attempt to prevent them from remating (Andersson & Simmons, 2006; Arnqvist & Rowe, 2005).

The level of control that males exert over females during the post-copulatory period varies across species. In many cases, males have physical adaptations—such as specialized appendages or structures—to physically restrain females during mate guarding, ensuring that their sperm are the ones that fertilize the eggs. For instance, water striders (*Gerris odontogaster*) males physically restrain females by clasping onto them for extended periods, riding passively on their backs. However, not all species have these physical adaptations. In some bird species, such as bluethroats (*Luscinia svecica*), males rely on behavioral strategies, like following the female closely during her fertile period to prevent remating (Harts et al., 2016). This strategy, though effective, has its limitations. It requires constant vigilance and can be energetically costly, leaving room for error. If the male fails to stay close or is distracted, the female may remate, reducing his reproductive

success. Thus, when males are limited in their ability to physically control female behavior after copulation, how can they ensure their reproductive success? What alternative strategies might evolve to overcome these challenges?

These questions led us to investigate the Pacific field cricket (*Teleogryllus oceanicus*), where, despite the absence of physical adaptations for mate guarding, males may use behavioral tactics to enhance their reproductive success. While males cannot physically control female movement, we observed an intriguing post-copulatory behavior in which some males produced a song immediately after mating, something not typically seen in crickets, where males usually refrain from singing until they produce a new spermatophore at least an hour later (Chipchase et al., 2021; Dowling & Simmons, 2012; Loher, 1974). This behavior intrigued us, as it appeared to challenge the typical understanding of post-copulatory behavior in these insects. We hypothesized that this post-copulatory song could serve as a mate-guarding tactic, attracting females back to the male and reducing the likelihood of her remating with other males, thus helping to secure the male's reproductive success.



Figure 0. A male Pacific field cricket, *Teleogryllus oceanicus*. Image credit: Jon Richardson.

In Chapter 1, we explore how males can influence post-copulatory female choice through post-copulatory song as a mate-guarding tactic (Hagg et al., 2024). While some species rely on physical restraint to control female movement, others, like the Pacific field cricket, *Teleogryllus oceanicus*, do not have such adaptations. In this species, some males continue to produce the calling song used for long-distance mate attraction after spermatophore transfer, despite being unable to mate again. We test the hypothesis that this post-copulatory song serves as a mate-guarding

strategy. Our results show that females exposed to post-copulatory song are more likely to remain near the male and retain their spermatophore, increasing the male's chances of fertilizing their eggs. This study provides the first evidence of post-copulatory song in *T. oceanicus* and suggests that it may be an effective tactic for mate guarding.

In Chapter 2, we explore how perceived rival male competition risk influences the use of post-copulatory mate-guarding song in male Pacific field crickets (*Teleogryllus oceanicus*) (Hagg & Zuk, *under review*). Additionally, we examined whether exposure to male competitors' calls during rearing or before mating influenced male behavior. Our results show that males exposed to acoustic cues of male competition during rearing or immediately before mating did not show an increased likelihood of singing post-copulatory songs. However, among those males that did sing, those reared with song had a delayed onset and spent less time singing overall compared to males raised in silence.

In Chapter 3, we investigate the potential physiological trade-offs associated with post-copulatory song and its relationship with sperm quality (Hagg, Lindsey, & Zuk, *in prep*). Males face a balancing act between investing in reproductive behaviors like mate guarding and maintaining high-quality sperm, as both require significant energetic resources. We hypothesize that males with lower sperm viability may compensate by investing more in post-copulatory song, an alternative strategy to increase their chances of fertilization. Our results reveal that males producing post-copulatory song transferred ejaculates with significantly lower sperm viability compared to non-singing males, suggesting a trade-off between sperm quality and mate-guarding behavior.

Broadly, these findings provide valuable insight into the complex strategies males employ to maximize their reproductive success. By examining how post-copulatory song functions as a mate-guarding tactic in the Pacific field cricket, this research reveals the intricate ways in which males respond to sexual conflict and attempt to retain control over fertilization. It emphasizes the adaptive significance of post-copulatory behaviors and highlights both physiological and behavioral adjustments males make in response to rival competition and variation in ejaculate investment. This work informs our understanding of signal evolution in the context of sexual conflict, particularly how a signal that originally evolved for mate attraction may be co-opted for post-copulatory mate guarding. Together, these findings show how competition, sperm quality, and behavioral plasticity interact to shape male reproductive strategies and influence reproductive outcomes, ultimately driving the dynamics of sexual selection.

Chapter 1

Postcopulatory song as a mate-guarding tactic in the Pacific field cricket, *Teleogryllus oceanicus*

Abstract

Males can bias insemination and fertilization in their favour by guarding females. In some species, males physically constrain females, while in others, female movement is not as directly controlled. In the Pacific field cricket *Teleogryllus oceanicus*, some males produce the same calling song used in long-distance mate attraction after spermatophore transfer, even though they cannot mate again. We tested the idea that this postcopulatory song serves as a mate-guarding tactic. Females that heard postcopulatory song were more likely to stay close to the male and retain their spermatophore, thus increasing the likelihood of the male fertilizing their clutch. To our knowledge, our results provide the first evidence of postcopulatory song in *T. oceanicus* and suggest that it may be an effective mate-guarding tactic.

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Introduction

Mate guarding of females by males is widespread and has been studied across animal taxa (e.g. amphibians: Cooper & Vitt, 1997; Rueda-Solano et al., 2022; reptiles: Ancona et al., 2010; Fitzgerald & Shine, 2018; Olsson, 1993; birds and mammals: Birkhead, 1979; Buss, 2002; Girard-Buttoz et al., 2014; Harts et al., 2016; Poole, 1989; crustaceans: Cruz-Rivera & Hafez, 2023; Shadrin et al., 2021; Takeshita & Henmi, 2010; insects: Alcock, 1994; Rodríguez-Muñoz et al., 2011; Vellnow et al., 2020). Mate guarding is a behaviour where males attempt to remain in close proximity to the mated female immediately after mating (Elias et al., 2014). The main function of this behaviour is to control the insemination of females and the fertilization of their eggs by limiting postcopulatory female mate choice and competitor male access to the reproductively active female (Andersson, 1994). When females mate multiply, the initial male's sperm must compete for fertilization with sperm from subsequent males, potentially leading to a reduction in his paternity share (Simmons, 2001). Males that ensure their mate is inseminated by their sperm and not by the sperm of competing males will be favoured by selection (Parker, 1970). Sexual selection thus favours adaptations in males, like mate guarding, that increase reproductive success (Birkhead & Møller, 1992).

Although mate guarding can benefit males, it can be costly to females. Mating multiply may provide direct benefits to females (e.g. increased resources from males) or indirect benefits (e.g. genetic benefits) to their offspring (Fedorka & Mousseau, 2002; Jennions & Petrie, 2000; Ridley, 1988; Zeh & Zeh, 2001). Prematurely terminating sperm transfer may benefit the female by limiting receipt of sperm from lower-quality males or the amount of potentially toxic substances that pass into the reproductive tract (Eberhard, 1996; Parker, 2006). Guarded females may be limited in their abilities to prematurely end sperm transfer and mate with additional males (Arnqvist & Nilsson, 2000; Bateman & MacFadyen, 1999; Bussière et al., 2006; Parker & Vahed, 2010; Sakaluk, 1991; Simmons, 1986). Therefore, females may benefit from avoiding male mate-guarding attempts (Cordero & Eberhard, 2003). Thus, if males benefit from mate guarding but females benefit from multiple mating, then there is potential for sexual conflict over mate guarding (Andersson & Simmons, 2006 ; Arnqvist & Rowe, 2005; Birkhead & Pizzari, 2002; Chapman et al., 2003; Parker, 1979).

In some species, mate guarding may be difficult for females to circumvent. In many insects, males have evolved adaptations that allow them to physically restrain females. For example, damselfly males remain in the tandem position following copulation, holding the female with their

claspers while she oviposits (Cordero, 1990; Stoks et al., 1997; Waage, 1984). In tiger beetles, males grasp females with their jaws and travel with their partner following copulation (Kraus & Lederhouse, 1983). In soapberry bugs, males may prolong copulation beyond sperm transfer by remaining connected to the female for as long as 11 days (Carroll, 1991). In these cases, females may not be able to avoid male mate-guarding behaviours.

However, in other insects, such as crickets, males do not have physical structures that allow them to control female movement. Instead, crickets rely on visual, olfactory or tactile monitoring to mate-guard, which offers an opportunity for females to circumvent mate guarding (Alexander, 1961; Huber, 1955; Loher & Rence, 1978). Mate guarding typically includes antennating females, producing body judders and aggressive movements in front of and towards females (Sakaluk, 1991; Simmons, 1991; Wynn & Vahed, 2004). Males attempt to stay in close proximity to the female to prevent her from removing the spermatophore, a capsule containing sperm, while it drains into the female's sperm storage organ, the spermatheca (Alexander, 1961; Hockham & Vahed, 1997). Males cannot assure insemination or fertilization success after mating (Khalifa, 1950). In crickets, where males are unable to control female movement postcopulation and cannot directly influence their own reproductive success through physical restraint, how do males guard when females have the space to avoid it?

Although mate guarding has been studied in crickets, most previous experiments have been performed in small arenas (typically several cricket body lengths (~50 mm) in size, i.e. 5 × 11 cm) (Parker & Vahed, 2010; Ritz & Sakaluk, 2002; Ryan & Sakaluk, 2009; Tuni et al., 2013). Although two studies of mate guarding in *Gryllus bimaculatus* (Simmons, 1986) and *Gryllus campestris* (Rodríguez-Muñoz et al., 2011) used larger spaces, in these species, females spend more time near males because the males provide a valuable resource, a burrow, unlike other crickets in which females leave the male to oviposit (Sakaluk, 1991; Simmons, 1991; Wynn & Vahed, 2004). Studies that use small arenas allow us to document male mate-guarding behaviours at close range but do not allow females the space to avoid male mate-guarding attempts. In the field, female crickets may freely walk away from males after mating, limit sperm transfer by removing the attached spermatophore and remate with additional males (Bateman & MacFadyen, 1999; Bussiégre et al., 2006; Evans, 1988; Loher & Rence, 1978; Parker, 2009). Therefore, studying mate-guarding behaviours in small spaces may limit our understanding of mate-guarding interactions between males and females. We set out to understand male mate-guarding behaviours under more realistic conditions, where female movement is less constrained, to uncover the extent to which males are

able to influence postcopulatory female mate choice. We examined mate guarding in the Pacific field cricket, *Teleogryllus oceanicus*, in a large arena (over 30 cricket body lengths in size, more than 20× larger than previous studies), allowing female crickets greater freedom to leave the guarding male by providing more space.

In our preliminary experiments, some *T. oceanicus* males produced songs immediately after mating. It has been long believed that male crickets will not produce calling song (for mate attraction) until a new spermatophore has been produced and is ready in the genital chamber (Chipchase et al., 2021; Dowling & Simmons, 2012; Loher, 1974; Nagao & Shimozawa, 1987). This process takes approximately 1 h after mating for the genus *Teleogryllus* (Loher, 1974). What is the function of postcopulatory song? We hypothesized that male crickets use postcopulatory song as a mate-guarding tactic. Because females are still phonotactic after mating, the postcopulatory song may provide a stimulus for the females to return to the male. Here, we document postcopulatory song in *T. oceanicus* for the first time and examine its effect as a mate-guarding tactic. We also examined how postcopulatory song influences sperm transfer (by measuring spermatophore removal) and female remating (by measuring female time spent in proximity to the calling male).

Methods

Study system

We used *T. oceanicus* from a laboratory colony that originated from the Hawaiian island of Oahu. The population was established using approximately 100 crickets collected from the University of Hawaii-Manoa campus in 1993 and 1994 (Zuk et al., 1995). Each year the population is supplemented using offspring of multiple wild-caught females from Oahu to maintain a minimum of 100 breeding individuals in the colony at all times. Crickets are housed with ~25 individuals per container and colonies are consistently mixed to avoid inbreeding.

Experimental set-up

We reared the crickets in Caron Insect Growth Chambers (model 6025, Caron Products, Marietta, OH, U.S.A.) at 75% humidity, 25 °C and on a 12:12 h photo-reversed light:dark cycle. Prior to final moult, we placed juvenile males and females in plastic containers (35.9 × 20 × 12.4 cm) with no more than 10–12 same-sex individuals to keep them unmated. Crickets had an egg carton for shelter, food (Teklad rabbit chow) and water ad libitum in damp cotton pads. Upon eclosion, we

housed crickets in individual 118 ml plastic cups with shelter, food and water. Adult crickets are considered sexually mature 6 days after the final moult (Bailey & Zuk, 2008), so we used individuals 7–9 days posteclosion for the mate-guarding trials.

We mated both male and female subjects before the mate-guarding trial because most field crickets in the wild are not virgins (Bretman & Tregenza, 2005). Previous work has found that mating experience affects *T. oceanicus* female mating behaviours (Tanner et al., 2019), which therefore could also influence postcopulatory behaviours like spermatophore removal in females or male mate-guarding intensity. Therefore, crickets were mated 6 days posteclosion as sexually mature crickets: the subject and a haphazardly chosen cricket of the opposite sex from the Oahu laboratory colony were placed in a 118 ml plastic cup for 15 min and then checked for a successful spermatophore transfer. Each virgin was given two opportunities to mate on the sixth day following eclosion prior to the mate-guarding trial. If an individual did not mate after two attempts, the individual was returned to the Oahu laboratory colony and not used ($N = 78$). After a cricket's first successful mating, it was used for the mate-guarding trial 24–72 h later. We included 75 males and 75 females in mate-guarding trials.

Mate-guarding trials

We conducted 75 mate-guarding trials in a 22–26 °C anechoic room under red lighting. During the trials, we placed a male and female in a large mating arena ($1.6 \times 1.6 \times 0.3$ m) on the floor. To measure movement and proximity, the bottom of the arena was marked with a 50×50 mm grid (the average body length of a cricket including the antennae). The male and female were given 15 min to mate in the arena. Following the successful transfer of a spermatophore, we recorded male and female behaviours for 40 min, the time needed for the transfer of sperm from the spermatophore to the female spermatheca, as well as average *T. oceanicus* mate-guarding length (Simmons et al., 2003; Tuni et al., 2013). If the male and female did not mate within 15 min, we returned them to their individual cups and gave them an additional mating attempt with a new partner. If no mating occurred on the second attempt, the crickets were excluded from the experiment ($N = 35$).

Once we determined visually that the male had successfully transferred a spermatophore and the female dismounted the male, the 40 min mate-guarding trial began. We measured whether males produced postcopulatory song, the total time females spent within close proximity to the male (50 mm, the average length of a cricket), as well as whether and when the attached

spermatophore was removed by the female. We measured pronotum width to the nearest 0.01 mm in both males and females as an estimate of body size. Individuals were only tested once. Both of the individuals were then returned to the Oahu laboratory colony after the mate-guarding trial was completed. We used a Student's *t* test to compare the amount of time females spent near males that did or did not produce postcopulatory song. We used a chi-square test to compare the presence or absence of the spermatophore at the end of trial with whether or not females heard a postcopulatory song from the guarding male. We conducted all statistical analyses of behaviour using R (version 4.1.2; R Core Team, 2021).

Ethical note

Our research adhered to ASAB/ABS Guidelines for the Use of Animals in Research and the legal requirements under the Animal Welfare Act of the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS permit no. P526P-21-07108 to M. Zuk).

Song analysis

To test whether postcopulatory song was the same as precopulatory calling song used for mate attraction, we compared pre- and postcopulatory song components for each male. After 5 min of acclimation to the mating arena in the anechoic room, a Sennheiser MK 600 shotgun microphone was directed towards the male at close range for song recording with a Marantz Professional PMD661 MK II recorder. When the male began singing, all precopulatory songs were recorded. After a successful mating (i.e. a spermatophore was transferred and attached to the female), any postcopulatory songs produced by the male were recorded. Precopulatory calling song in *T. oceanicus* consists of a 'long chirp' segment of between three to nine pulses or wing strokes followed by a series of 'short chirps' consisting of two pulses (Zuk et al., 1993, 1998, 2001).

Out of the 40 singing males, 17 males produced five or more song bouts pre- and postcopulation. We used five song bouts as the minimum threshold to be included in song analysis. We compared precopulatory to postcopulatory song in these 17 males. Earlier studies used eight calling song variables for analysis in *T. oceanicus*: total song duration, long chirp duration, long chirp singular pulse duration, interval between the long chirp pulses, total interval between the long and short chirp, short chirp duration, short chirp singular pulse duration and the interval between the short chirp pulses (Zuk et al., 2008). Although we would ordinarily use a multivariate analysis,

such as a discriminant analysis, to compare the songs using all of the variables simultaneously, we did not do so here because the sample size of 17 relative to the eight predictor variables violates the assumptions for the test. For discriminant function analysis, the ratio of the number of samples to the number of predictor variables is suggested to be 5:1 (O'Rourke et al., 2013). We therefore chose to compare only the two most important components of the song for female preference, as shown in earlier studies (long chirp duration and short chirp interpulse interval shown in Fig. 1) (Simmons et al., 2001; Zuk et al., 2008).

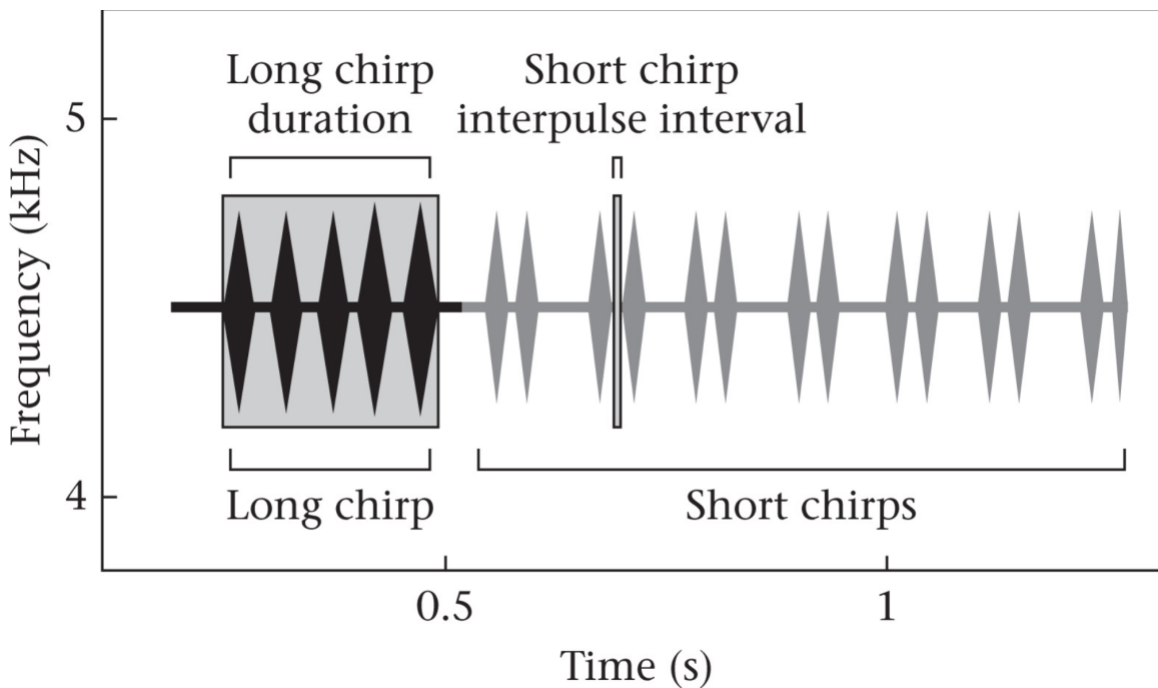


Figure 1.1. Simplified *T. oceanicus* song spectrogram with song analysis measures, long chirp duration and short chirp interpulse interval.

Long chirp duration was measured using each male's second song, beginning at the first long chirp pulse to the end of the last long chirp pulse. Short chirp interpulse intervals were scored between the second and third short chirp pulse in the male's second song by measuring the end of one pulse to the beginning of the next in the short chirp pair. We measured temporal components of each male's precopulatory calling song and postcopulatory song using Raven Pro: Interactive Sound Analysis Software (Version 1.6.4, Cornell Lab of Ornithology, <https://ravensoundsoftware.com/software/raven-pro/>) and compared the means of the two variables using a paired *t* test.

Results

Roughly half of the 75 males (53.3%, $N = 40$) produced a postcopulatory song after successfully transferring a spermatophore to the female. The majority of these males (90%, $N = 36$) sang within 15 min after mating, with half of the males singing in the first 5 min (52.5%, $N = 21$). There were 17 males (42.5%) that produced five or more song bouts and six males (15%) that produced just one song bout postcopulation. Male body size had no effect on the likelihood to produce postcopulatory song ($t_{73} = -0.43$, $P = 0.66$). Female body size also had no effect on the likelihood of male postcopulatory song ($t_{73} = -0.03$, $P = 0.97$). Logistic regression did not show a significant interaction between male and female size influencing the likelihood that the male produced a postcopulatory song (logistic regression: $\chi^2 = 1.22$, $P = 0.27$).

Of the 75 females, 68 (90.7%) moved >50 mm (the average body length of a cricket) away from males within 2 min after the conclusion of copulation. However, when males produced postcopulatory song during the mate-guarding period (40 min), females stayed within 50 mm of the males for a significantly longer time than when males did not produce postcopulatory song ($t_{73} = -3.32$, $P = 0.001$; Fig. 2). To determine whether postcopulatory song impacted females' removal of the spermatophore, we assessed the number of females who removed their spermatophore during the mate-guarding period. In four pairings in which the male used postcopulatory song, they did so after the female had removed the spermatophore. We considered these four males as 'no postcopulatory song', but only after the female had already removed their spermatophore.

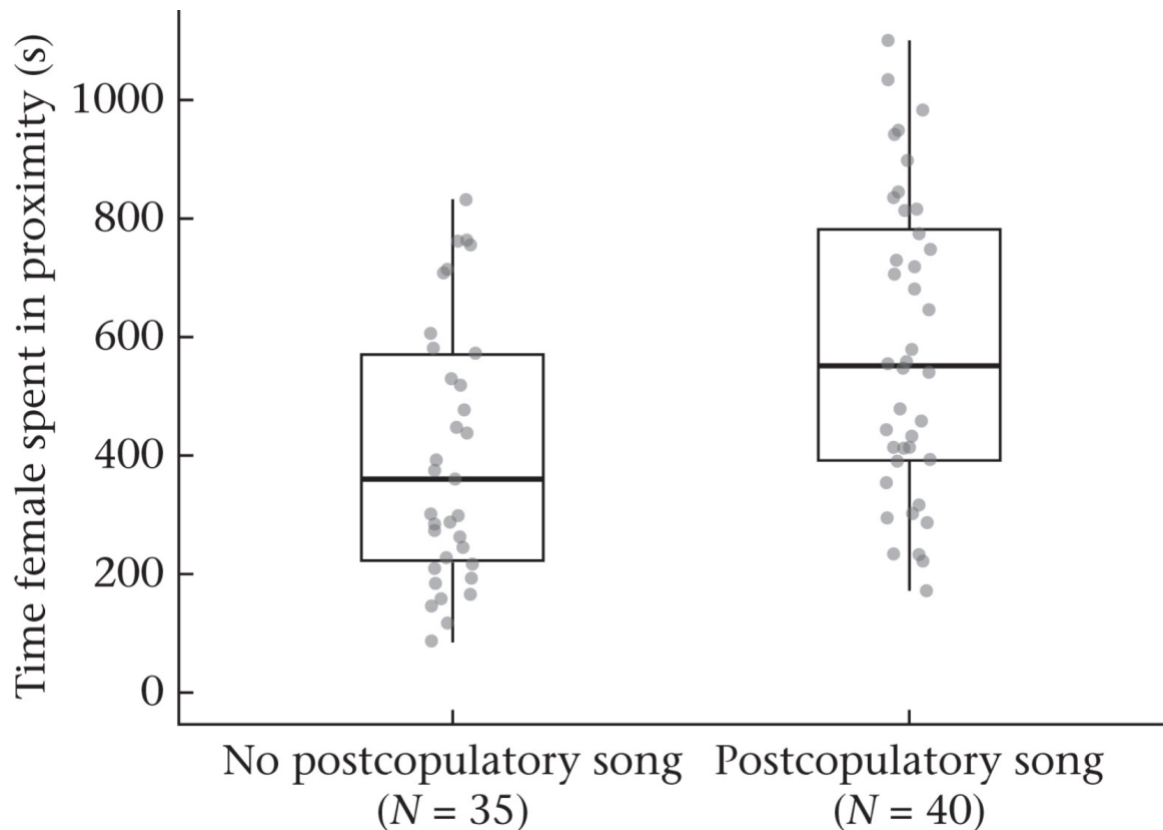


Figure 1.2. Time (seconds) *T. oceanicus* females spent in close proximity (<50 mm) to males who did or did not produce postcopulatory song. Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5× the interquartile range from the 25th and 75th percentiles.

Females were significantly more likely to retain males' spermatophores when the males produced postcopulatory song and more likely to remove the spermatophore where song was absent (41% removed without song compared to 15% removed with song) ($\chi^2_{21} = 5.19, P = 0.02$; Fig. 3). The earliest a spermatophore was removed after successful attachment was 6 min and the latest was 38 min ($N = 22, \bar{X} \pm SD = 1196 \pm 418.52$). Female body size had no effect on the time spent in proximity to the male ($36.57 \pm 45.22; t_{73} = 0.81, P = 0.42$) or on the likelihood of removing the spermatophore ($0.58 \pm 0.39; z_{73} = 1.485, P = 0.14$). Additionally, male body size had no effect on spermatophore removal (logistic regression: $\chi^2 = 0.01, P = 0.99$) and neither did the interaction of male and female body size ($\chi^2 = 0.02, P = 0.96$).

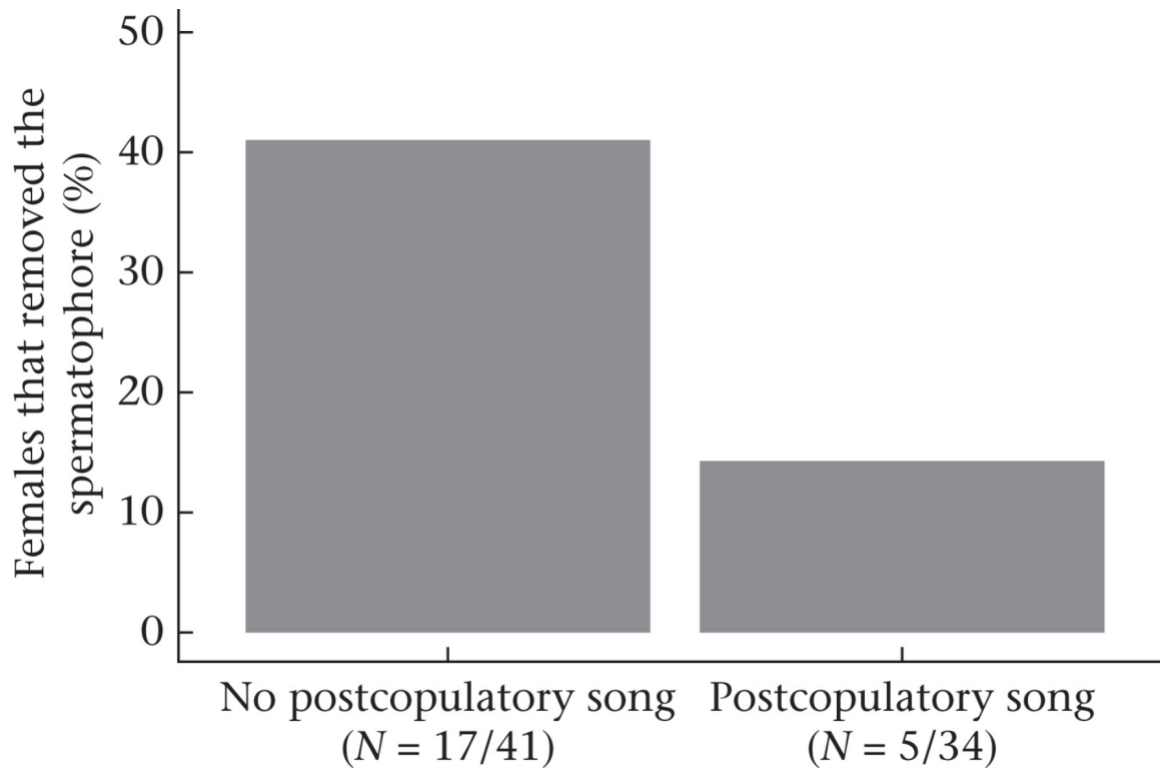


Figure 1.3. Percentage of *T. oceanicus* females who removed their spermatophore when paired with males that did or did not produce postcopulatory song.

There were no statistically significant differences between males' precopulatory or postcopulatory long chirp duration or interpulse interval (long chirp duration: $t_{16} = 1.25$, $P = 0.23$; interpulse interval: $t_{16} = 1.17$, $P = 0.26$).

Discussion

Here, we document postcopulatory song in *T. oceanicus*. We confirm that this postcopulatory song is structurally similar to the precopulatory calling song used by males to attract females for mating. We also provide the first empirical evidence that male postcopulatory song functions as a mate-guarding tactic. As is expected if postcopulatory song serves as a mate-guarding tactic, females who were presented postcopulatory song spent more time in close proximity to the singing males (Fig. 2) and were more likely to retain their spermatophores (Fig. 3). Our results show that males who sing postcopulatory song keep mated females nearby after mating for a longer period. Therefore, males who sing may prevent females from mating with a new male and prematurely removing their spermatophore and/or have greater chances of remating with the female (Arnqvist

& Nilsson, 2000). Although spermatophore attachment time and the number of sperm transferred do not guarantee fertilization (Hall et al., 2010; Simmons et al., 2003; Tuni et al., 2013), all else being equal, males that increase the time their spermatophore is attached still have a greater likelihood of reproductive success than males whose spermatophores are quickly removed (Parker & Vahed, 2010).

If postcopulatory song is a mate-guarding tactic, then why do not all males call after mating? The use of postcopulatory song may also depend on the social environment. Males may be using environmental cues to assess the level of mate availability and potential sperm competition to inform postcopulatory mate-guarding tactics (Bailey et al., 2010). If the risk of sperm competition is high, it may benefit the male to invest more in mate guarding by producing postcopulatory song to increase sperm transfer and limit female remating (Simmons, 2001). If the risk of sperm competition is low, it may benefit the male to invest less in mate guarding and more time and energy on additional mate acquisition (Simmons et al., 2007). In addition to the level of sperm competition, the use of postcopulatory song may depend on the quality of the female. If the female is of higher quality, it may benefit the male to invest more into mate guarding, whereas if the female is of lower quality, then it may benefit the male to spend that time and energy searching for a new mate instead of guarding (Thomas & Simmons, 2007). Female quality and male quality could also interact, where high-quality males only guard high-quality females, whereas low-quality males guard every mated female (Bateman et al., 2001). A high-quality male who is preferred by females may put energy into searching for mating opportunities rather than mate guarding (Magrath & Komdeur, 2003). On the contrary, a lower-quality male who has limited opportunities to mate may benefit by investing in guarding behaviours to bias fertilization (Kokko & Morrell, 2005). However, all of these explanations are unlikely to explain why only half of the males sang in our study because, in our experiment, we used males and females of similar age, body condition and mating experience and we reared them in the same social environment.

Instead, postcopulatory song may be a reproductive trade-off that males are making. Postcopulatory song, like precopulatory calling song, is energetically costly (Bertram et al., 2022; Hoback & Wagner, 1997). The cost of singing means there will be trade-offs between energy spent producing this postcopulatory song with the potential benefits (e.g. sperm transfer assurance, reduce remating with competing males, facilitate remating with same male). Using postcopulatory song to mate-guard may therefore be a plastic behaviour that is used variably across an individual

male's lifetime. Alternatively, it may be that some males experienced slight disturbances in their rearing conditions during the experiment that affected their likelihood to produce postcopulatory song. Therefore, it is still largely unknown why only half of the males produced postcopulatory song if it is an effective mate-guarding behaviour.

Post-copulatory song presents potential for sexual conflict over male guarding and female mate choice. Females benefit by mating with multiple males, but they may lose mating opportunities with additional males by responding to postcopulatory song (Peretti & Aisenberg, 2015). By responding to postcopulatory song, females spend more time being guarded and have less control over sperm utilization and a reduced ability to mate with other males. Given these costs, we might expect selection to favour females that ignore postcopulatory song. However, we found that *T. oceanicus* males are using the same song used for mate attraction as a postcopulatory mate-guarding tactic. Thus, if females are unable to distinguish between these two structurally similar songs and ignore male calls altogether, they will not be phonotactic and have very low odds of mating with any males (Pollack & Hedwig, 2017). It may be that, for females, the cost of not responding to song outweighs the costs of being guarded and thus positive phonotaxis, even postcopulation, is evolutionarily favoured (Dougherty, 2021).

In mating systems dominated by female choice, where males lack physical adaptations to control female movement, males may take advantage of female behaviours like phonotaxis that are already well established. Postcopulatory song use as a mate-guarding tactic may therefore be an example of sensory exploitation (Endler & Basolo, 1998; Ryan, 1990). Sensory exploitation is typically defined as a novel trait that exploits a pre-existing sensory bias in the receiver (Stevens, 2013). Males using precopulatory calling song immediately after mating for the purpose of guarding instead of mating is using an already existing male trait that takes advantage of pre-existing phonotaxis behaviour in females in order to increase reproductive success in males. For example, in the long-tailed dance fly *Rhamphomyia longicauda*, females compete for males that provide a highly desirable nuptial gift (Funk & Tallamy, 2000). In this species, males prefer females with swollen abdomens, likely because abdomen size is perceived to be a good indicator of egg maturity and fertilization success (Funk & Tallamy, 2000). However, females exploit this male preference for larger females by swallowing air to 'puff up' their abdomen (Funk & Tallamy, 2000). Therefore, females gain the necessary sustenance from the nuptial gifts needed to complete egg

development to increase their own reproductive success by exploiting male preferences and response.

Our discovery of postcopulatory song is particularly interesting when considering Hawaiian populations of *T. oceanicus*, in which a proportion of males are unable to sing (Zuk et al., 2006). In some Hawaiian populations of *T. oceanicus*, acoustically orienting parasitoid flies (*Ormia ochracea*) deposit larvae on and around calling males, which burrow into the male, then grow and develop and eventually emerge, killing the male cricket (Adamo et al., 1995; Zuk et al., 1993). This natural selection pressure against sexual signalling has led to the loss of the necessary structures to produce song through a rapidly spreading novel mutation (Zuk et al., 2006). These males are also unable to produce postcopulatory song. Our results suggest that silent males will be less effective at mate guarding, with a greater chance that females will remove their spermatophores or mate with other males. Males unable to produce postcopulatory song may have lower reproductive success and thus are less likely to pass on their genes to the subsequent generations. Thus, although natural selection favours the allele that renders males silent, as they avoid parasitism and likely have greater survival, postcopulatory song is yet another factor that strengthens sexual selection for singing males (Heinen-Kay et al., 2020; Heinen-Kay, Strub, et al., 2019; Heinen-Kay, Urquhart, et al., 2019). Our results may also help explain why the *flatwing* allele for signal loss is not driven to fixation.

Chapter 2

Lack of perceived sperm competition risk increases post-copulatory song in Pacific field crickets

Abstract

Mate guarding enables males to bias fertilization, increase their share of paternity, and thereby enhance reproductive success. When the risk of sperm competition is high, males may benefit from investing more in guarding behaviors. While some species physically restrain females during guarding, others use more passive tactics. Male Pacific field crickets (*Teleogryllus oceanicus*) sing after mating to entice females to stay, even though they cannot mate again during this time period. Post-copulatory song effectively keeps females in proximity, prolongs spermatophore attachment, and ultimately enhances male reproductive success. We investigated whether cues about the risk of sperm competition determine male investment in post-copulatory song. Additionally, we studied whether hearing cricket calls either during rearing or immediately before mating modified the listeners' behavior. Males exposed to acoustic cues of male competitors during rearing or immediately before mating were not more likely to sing post-copulatory songs. However, among those males that did sing post-copulatory songs, those exposed to song during rearing had a greater latency to begin singing and overall spent less time singing post-copulation than males exposed to silence.

Introduction

Mate guarding is a taxonomically widespread behavioral strategy employed by males to prevent their mates from prematurely ending sperm transfer and engaging in copulations with rival males (Alcock, 1994). This strategy aims to reduce the likelihood of sperm competition, where sperm from multiple males compete to fertilize the same female's eggs (Parker, 1970). Mate guarding behaviors vary widely across species and may involve physical proximity to the mate or aggressive defense against rival males to maintain exclusive access to the mate (Neff and Svensson, 2013). For instance, male baboons (*Papio cynocephalus*) carefully track female movements and display defensive behaviors toward rival males attempting to approach fertile females (Alberts et al., 1996). In many species, males exhibit morphological adaptations that increase guarding efficiency. For example, male damselflies remain in tandem with the female by engaging abdominal claspers until the female oviposits (Cordero, 1990; Stoks et al., 1997; Waage, 1984). Mate guarding is commonly observed within species when mate competition is fierce (Birkhead & Møller, 1992). Male tree weta (*Hemideina crassidens* Blanchard) remove mates from locations where females have a high likelihood of encountering and mating rival males (Kelly, 2008). Consequently, investment in mate-guarding behaviors is likely contingent on the degree of sperm competition.

Sperm competition significantly influences post-copulatory mating behavior in males of many species (Bailey et al., 2010; Gray & Simmons, 2013). Despite its recognized importance in enhancing reproductive success by impacting fertilization outcomes (Simmons, 2001), the precise mechanisms by which sperm competition affects mate guarding remain unclear. Specifically, it is not well understood how males assess the risk of sperm competition and adjust their mate-guarding strategies accordingly. For instance, do males alter their guarding behaviors in response to cues about the presence of rival males, and if so, how does this affect their investment in post-copulatory behaviors? The post-copulation period is critical, as it represents a final opportunity for males to maximize their paternity (Parker, 1970). We asked how cues indicative of sperm competition influence male investment in mate guarding and post-copulatory behaviors.

Males use social cues, such as acoustic signals from surrounding competitors, to gauge the risk of sperm competition and adjust their mate-guarding behavior accordingly (Simmons & Shuker, 2014). These cues provide indirect information about the presence and density of rivals, allowing males to adapt their reproductive strategies in response. For instance, in lek-mating lesser wax moths (*Achroia grisella*), males exposed to playback of conspecific songs showed shorter

mating latencies and longer copulation durations compared to controls, suggesting that adaptive precopulatory mate guarding helps maximize reproductive success under intense competition (Jarrige et al., 2016). Similarly, male crickets (*Teleogryllus oceanicus*) exposed to high-density song environments, indicating a greater risk of competition, invest more in reproductive tissue and produce ejaculates with a higher percentage of viable sperm (Bailey et al., 2010; Gray & Simmons, 2013), demonstrating a plastic response to perceived sperm competition risk. By adjusting these behaviors in response to cues of sperm competition, males may better compete for fertilizations and achieve overall higher reproductive success.

In this study, I manipulate social cues at two key time points, during juvenile development and immediately before mating, to test how males adjust their reproductive strategies in response to perceived sperm competition. Social cues encountered during development may shape long-term mating strategies, while cues present just before mating could allow males to respond more flexibly to current conditions. Although acoustic signals experienced early in life have been shown to influence adult mating behaviors (Kasumovic & Brooks, 2011), the effects of social cues encountered immediately prior to mating remain less well understood (Swanger & Zuk, 2015). If males are more responsive to recent signals, this could enable plastic adjustment to rapidly changing social environments, especially important given that sperm competition intensity can vary substantially across time and space (Simmons, 2001). However, males may face constraints in modifying reproductive investment on short timescales. Conversely, if male behavior is shaped primarily by juvenile experiences, this would suggest a strategy based on early-life predictions of future competition. Disentangling these influences is challenging, as manipulations of one can confound the other (Engqvist & Reinhold, 2005). To address this, I experimentally isolate the effects of both early and immediate competition cues by manipulating acoustic environments during rearing and again just before mating. This design allows me to assess whether male reproductive strategies are shaped more by developmental context or by immediate social conditions.

Male crickets rely on a variety of strategies to maximize reproductive success post-copulation, including acoustic signals that help assess sperm competition and male density. While field crickets lack physical structures for controlling female movement after mating (Alexander, 1961), they employ behavioral tactics such as antennating females and engaging in aggressive displays to guard mates (Sakaluk, 1991; Wynn & Vahed, 2004). In the Pacific field cricket (*Teleogryllus oceanicus*), males uniquely use their calling song—a signal typically reserved for

mate attraction pre-copulation—as a mate-guarding tactic immediately following mating (Hagg et al., 2024). Due to the inability to produce another spermatophore for at least an hour after copulation (Chipchase et al., 2021; Dowling & Simmons, 2012; Loher, 1974; Nagao & Shimozawa, 1987), post-copulatory song likely serves to maintain female proximity and deter other potential mates, rather than attract new ones. Males producing this song after mating are more successful at keeping females close, which enhances their ability to guard them effectively (Hagg et al., 2024). Additionally, females exposed to post-copulatory song are more likely to leave the spermatophore attached for longer periods, further increasing male reproductive success (Hagg et al., 2024).

To investigate how the perception of sperm competition influences post-copulatory song as a mate-guarding tactic, we hypothesized that the use of song during mate guarding depends on the level of sperm competition risk. Specifically, we predicted that males exposed to conspecific male signals indicating higher sperm competition would invest more heavily in mate guarding and produce post-copulatory songs. To test this, we reared *T. oceanicus* juvenile males in either song-rich ('Song') or silent ('No Song') environments, isolating them before their hearing developed. This treatment allowed us to examine the effect of early exposure to sperm competition cues on mate-guarding behavior. In addition to the developmental acoustic treatment, males were exposed to 'Song' (high sperm competition) or 'No Song' (low sperm competition) environments one hour before mating to assess how immediate cues affect mate-guarding strategies. Investigating the influence of these real-time cues is particularly interesting because they reflect current sperm competition risk, which could enable males to adjust their behaviors dynamically in response to the immediate competition level.

Methods

Study system

We used *T. oceanicus* from a laboratory colony that originated from the Hawaiian island of Oahu. The population was established using approximately 100 crickets collected from the University of Hawaii-Manoa campus in 1993 and 1994 (Zuk et al., 1995). Each year the population is supplemented using offspring of multiple wild-caught females from Oahu to maintain a minimum of 100 breeding individuals in the colony at all times. Crickets are housed with ~25 individuals per container and these containers are consistently mixed to reduce inbreeding. Mating behaviors are influenced by circadian rhythms, with males and females typically being receptive to mating during

the dark hours (Loher & Orsak, 1985). All mating trials were conducted during the receptive window of time, specifically within the dark hours dictated by their circadian rhythm.

Experimental set-up

We reared crickets in Caron Insect Growth Chambers (model 6025, Caron Products, 128 Marietta, OH, U.S.A.) at 75% humidity, 25 °C and on a 12:12 photo-reversed light:dark cycle. Male crickets were isolated in individual 118 mL plastic cups as fourth (penultimate) instars when the primary hearing organ is first developed and functional (Ball et al. 1989). Crickets had an egg carton for shelter, food (Teklad rabbit chow), and water *ad libitum* in damp cotton pads. We used a 2x2 factorial design to examine the effects of acoustic rearing and immediate environments on post-copulatory song production, haphazardly assigning individuals to one of four treatments: (1) acoustic rearing environment ('Song' or 'No Song') and (2) acoustic immediate environment ('Song' or 'No Song').

Rearing environment

We isolated the penultimate instar males into one of two acoustic rearing environments: 'Song' or 'No Song'. Each environment consisted of an incubator on the same photo-reversed 12 L : 12 D cycle. In the 'Song' incubator, an average Oahu male calling song, constructed from >20 males recorded in the wild was broadcasted from Sony SRS-M30 speakers during the dark phase of the light:dark cycle, when *T. oceanicus* is active (Zuk et al. 2006). There was one speaker on each of the three shelves with 5-10 crickets surrounding the speaker at a given time (Balenger et al., 2016, 2018). Song intensities experienced by male crickets in the 'Song' environment ranged from 70 to 75 dB, which are typical of a male cricket singing from 50 cm (Bailey & Zuk, 2008, Bailey et al., 2010; Simmons et al. 2001). In the rearing 'No Song' incubator, the males were individually housed in identical fashion and remained in silence until we tested them. When males in the 'No Song' incubator eclosed and began producing song, they were moved to a separate incubator and placed inside foam boxes to minimize song exposure until testing. Males' positions in the 'Song' incubator were randomly rotated every other day to ensure that they experienced each level and angle from the speaker during rearing. Males in the 'No Song' environment were similarly manipulated on a daily basis to minimize handling differences between the treatments. Adult crickets are considered sexually mature 6 days after the final molt (Bailey & Zuk, 2008), so we used individuals 7-10 days post-eclosion for the mate-guarding trials.

Immediate environment

Following rearing in the acoustic environment, the male was placed in an immediate environment 1 hour before the mating trial. Half of the individuals housed in the acoustic rearing environment ‘Song’ were then individually placed in a foam box within a silent incubator for 1 hour prior to the mating trial. The same was true for the ‘No Song’ individuals where half remained in silence and the other half experienced 1 hour of song from the “Song” incubator. The experiment thus contained four groups: No Song-No Song (NN, n = 29), No Song-Song (NS, n = 29), Song-No Song (SN, n = 29), Song-Song (SS, n = 29).

Mate-guarding trials

We conducted mate-guarding trials in a 22–26 °C anechoic room under red lighting. After acoustic exposure was complete, we placed the male and an adult female from the Oahu population under a 118 mL plastic cup in a large mating arena (1.6 m x 1.6 m x 0.3 m) on the floor. To measure movement and proximity, the bottom of the arena was marked with a 50 mm by 50 mm grid (the average body length of a cricket including the antennae). Females were isolated from the population 1-3 days before the mating trial to increase the likelihood of sexual receptivity. The male and female were given 15 minutes to mate in the arena. If there was no successful mating, the male was given two additional attempts with different females (15 minutes each). If no mating occurred after 45 minutes (i.e, 3 females), the male was excluded from the experiment. Following the successful transfer of a spermatophore, the plastic cup was lifted and the male and female behaviors were recorded for 15 minutes. Over 90% of males who produce post-copulatory song do so within the first 15 minutes (Hagg et al., 2024).

Once we visually determined that the male had successfully transferred a spermatophore and the female dismounted the male, the 15-minute mate-guarding trial began. We observed the crickets directly, ensuring minimal disturbance by keeping a sufficient distance so as not to interfere with their behavior. We measured whether males produced post-copulatory song, when and for how long, the total time females spent within close proximity to the male (50 mm, within physical contact range using antennae), as well as if and when the attached spermatophore was removed by the female. The total duration of singing was measured by noting each instance with a stopwatch. We measured pronotum width to the nearest 0.01 mm in both males and females as a proxy for body size. After the mate-guarding trials, mated males and females were returned to the Oahu lab colony.

Statistical analyses

We used a binary logistic regression to test the hypothesis that the risk of sperm competition influences the likelihood of males singing post-copulatory songs. Our model included singing presence (0= no, 1= yes) as the response variable and acoustic rearing and immediate environments as fixed factors. We used two separate ANOVAs to test whether latency to sing and total time singing depended on the rearing or immediate environments or their interaction. Latency to sing and total time singing were log-transformed to meet the assumption of normally distributed residuals. Fixed effects included in the models were the rearing environment ('Song' or 'No Song'), the immediate acoustic environment ('Song' or 'No Song'), and their interaction. We explicitly tested the assumptions of normality and homogeneity of variances through these transformations and diagnostics before conducting the ANOVAs. Means are presented \pm 1 standard error of the mean. All statistical analyses were performed using R (version 4.1.2; R Core Team 2021). We predicted that if males from song-rich environments began singing sooner and sang for longer durations, it would support the hypothesis that the risk of sperm competition influences the likelihood of males singing post-copulatory songs.

Ethical note

Our research adhered to the guidelines for the use of animals in research and the legal requirements under the Animal Welfare Act of the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS permit no. P526P-21-07108 to M. Zuk).

Results

We assessed the mating and post-copulatory behaviors of 116 male crickets, 29 individuals per treatment group: NN, NS, SN, SS. We confirmed copulation in all 116 pairs. Roughly a third of the 116 males (35%, $n = 41$) produced a postcopulatory song after successfully transferring a spermatophore to the female. When males produced postcopulatory song during the mate-guarding period (15 min), females stayed within 50 mm of the males for a significantly longer time than when males did not produce postcopulatory song ($t_{106.58} = -2.47$, $P = 0.01$, time in proximity for non-singers = 41.01 ± 9.59 s; time in proximity for singers = 73.71 ± 9.15 s), confirming previous results (Hagg et al., 2024). Across all treatments, the distribution of males singing post-copulatory songs within 15 minutes was as follows: NN = 9, NS = 12, SN = 8, and SS = 12.

The interaction between the acoustic rearing environment and the immediate acoustic environment (binary logistic regression: $Z_{115} = 0.21$, $P = 0.83$) was not significant, indicating that the treatments did not affect the likelihood of males producing post-copulatory songs (Fig.1). Neither the acoustic rearing environment alone (binary logistic regression: $Z_{115} = -0.29$, $P = 0.77$) nor the immediate acoustic environment alone (binary logistic regression: $Z_{115} = 0.82$, $P = 0.41$) significantly influenced whether a male produced post-copulatory songs.

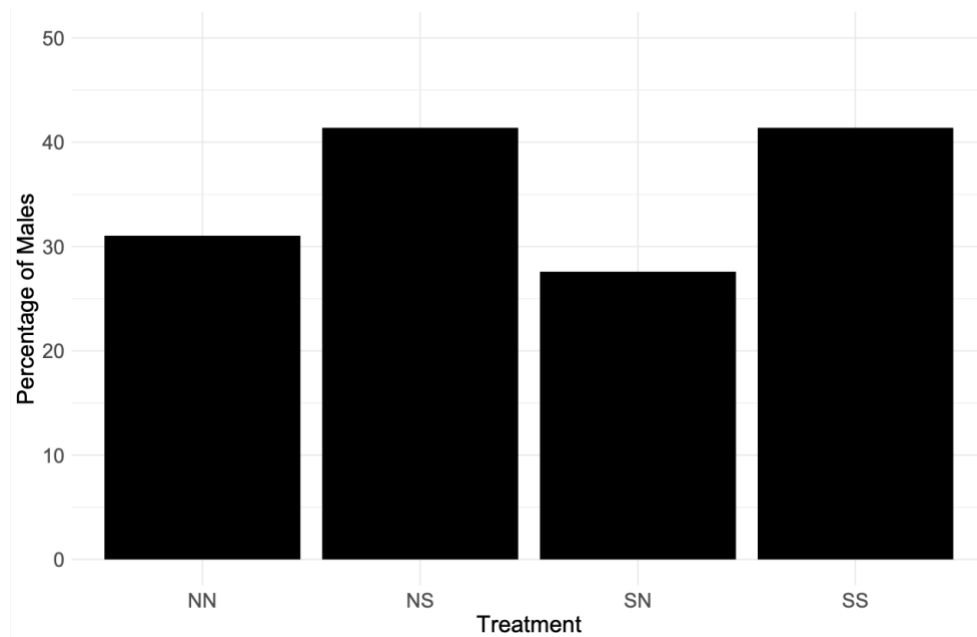


Figure 2.1. Percentage of males that sang post-copulatory mate-guarding song ($n = 29$ per group). NN = Rearing No Song and Immediate No Song. NS = Rearing No Song and Immediate Song. SN = Rearing Song and Immediate No Song. SS = Rearing Song and Immediate Song.

However, for males that did produce the post-copulatory song, the rearing environment influenced the latency to start singing post-copulation. There was no significant interaction between the rearing and immediate environments on the latency to start singing ($F_{(1,38)} = 0.01$, $P = 0.95$) (Fig. 2). Males reared in an environment without song began singing post-copulatory song more quickly than males reared in song-rich environments ($F_{(1,38)} = 5.48$, $P = 0.02$; NoSong: 178 ± 37.35 s, Song: 342.62 ± 60.89 s). The immediate environment did not significantly affect the time to start singing ($F_{(1,38)} = 1.28$, $P = 0.31$; NoSong: 302.22 ± 60.53 s, Song: 228.87 ± 47.61 s).

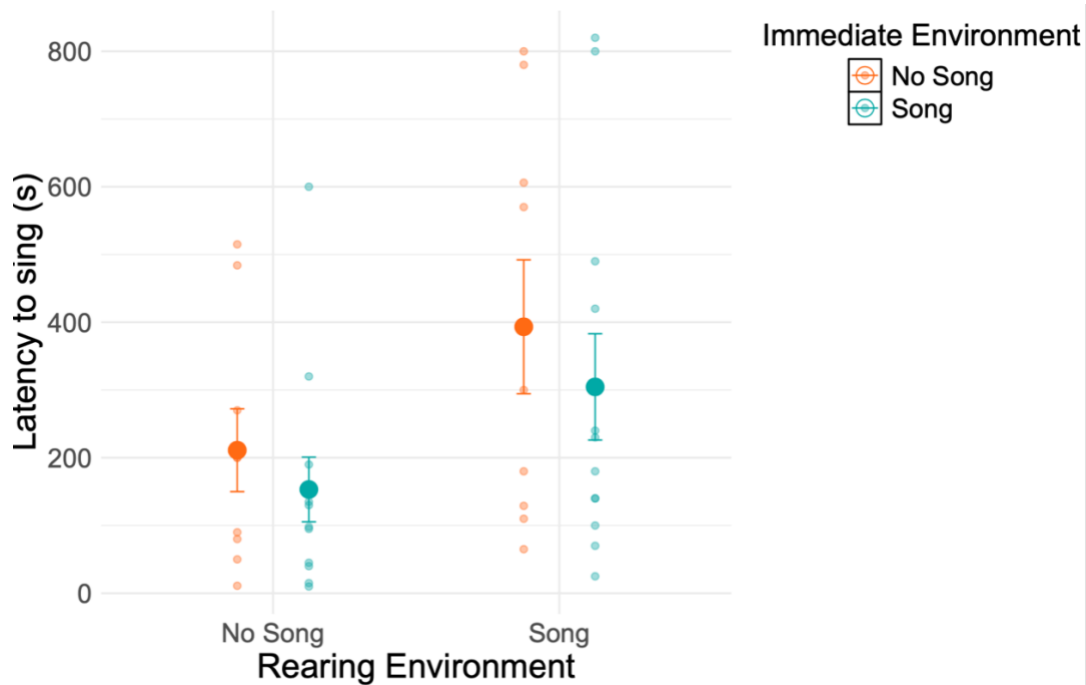


Figure 2.2. Time (seconds) *T. oceanicus* males took to begin singing post-copulatory song (~35%, n = 41 singers total: NN= 9, NS= 12, SN= 8, SS= 12), dependent on rearing environment and immediate exposure to conspecific male acoustic signals indicating high (song) or low (no song) risk of sperm competition. Mating trials were 15 minutes / 900 seconds total. Large circles depict the mean values with standard error bars for rearing treatments. Small circles represent individual data points as raw, untransformed data.

Additionally, the rearing environment significantly influenced the duration of singing when males did sing post-copulatory songs. There was no significant interaction between the rearing and immediate environments on time spent singing ($F_{(1,38)} = 0.31$, $P = 0.58$) (Fig. 3). Males reared in environments without song sang for longer than males reared in song-rich environments ($F_{(1,38)} = 6.38$, $P = 0.02$; NoSong: 142.62 ± 36.56 s, Song: 33.05 ± 9.53 s). The immediate environment did not significantly affect the amount of time males spent singing ($F_{(1,38)} = 1.59$, $P = 0.21$; NoSong: 108.61 ± 29.99 s, Song: 72.25 ± 28.13 s).

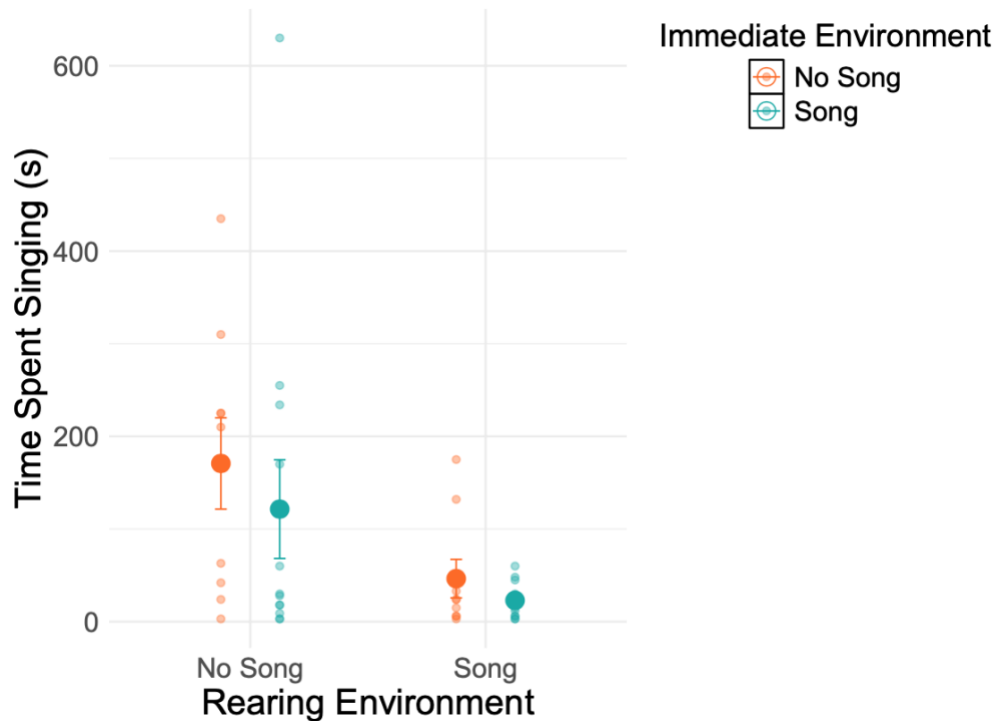


Figure 2.3. Total time (seconds) *T. oceanicus* males spent singing post-copulatory song (~35%, n = 41 singers total: NN= 9, NS= 12, SN= 8, SS= 12), dependent on rearing environment and immediate exposure to conspecific male acoustic signals indicating high (song) or low (no song) risk of sperm competition. Mating trials were 15 minutes / 900 seconds total. Large circles depict the mean values with standard error bars for rearing treatments. Small circles represent individual data points as raw, untransformed data.

Discussion

Our study investigated whether males adjust their mate-guarding behaviors, specifically post-copulatory song production, in response to cues indicating different levels of sperm competition risk. Contrary to our initial hypothesis, exposure to sperm competition cues through male acoustic signals did not increase the likelihood of males producing post-copulatory songs (Fig. 1). Interestingly, males reared in song-rich environments, indicative of high sperm competition risk, exhibited a longer latency to begin post-copulatory song (Fig. 2) and a shorter duration of singing compared to males reared in environments without conspecific male song (Fig. 3). In contrast, males reared in environments without acoustic cues from competitors sang more quickly and for longer periods while guarding. This counter-intuitive finding suggests that in high-risk environments, males may be conserving energy by minimizing post-copulatory song effort,

possibly reallocating resources to other reproductive strategies or seeking additional mates. Males in environments perceived to have fewer competitors and lower sperm competition risk might invest more in prolonged mate guarding, as the benefits of maintaining a mate outweigh the costs of extended singing. Conversely, in high-density environments, conserving energy for other reproductive investments could be more advantageous, reflecting a trade-off where males prioritize other forms of reproductive investment over prolonged singing. Additionally, we confirmed that post-copulatory song is an efficient strategy, as males who sang kept females in close proximity for a longer amount of time following mating (Hagg et al., 2024).

Our results challenge Alcock's (1994) hypothesis that mate guarding intensity increases with a more male-biased operational sex ratio. We found that sperm competition risk did not increase the presence of post-copulatory song; in fact, males exposed to high sperm competition risk had longer latencies to sing and shorter song durations. This suggests that, rather than intensifying mate guarding, males in high sperm competition environments prioritized energy conservation or seeking additional mating opportunities. While it is possible that high-density environments signal a level of competition that leads males to adopt a stealthier strategy (Knell, 2009), we think this is unlikely. Males in this species have little control over female movement without post-copulatory song, as females tend to leave after mating, making song critical to keeping the female nearby (Hagg et al., 2024).

Contrary to Alcock's (1994) hypothesis, we hypothesize that in environments with fewer competitors and lower sperm competition risk, male crickets may invest more in prolonged mate guarding due to perceived low mate availability (Parker, 2020). The trade-off between the costs of guarding and the effort needed to find a new mate influences this behavior, making extended mate guarding more beneficial when mates are scarce (Simmons, 2001; Tanner et al., 2019). For instance, in snapping shrimp (*Alpheus angulatus*), males in female-biased ratios are more likely to abandon recently mated females if they anticipate encountering new mates (Mathews, 2002). This suggests that when mates are scarce, maintaining a mate becomes more valuable. Similarly, in crickets, post-copulatory song use may be energetically costly (Hoback et al., 1997). Males use long-distance calling songs to attract females, followed by close-range guarding behaviors such as body judders and antennal fencing to keep the female close. In low male density environments with fewer competitor signals, investing in post-copulatory song could be more advantageous, as it helps to keep the female nearby, prevent her from removing the spermatophore, and increase the chances of remating with the same female. Conversely, in high-risk environments with many male

competitors, males may adopt a conservative approach by conserving energy and forgoing prolonged singing in order to produce a new spermatophore and avoid short term energy costs and predatory threats (Ramm & Stockley, 2014).

The observed variation in post-copulatory song behavior, (i.e., which males sang and which males did not sing), likely reflects a trade-off between the cost of singing and the perceived level of sperm competition. Males may adjust their strategies based on the relative costs and benefits of post-copulatory song versus other reproductive investments. Males perceiving increased sperm competition risk may allocate resources to other strategies, such as enhancing reproductive tissue or ejaculate quality (Bailey et al., 2010; Gray & Simmons, 2013), rather than investing in post-copulatory song. Research has shown that males reared in song-dense environments invest more in reproductive tissue (Bailey et al., 2010) and produce ejaculates with a greater percentage of viable sperm (Gray & Simmons, 2013). This strategic shift suggests that enhancing one aspect of reproduction often requires reducing investment in another. Such trade-offs indicate that reproductive strategies can coexist within a species based on environmental conditions and individual constraints. If males exhibit significant plasticity in sperm production, this may reduce the need for plasticity in mate-guarding behaviors like post-copulatory song. Males with lower-quality sperm might compensate with behaviors such as post-copulatory song, while those focusing on enhancing sperm quality might prioritize other traits.

While perceived sperm competition risk did not influence the likelihood of males producing post-copulatory songs, it did affect the latency and duration of singing when it occurred. This highlights that early developmental cues have a more substantial impact on shaping long-term behavioral strategies than immediate pre-mating cues, indicating that early life experiences can have enduring effects on reproductive tactics (Hoverman & Relyea, 2007). It is surprising that immediate environmental cues, which provide an accurate depiction of current sperm competition risk (i.e., short-term, real-time risk), do not influence post-copulatory song production, especially in rapidly changing environments where one might expect males to optimize fitness by adjusting behaviors based on environmental and social contexts (Snell-Rood, 2013). Perhaps constraints exist on rapid behavioral adjustments, likely due to the costs of maintaining plasticity and varying selective pressures (Botero et al., 2015). Once established during early development, some phenotypic traits may be resistant to modification later in life due to these developmental constraints (DeWitt et al., 1998). This robustness in strategies developed during rearing could explain why certain behaviors, like mate guarding duration in bulb mites, remain resistant to short-

term changes in sperm competition risk (Skwierzyńska et al., 2018). Therefore, while male crickets can assess sperm competition risk, their ability to adjust mating strategies in response to immediate environmental changes may be limited by developmental constraints on how and when information is integrated into behavioral responses.

In the case of *T. oceanicus*, crickets may not always receive accurate cues about sperm competition risk from competitor male acoustic signals. In some Hawaiian populations, a mutation has resulted in males lacking song-producing wing structures, making a proportion of males silent and unable to produce songs (Zuk et al., 2006). This adaptation protects them from acoustically-orienting parasitoids but limits their ability to attract females and engage in mate guarding (Heinen-Kay et al., 2020; Heinen-Kay, Strub et al., 2019; Heinen-Kay, Urquhart et al., 2019). Silent males are less effective at mate guarding due to their inability to produce post-copulatory songs (Hagg et al., 2024). Consequently, in populations with silent males, acoustic signals may not accurately reflect the social environment or sperm competition risk, suggesting that other cues, such as cuticular hydrocarbons or physical interactions, may offer a more reliable indication of mate availability and competition risk (Thomas & Simmons, 2009). This shift in cue reliability may favor males that rely on these more accurate physical cues over acoustic signals.

When acoustic signals are absent, masked, or altered, it can lead to a mismatch between perceived and actual competition. For example, electric fish (*Brachyhypopomus pinnicaudatus*) use 'cloaking' strategies to avoid detection, leading other males to underestimate competitor density (Stoddard & Markham, 2008). Similarly, light pollution disrupts firefly (*Photuris versicolor*) signaling, causing inaccurate perceptions of competition despite unchanged population density (Firebaugh & Haynes, 2016). Such disruptions can distort perceived competition and influence suboptimal reproductive strategies. In *T. oceanicus*, the presence of silent males may mislead males about competition levels, potentially impacting mating behaviors. Future studies could explore how the use of post-copulatory song as a mate-guarding tactic differs across populations with varying levels of signal reliability.

Chapter 3

Tradeoff between sperm quality and post-copulatory mate-guarding song use in the Pacific field cricket, *Teleogryllus oceanicus*

Abstract

Males often face trade-offs between competing reproductive investments, such as ejaculate quality and mate-guarding behaviors. In the Pacific field cricket (*Teleogryllus oceanicus*), some males produce a post-copulatory acoustic signal that resembles their long-distance calling song, despite being temporarily incapable of remating. We tested whether post-copulatory song is associated with sperm viability. Although males that sang after mating showed lower sperm viability than non-singers, overall viability values were unusually low and highly variable. The majority of males had fewer than 500 sperm, and viability scores were unusually low (~4-6%). These patterns likely reflect methodological limitations, including delayed sperm staining and longer exposure to saline buffer, that complicate interpretation of the results and preclude strong conclusions about biological differences in sperm viability.

Introduction

In species where females mate with multiple males, the sperm from those males must compete to fertilize the available eggs (Simmons, 2001). This process, termed sperm competition, has led to the evolution of male traits including mate guarding, the production of anti-aphrodisiac scents, or the formation of copulatory plugs and other physical barriers to block rival males (Birkhead & Møller, 1992). Males may also adapt to sperm competition by increasing mating frequency, prolonging copulation, or modifying sperm quantity or quality to enhance fertilization success (Wigby & Chapman, 2004). Understanding how males balance these divergent reproductive strategies offers critical insight into the evolutionary trade-offs that shape sexual behavior, energy allocation, and reproductive success under intense selection pressure.

Mate guarding is a well-documented strategy to limit sperm competition, wherein males protect females from rivals through physical restraint or territorial behaviors (Alcock, 1994). It can also help prevent interruption of sperm transfer and discourages females from prematurely ending copulation (Bussiégre et al., 2006; Hockham & Vahed, 1997). However, mate guarding can be costly, as it may reduce a male's opportunities to forage or pursue additional mating opportunities (Simmons et al., 2003). As a result, males may adjust their mate-guarding investment based on the perceived risk of sperm competition. In species where guarding reliably deters rivals or ensures prolonged sperm transfer, males may reduce investment in reproductive tissues. For instance, in salt-marsh amphipods, *Gammarus duebeni*, males exposed to intense competition produced fewer and shorter sperm over time, prioritizing mate guarding over sperm production (Arundell et al., 2014). Conversely, when mate guarding is less effective (such as in species with internal sperm storage, sperm mixing, or cryptic female choice), males taxa often invest more in ejaculate quality and quantity to boost their chances of successful fertilization (Delbarco-Trillo, 2011).

Males can rapidly adjust their energetically costly ejaculate investment, modulating sperm output or quality, in response to perceived sperm competition risk or future mating opportunities (Hayward & Gillooly, 2011). For example, in Arctic charr (*Salvelinus alpinus*), dominant males produce fewer sperm with lower velocity but higher sex steroid concentrations, reflecting reduced investment in sperm following dominance acquisition, supporting models predicting that subordinate or less-favored males invest more in ejaculate quality (Rudolfson et al., 2006). However, which sperm traits are most strongly selected for can differ markedly across

taxa. In Atlantic salmon (*Salmo salar*), sperm velocity is the strongest predictor of fertilization success, while traits like sperm number, length, and longevity are less important (Gage et al., 2004). In the freshwater snail (*Viviparus ater*), larger nonfertile oligopyrene sperm play a critical role in paternity success (Oppliger et al., 2003). Additionally, males of many species can adjust sperm motility and swimming speed to improve fertilization chances (Wedell et al., 2002).

Investment in sperm is not without trade-offs, particularly when combined with mate guarding efforts. This trade-off is central to sperm competition theory, which assumes males have a fixed resource pool for both attracting females and securing fertilizations (Parker, 2006). In species where mate guarding effectively prevents further copulations, males may prioritize guarding and reduce sperm investment. In contrast, when guarding is less effective or sperm competition is high, males may shift focus to sperm investment, even if it means decreasing mate guarding efforts (Simmons & Emlen, 2006). This trade-off is evident in species with alternative male strategies, where non-guarding males often invest more in sperm production due to greater sperm competition pressures. In species with alternative mating tactics, like the Mediterranean wrasse (*Symphodus mediterraneus*), non-guarding males invest more in sperm quality as a compensatory strategy, since they cannot monopolize mates through guarding. In contrast, guarding males invest less in ejaculates, relying instead on their ability to keep rivals away (Alonzo & Warner, 2000).

Crickets are a useful system for examining how males balance competing post-copulatory strategies, such as mate guarding and sperm investment. Females are capable of storing sperm, and frequently mate with multiple males, creating intense post-copulatory sexual selection (Vaughan, 1995). In response, males employ both behavioral and physiological tactics to maximize their fertilization success. For example, they adjust sperm quality in response to the level of sperm competition, producing ejaculates with a higher proportion of viable sperm under moderate risk, but reducing this investment when competition becomes intense (Simmons et al., 2007; Thomas & Simmons, 2007). These relationships extend to other energetically costly reproductive traits: males that invest more in courtship song tend to produce higher-quality ejaculates (Simmons et al., 2010), while allocation to attractive cuticular hydrocarbon profiles can reduce sperm quality and competitive fertilization success (Thomas & Simmons, 2009). Together, these findings suggest that males must strategically allocate limited energetic resources across multiple reproductive tactics in response to social and environmental cues.

After male crickets transfer a spermatophore, a capsule containing sperm, to the female during mating, males remain in close proximity to prevent its premature removal before sperm transfer is complete (Alexander, 1961; Hockham & Vahed, 1997). Guarding behaviors include antennal contact, body judders, aggressive posturing back and forward, and headbutting (Sakaluk, 1991; Simmons, 1991; Wynn & Vahed, 2004; Bussiere et al., 2006). While cricket song is typically associated with pre-copulatory mate attraction, we recently discovered that males from Hawaiian populations of the Pacific field cricket (*Teleogryllus oceanicus*) produce this same acoustic signal immediately after mating, well before they are physiologically capable of generating a new spermatophore, a process that takes over an hour in *Teleogryllus* species (Loher, 1974; Nagao & Shimozawa, 1987; Chipchase et al., 2021). This timing rules out a mate attraction function, which had been the signal's only previously suggested role. Instead, our recent work shows that post-copulatory song functions as a mate-guarding tactic, increasing the likelihood that phonotactic females remain nearby and retain the spermatophore longer (Hagg et al., 2024). Prolonged attachment enhances sperm transfer and fertilization success, making this behavior a critical component of male post-copulatory strategy. However, despite its apparent importance, the relationship between post-copulatory song and other male reproductive investments, particularly sperm quality, remains unknown.

In this study, we test the hypothesis that *T. oceanicus* males strategically allocate resources between post-copulatory acoustic signaling and sperm quality. Specifically, we predict that males investing more in sperm quality will not produce post-copulatory song, whereas those producing lower-quality ejaculates will increase their investment in song. By quantifying song traits (presence, latency, duration) alongside sperm viability, we provide the first test of how these strategies co-vary. Here, we examine the trade-offs shaping male reproductive investment and the integrative nature of post-copulatory sexual selection.

Methods

Study system

We used crickets from a large, outbred laboratory population originally derived from eggs laid by wild-caught females from Hilo, Hawai'i. This population is supplemented annually with wild-caught eggs to maintain genetic diversity. Crickets were housed in multiple 15 L plastic containers at consistent densities of approximately 25 adults per container. Each container provided ad libitum access to rabbit food (Teklad), moist cotton for water and oviposition, and

cardboard egg cartons for shelter. All crickets were kept in a Caron Insect Growth Chamber set to 26°C with 75% relative humidity and a photo reversed 12:12 light–dark cycle. All crickets used in this study were isolated at the penultimate instar stage, prior to eclosion and sexual maturation, to ensure unmated status for mating trials (Bailey & Zuk, 2008).

Experimental set-up

Male crickets were isolated in individual 118 mL plastic cups as fourth (penultimate) instar nymphs. Each male was housed in a 118 mL plastic cup, equipped with food (4-5 pellets), an egg carton for shelter, and a water vial. The cups were changed twice a week to maintain cleanliness and ensure that the crickets had access to fresh food and water. Each cricket was isolated with a label indicating the date of isolation. Adult crickets are considered sexually mature 6 days after the final molt (Bailey & Zuk, 2008), so we used individuals 7–9 days post-eclosion for the mate-guarding trials. Adult females were isolated 1–3 days prior to the trial to increase sexual receptivity.

Mating trials and mate-guarding observations

We conducted mate-guarding trials in a 22–26°C anechoic room under red lighting to minimize disturbance. A sexually mature male and female were placed under a 118 mL plastic cup in a large mating arena (1.6 m x 1.6 m x 0.3 m), with the arena floor marked with a 50 mm by 50 mm grid to track locations. The male and female were given 15 minutes to mate. If mating was unsuccessful, the male was given two additional opportunities on subsequent days, with each attempt lasting 15 minutes. On the second day, the male was paired with a different female for 15 minutes, and if no mating occurred, a third attempt was made on the third day with a new female. If no mating occurred after the third attempt, the male was excluded from the experiment.

After successful spermatophore transfer was visually determined, the plastic cup was removed. We collected the spermatophore from the female using forceps. The spermatophore was placed in a 1.7 mL microcentrifuge tube containing 50 μ L of Grace’s insect solution (Catalog number:11605094) and then opened with sharp forceps. The female was quickly returned to the arena and the 15-minute mate-guarding trial began. This period was crucial as over 90% of males that produce post-copulatory song do so within the first 15 minutes (Hagg et al., 2024). During the mate-guarding trial, we recorded whether the male produced post-copulatory song, and the timing and duration of song production. Pronotum width was measured to the nearest 0.01 mm in

both males and females as an estimate of body size. Once the trial was completed, the male and female were returned to the colony and excluded from future use.

Sperm viability assessment

Following established protocols for assessing sperm quality in this species (Simmons, 2012; Simmons, Tinghitella, & Zuk, 2010; Thomas & Simmons, 2009), we assessed sperm viability using the LIVE/DEAD™ Sperm Viability Kit (Molecular Probes, L-7011), which uses fluorescence-based staining to differentiate between live and dead sperm cells. Following mating (see above), spermatophores were removed from the female and placed into a 1.7 mL tube containing 50 μ L of Grace's Insect Medium (Gibco™, Grand Island, New York, USA). The spermatophore was cracked open by pinching five times with fine forceps to ensure sperm were released into the medium. A 20 μ L aliquot of sperm in Grace's medium was then transferred to a new amber, light-resistant tube. To stain the sperm, 1.3 μ L of a 1:50 dilution of SYBR® 14 nucleic acid stain (final concentration: 1.27 μ M) was added. This stain labels live sperm with green fluorescence. The sample was incubated for 10 minutes in the dark. After incubation, 2.5 μ L of propidium iodide (final concentration: 157.5 nM) was added. This dye labels nucleic acids of membrane-compromised sperm with red fluorescence. The sample was incubated for another 10 minutes in the dark. After the final incubation, 10 μ L of the stained sperm mixture was loaded onto a hemocytometer (MilliporeSigma, Millicell Disposable Hemocytometer, Catalog No. MDH-4N1). All steps were performed in the dark at room temperature. After each step, the sample was gently mixed using three short pulses on a benchtop vortexer.

Imaging

Samples were imaged on an ECHO Revolve R4 Fluorescence Microscope fitted with an extralong working distance (ELWD) universal condenser, motorized LED fluorescence CMOS monochrome camera, FITC and TxRED LED and filter sets, and a 10x PLAN Fluorite objective lens. For each sample, 16 images were acquired (four images for each of four major grid squares). The acquisition settings for FITC, TxRED, and transmitted light were consistent across all samples. All images were blinded prior to analysis to eliminate observer bias, and manually counted. To assess repeatability, I recounted 80 previously analyzed images, selected from the image sets of five randomly chosen specimens.

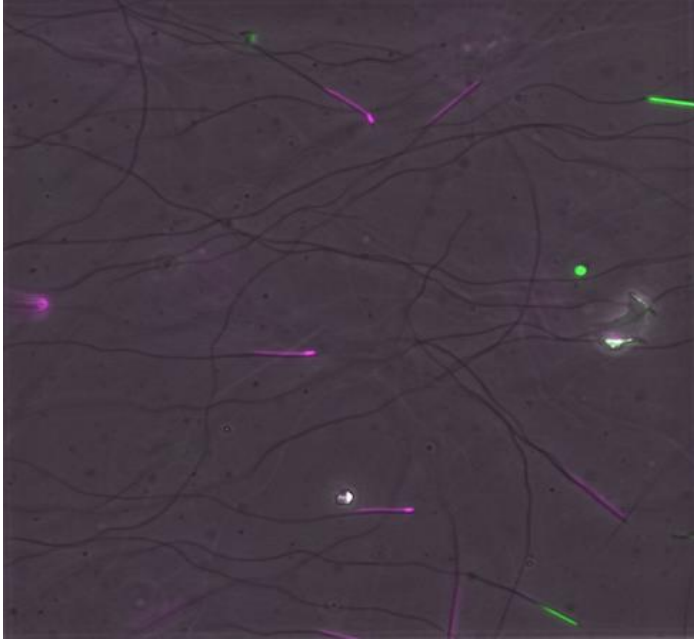


Figure 3.1. Example fluorescence microscopy image (x10 magnification) of *Teleogryllus oceanicus* sperm stained with SYBR® 14 (live, green) and propidium iodide (dead, red) using the LIVE/DEAD™ Sperm Viability Kit.

Statistical analyses

To determine whether males that produced post-copulatory song had more viable sperm or a greater number of sperm, we compared the percent of live sperm and the total number of sperm between singers and non-singers using t-tests. To assess what factors predicted the likelihood of producing a post-copulatory song, we used a binomial logistic regression with male and female body size, the number of females a male had previously encountered, and time to mate as predictors. To test whether the relationship between sperm production and viability differed based on postcopulatory singing behavior, we used a linear model including total sperm count, singing status, and their interaction to predict percent live sperm. To determine whether sperm viability was associated with post-copulatory song traits (time to start singing/latency, song duration), body size (male and female), time to mate, or number of prior female exposures, we ran a multiple linear regression with percent live sperm as the response variable. Model assumptions were checked using diagnostic plots, and no distributions violated the assumptions of the tests. All statistical analyses were performed in R (version 4.1.2; R Core Team, 2021).

Ethical note

Our research adhered to the guidelines for the use of animals in research and the legal requirements under the Animal Welfare Act of the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS permit no. P526P-21-07108 to M. Zuk).

Results

Post-copulatory song

The counts of live, dead, and total sperm, as well as the percentage of live sperm, showed $\geq 95\%$ consistency, indicating high intra-observer reliability. Exactly half of the 86 males (50%, $n = 43$) produced a post-copulatory song after successfully transferring a spermatophore to the female. Males who did sing, sang an average of 46.39 ± 12.34 seconds (mean \pm se) during the 15-minute mate-guarding trial. Neither male size (estimate \pm se = -0.72 ± 0.76 , $z_{85} = -0.95$, $P = 0.34$), female size (estimate \pm se = -0.03 ± 0.89 , $z_{85} = -0.03$, $P = 0.98$), the number of females a male was exposed to before mating (estimate \pm se = -0.75 ± 0.44 , $z_{85} = -1.72$, $P = 0.09$), nor the time it took the male to mate (estimate \pm se = -0.001 ± 0.0008 , $z_{85} = -1.37$, $P = 0.17$) significantly affected the likelihood of producing a post-copulatory song.

Sperm viability

Males who did not produce a post-copulatory song had a significantly higher percentage of live sperm compared to males who did sing (estimate \pm se = 2.52 ± 1.06 , $t = 2.41$, $df = 80.20$, $P = 0.018$; Fig. 1). The mean percentage of live sperm was 6.74 ± 0.82 for males who did not sing, and 4.22 ± 0.65 for those who did (Fig. 1).

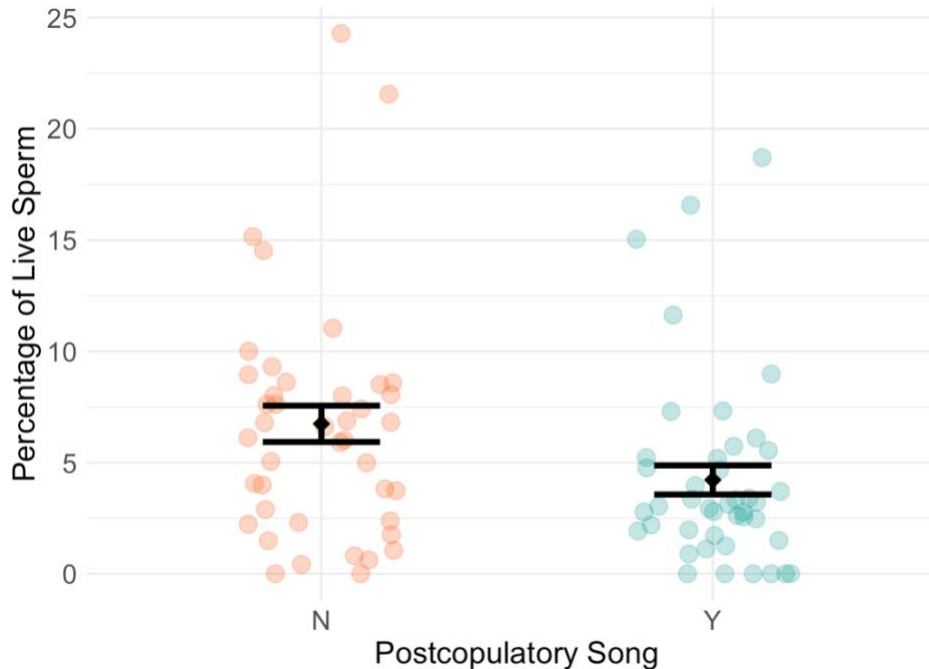


Figure 3.2. Percentage of live sperm in *Teleogryllus oceanicus* males that did or did not produce a post-copulatory mate-guarding song ($n = 86$; $N = 43$, $Y = 43$).

We tested whether total sperm number predicted sperm viability and whether this relationship differed based on males being either post-copulatory singers or non-singers. A linear model including an interaction between total sperm number and singing revealed a significant interactive effect (estimate \pm SE = 0.011 ± 0.005 , $t_{82} = 2.08$, $P = 0.041$), indicating that the relationship between sperm number and percent live sperm depended on whether males sang post-copulation. Specifically, males who sang had a lower average percentage of live sperm (estimate = -4.84 ± 1.64 , $P = 0.004$), but among singers, sperm viability increased more steeply with total sperm count. To confirm the robustness of this effect, we reran the model excluding one individual (ID = H174) with an unusually high sperm count ($>1,000$). The interaction between total sperm and singing remained significant (estimate \pm SE = 0.017 ± 0.006 , $t_{81} = 2.82$, $P = 0.006$), and the negative effect of singing on sperm viability was even stronger (estimate = -5.97 ± 1.69 , $P = 0.0007$) (Fig. 2). These results suggest that males who sing after mating tend to have lower sperm viability overall, but among those males, higher sperm counts are more strongly associated with increased viability.

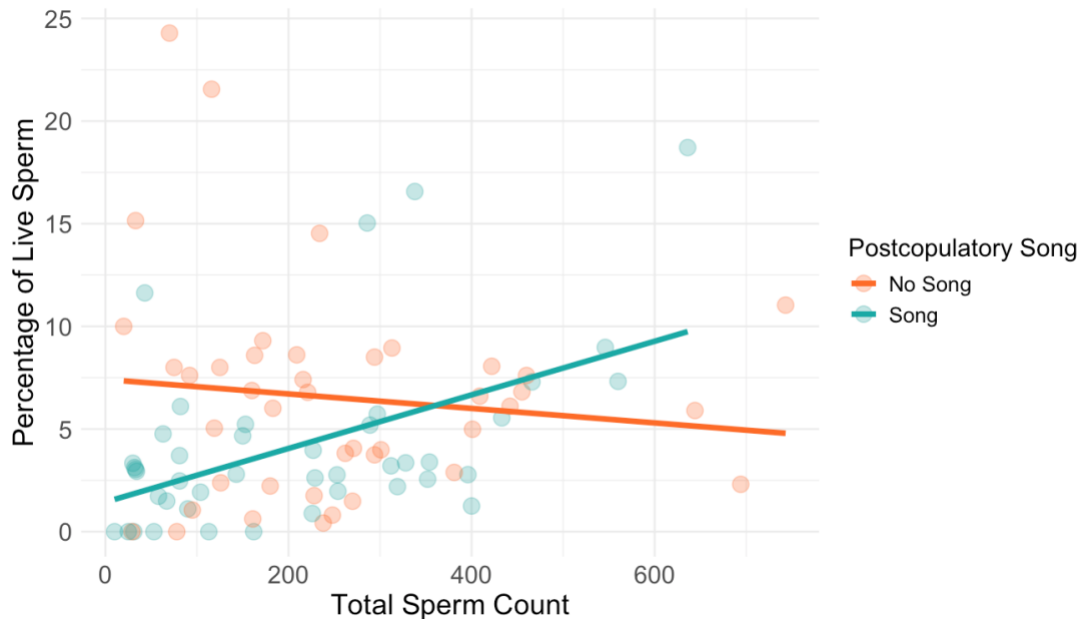


Figure 3.3. Relationship between total sperm count and the percentage of live sperm in *Teleogryllus oceanicus* males ($n = 86$). One outlier with a total sperm count exceeding 1,000 was removed to improve visualization and model fit.

The total number of sperm was not significantly related to post-copulatory song use (estimate \pm se = 272.23 ± 27.12 for non-singers, 212.53 ± 28.87 for singers, $t = 1.46$, $df = 79.86$, $P = 0.15$). Post-copulatory song traits were not associated with differences in sperm viability as neither the time to start singing (latency) (estimate \pm se = -0.005 ± 0.006 , $t_{36} = -1.35$, $P = 0.18$), time spent singing (estimate \pm se = 0.003 ± 0.005 , $t_{36} = 0.68$, $P = 0.50$), nor time to mate (estimate \pm se = -0.002 ± 0.003 , $t_{36} = -0.84$, $P = 0.41$) were associated with significant differences in percentage of live sperm. Male body size (estimate \pm se = -0.05 ± 2.33 , $t_{36} = -0.02$, $P = 0.98$), female body size (estimate \pm se = 3.57 ± 2.90 , $t_{36} = 1.23$, $P = 0.23$), and the number of females a male was exposed to prior to mating (estimate \pm se = -2.69 ± 1.53 , $t_{36} = -1.75$, $P = 0.09$) also had no significant effect on the percentage of live sperm.

Discussion

This study investigated the potential relationship between post-copulatory song and sperm viability in *Teleogryllus oceanicus*. Although we observed lower sperm viability in males that sang after mating compared to those that did not, these findings should be interpreted with considerable skepticism. The absolute difference in viability between singers (4.22%) and non-

singers (6.74%) was minimal, and more critically, both values were dramatically lower than what has been consistently reported in previous research. Prior studies have found sperm viability ranging from 48–93% (mean ~78%) when assessed directly from seminal fluid (Simmons & Beveridge, 2011). Given this substantial discrepancy, the pattern we observed is likely not a reflection of true biological variation, but rather an artifact of methodological limitations.

One likely contributor to these atypically low values is the timing of sperm staining. Unlike previous studies that stained sperm immediately after ejaculation, we began staining approximately 30 minutes post-spermatophore transfer. This approach was intended to align with the natural timeframe of sperm transfer in *T. oceanicus* (Simmons et al., 2003; Tuni et al., 2013), but evidence from related species suggests that sperm degrade rapidly in saline solution, with viability declining drastically after 10 minutes (Gress & Kelly, 2011). Given this, it is highly probable that the removal of sperm from protective seminal fluid and prolonged exposure to saline buffer significantly reduced viability across all samples. Future studies will directly assess how sperm viability changes over time in saline and seminal fluid to confirm these results.

In addition, our sperm counting method diverged from standard protocols. Rather than selecting the first 500 sperm (Simmons & Beveridge, 2011), we counted all sperm within a standardized hemocytometer grid. While this avoided sampling bias, it introduced variability due to low sperm counts in most males (mean = 243, range = 10–1,059). Potential clumping of sperm, which was not controlled for, may have further confounded both count accuracy and viability estimation. Given these compounding issues, it is difficult to assign any biological meaning to the viability values we obtained.

Moreover, sperm viability represents just one facet of ejaculate quality. Characteristics such as sperm number, motility, and the biochemical makeup of seminal fluid also influence fertilization potential and may vary independently. It is possible that post-copulatory song is related to other components of ejaculate investment not captured by our methods. Future research should adopt a broader approach to evaluating ejaculate traits in relation to post-copulatory song behavior.

In conclusion, while we initially explored whether post-copulatory song might be linked to changes in sperm viability, methodological limitations appear to be the dominant influence on

the values we recorded. These preclude any strong inferences about a biological relationship between post-copulatory song and sperm viability. Instead, our findings underscore the importance of methodological rigor when evaluating delicate traits like sperm viability and highlight areas for refinement in our future work.

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