Title: The value in the details: Copulatory courtship behavior in soldier flies and bean beetles

Abstract

William Eberhard's legacy attests to the value of detailed behavioral observations. The significance of this descriptive approach can be illustrated by the phenomenon of copulatory courtship, or courtship that occurs during and following copulation. Through meticulous descriptions of copulatory behavior in numerous arthropods, Eberhard has demonstrated that copulatory courtship is common and widespread, and is likely a behavior shaped by cryptic female choice. Here I will highlight the importance of detailed observations of copulatory behaviors in both an understudied tropical species and a model organism. In the soldier fly *(Merosargus cingulatus)*, males perform copulatory courtship by waving their hind legs and tapping the female abdomen during copulation. In the absence of this behavior, females fail to oviposit after copulation, which is one mechanism of cryptic female choice. In bean beetles (*Callosobruchus maculatus*)*,* males tap the female body with their antennae immediately before intromission. The courtship function of this antennation behavior is being investigated, and the results presented here support a role in copulatory courtship. These examples demonstrate that careful observations can generate novel insights, even in well-studied species.

Key words: Copulatory courtship, cryptic female choice, antennation, behavioral observations

Introduction

William Eberhard has had an immeasurable influence in the fields of evolutionary biology and sexual selection. He has also had an inordinate influence on the career paths of countless biologists as a mentor and educator. I am one of many students who were fortunate to benefit from his mentorship, and working with Bill Eberhard has profoundly shaped my research program. As an undergraduate student in Brazil, I was drawn to Bill Eberhard's work on cryptic female choice and copulatory courtship: Not only was Bill Eberhard shedding light into a fascinating and markedly understudied topic, but he was also conducting simple and elegant experiments that required few resources, could be accomplished under field conditions, and relied mostly in detailed observations of behavior (Eberhard 2009, Aisenberg and Eberhard 2009, Peretti and Eberhard 2010, Eberhard 2015). By focusing on a variety of mostly understudied tropical arthropod species, Bill Eberhard illustrated that cryptic female choice and copulatory courtship are not just important phenomena in sexual selection, they are common: If you seek, through detailed and careful observations of behavior, you are likely to find them – perhaps even in species in your own backyard (Eberhard 1991, Eberhard 1994, Eberhard 1996). This paradigm was extremely appealing to an undergraduate student in a Latin American institution with relatively few resources but a vast, accessible diversity of arthropods.

Later on, as a graduate student, I had the opportunity to work at the Smithsonian Tropical Research Institute in Gamboa, Panama. I had observed what I believed to be copulatory courtship behavior in a soldier fly species (Barbosa 2009). This became the focus of my dissertation, and Bill Eberhard became my mentor at STRI and a member of my thesis committee. I had the pleasure to conduct field work in his backyard in Gamboa for multiple summers, and being trained by Bill Eberhard deeply shaped my work ever since – both my research program, which has always involved detailed behavioral observations, and my commitment to undergraduate education. Here I will describe two case studies of copulatory courtship: one in an understudied tropical species, the soldier fly *Merosargus cingulatus* (Diptera: Stratiomyidae, figure 1A), and one in a model species for sexual selection studies, the bean beetle *Callosobruchus maculatus* (Coleoptera: Chrysomelidae, figure 1B).

Copulatory courtship

For a long time, the field of sexual selection focused solely on processes happening prior to copulation. However, the past few decades have accumulated a vast amount of evidence that processes during or after copulation play an important role in sexual selection. The role of sperm competition was quickly accepted as an important process shaping male physiology, morphology and behavior, as well as being an almost ubiquitous process among animals (Parker 1970, Birkhead 1998, Simmons 2001). Cryptic female choice, on the other hand, did not receive as much attention at first (Thornhill 1983), and the role it plays in sexual selection was controversial until recently (Birkhead 1998, Birkhead 2000, Kempenaers et al. 2000). Its importance for sexual selection has finally been acknowledged, thanks largely to Bill Ebehard's work which extensively document this phenomenon (Eberhard, 1993, 1996, 2001; Eberhard & Huber, 1998). Cryptic female choice has remained an important topic in sexual selection ever since (Brennan & Prum, 2012; Firman et al., 2017; Orbach et al., 2017).

Cryptic female choice has important evolutionary consequences, as it can shape behavior and morphology for both males and females (Eberhard 1996, Peretti and Aisenberg 2015). Perhaps one of the best examples of a trait that probably evolved as a result of cryptic female choice is copulatory courtship, or courtship that occurs during copulation. As pointed out by Bill

Eberhard, this behavior might seem paradoxical at first, since it occurs after a male has already obtained his mate (Eberhard 1991). Bill Eberhard demonstrated copulatory courtship to be widespread and established criteria for a behavior to be considered copulatory courtship. Copulatory courtship is a widespread behavior in arthropods: a survey of 131 species found that it occurs in 81% of them (Eberhard 1994). In addition, in over 80% of the species where copulatory courtship was observed, the male abandoned the female after mating, suggesting that the behavior was not under selection for having the female remate with that male, and supporting the idea that copulatory courtship is aimed at cryptic female choice (Eberhard 1994). Subsequent studies have shown that copulatory courtship affects several postcopulatory female processes, such as sperm usage, oviposition timing, sperm dumping and remating (Eberhard 1991, Tallamy et al. 2002, Barbosa 2009, Edvardsson and Arnqvist 2000, Peretti and Eberhard 2010). Evidence for copulatory courtship has been reported in insects (Brown et al. 1996; Eberhard 1994; Evardsson and Göran 2000; Tallamy et al. 2003), birds (Mateos and Carranza 1999; Borgia 1995), and amphibians (Doty and Welch 2001). In many species, particularly insects, females prefer more stimulating copulatory courtship (Ryan & Keddy-Hector, 1992). Still, copulatory courtship may remain hidden in well-studied species, as it entails subtle behaviors that could easily be undetected without careful, detailed observations.

Copulatory courtship in the soldier fly *Merosargus cingulatus*

The genus *Merosargus* (Diptera: Stratiomyidae) encompasses over one hundred species of soldier flies that are found in a variety of neotropical habitats (James and McFadden 1971). In several species, the larvae are known to be detritivores that develop on a variety of plant matter, such as *Heliconia* plants (Seifert and Seifert 1976, 1979), or a variety of decomposing fruits, recently cut grass, and fallen stems of succulents (Woodley 2001). The larvae of *Merosargus cingulatus* feed on a variety of decomposing vegetable matter, and adults aggregate near this substrate (Woodley 2001). Males defend territories continuously during the times of the day when females search for oviposition sites. When a female approaches the oviposition substrate, the territory holder attempts to grab her, and copulation ensues if he is successful. Females appear to be attracted by the oviposition site, and do not seem to resist mating once they are grasped by a male (F. Barbosa, personal observation).

There does not appear to be precopulatory signaling or courtship in this species, but there is copulatory courtship: a male typically grabs a female in flight, and following intromission, the mating pair lands on vegetation on or near that male's territory. Then, the male alternates between two behaviors for the entire duration of copulation: tapping the female's abdomen with his hind legs, which have contrasting black and white stripes, and waving his hind legs in the air (Barbosa 2009, 2011, 2012). Both males and females mate several times (often multiple times on the same day), and females oviposit after most, but not all, copulations, on or near the male's territory. When a female leaves a male's territory without ovipositing, another male will likely grab and mate with her before she has the chance to oviposit again (F. Barbosa, personal observation). This means there is the potential for cryptic female choice by female control of oviposition timing: a female may choose not to oviposit after mating with a less preferred male, a behavior which may decrease that male's fertilization success.

I tested this hypothesis through a field experiment where I compared the oviposition behavior of females that mated with males of two groups: individuals who did not perform copulatory courtship and individuals who did (Barbosa 2009). I set up oviposition substrate in the field, which attracted both males and females. I captured males, gave them a unique marking and assigned them to one of two treatments: manipulated males had black acrylic paint applied to their hind legs. The paint made it so that their legs became stiff and could not move during copulation. These males were able to hold territories, capture females and copulate, but they were not able to perform copulatory courtship. Control males were marked and handled like the treatment males, but did not have paint applied to their legs, allowing for normal movement during copulatory courtship. I then released these males and observed them once they returned to their territories.

When experimental males of either treatment obtained a mate, I video-recorded their copulations with a hand-held camcorder in the field and scored the female behavior afterwards. I found that when courtship was absent, females left the male's territory without ovipositing. None of the females who mated with manipulated males oviposited after mating, while all females who mated with controls did (Barbosa 2009). I also found through paternity analysis that in this species, the last male to mate with a female fertilized on average 83% of her eggs (Barbosa 2009). Consequently, when a female mated and left without ovipositing, that male would have a decreased reproductive success as she would likely mate with a different male before having the chance to oviposit again. Through a simple field experiment that entailed detailed observations, I demonstrated that female control of oviposition timing in these flies functions as a mechanism of cryptic female choice (Barbosa 2009).

Antennation behavior in the bean beetle *Callosobruchus maculatus*

The bean beetle *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) is an agricultural pest species that can be found worldwide throughout the tropical and subtropical zones. They feed and develop exclusively on beans: Mated females adhere eggs to the surface of dry beans, and larvae burrow into the beans after eclosion (Vamosi, 2005; K. Wilson, 1994). Larvae undergo a series of molts and pupation inside the bean. Finally, the winged adults emerge from the bean by chewing a window through the seed coat (K. Wilson, 1994). Adults have a limited lifespan of 10 to 14 days (Vamosi, 2005), during which time they primarily mate and lay eggs. Larval density is known to induce differential resource allocation in this species, inducing the development of dispersal morphs (large, functional wings and flight muscles relative to body size). Under low densities, individuals develop smaller hind wings and weak flight muscles, and are not capable of flight (Utida 1972).

Callosobruchus maculatus became a model system for sexual selection and sexual conflict thanks to a number of easily observable and quantifiable behaviors that occur during the interactions between males and females. Both sexes mate multiple times, females have multiple oviposition events throughout their life (Hotzy et al., 2012), and the mating sequence has been extensively studied and described in the literature. In this species, males do not appear to court or advertise to females at long range. Rather, when a male detects a female, he chases her and attempts to mount, while females often flee and attempt to resist male mating pursuits (Rup, 1986). Indeed, male harassment has been documented to have significant fitness costs in this species, including reduced longevity and lifetime offspring production (Bacon & Barbosa, 2020; den Hollander & Gwynne, 2009), and female choice is primarily expressed as overt resistance to harassment behaviors from males (Van Lieshout et al., 2014; C. J. Wilson & Tomkins, 2014).

When males attempt to mount females, they perform *antennation*, a behavior in which they rapidly tap the posterior end of the female abdomen and elytra with their antennae. This behavior has been previously described in the literature (Messina et al., 2007; Rup, 1986), but has received little attention and its function remains unknown. After antennation, the male inserts the aedeagus in the female, which inflates and has spines that anchor the male to the female's genital tract (Crudgington & Siva-Jothy, 2000; Dougherty et al., 2017; Rönn et al., 2007). Towards the end of copulation, the female vigorously kicks the male with her hind legs until they separate. Female latency to kicking is a measurement of female choice, where females allow preferred males to copulate for longer and therefore have longer latencies to kick (Van Lieshout et al., 2014; C. J. Wilson & Tomkins, 2014). Through extensive, detailed observations conducted in my research group, we observed that males perform this antennation consistently, although there is variation on the duration and the rate of tapping between individuals. We also observed that this looked different from how males and females move their antennae as they are sensing their environment, because it is performed at a regular rate once they mount the female (F. Barbosa, personal observation). Evidence from laboratory observations and previous studies conducted in my research group suggest that antennation may be a courtship behavior, as it meets several of the criteria outlined in (Eberhard, 1994): (1) it is a stereotyped, repeated behavior, although duration and rate of antennation vary within and between individuals; (2) it likely provides sensory stimulation to the female, as the antennae touch the female's body; (3) antennation does not seem to be necessary for the male to be able to physically mount or stay connected to the female; and (4) this antennation behavior is not performed in any other context besides immediately before intromission.

In a previous study, we tested whether males would provide more intense antennation in response to intense sperm competition cues. We had pairs mate in arenas containing filter paper where they created three competition treatments: no competition (zero males), low competition (one male competitor), and high competition (four male competitors). We added these males to the arena for 24 hours, then removed the males. Then, we added a focal male and female to the now empty arenas which still contained the filter paper that had the chemical cues with different competitor numbers. We video-recorded the matings and measured the male antennation rate and duration. We found that under high competition cues, males increased antennation duration (Figure 2A), which is consistent with a courtship function for antennation (O'Neall, Grossman and Barbosa, *unpublished*). Females who mated with these males with increased antennation duration had increased oviposition in the 24 hours following copulation (Figure 2B, O'Neall, Grossman and Barbosa, *unpublished*).

In a second study, we tested the hypothesis that competition for resources would determine the outcome of trade-offs between dispersal and reproduction in bean beetles (Gascoigne et al. 2022). We predicted that under high larval density, individuals would allocate more resources to dispersal and fewer resources to reproduction, developing smaller gonads. We induced differential resource allocation to dispersal traits by allowing beetles to develop under three larval density treatments: low, medium, and high, which corresponded to 1-3, 7-9, and 13+ eggs per bean respectively. Once adults emerged, we measured a suite of morphological and behavioral traits and investigated the covariances between them. Larval density has a well-reported effect on insect morphology and behavior, inducing the development of dispersal morphs in several wing-dimorphic species (aphids: Braendle et al., 2006; *Uraba lugens*: Johnson et al., 2017; *C. maculatus*: Utida, 1972). Larval density also affected multiple sexually-selected behaviors and traits in other insect species (*Achroia grisella*: Danielson-François et al., 2006; *Onthophagus taurus*: Emlen, 1994; *Drosophila melanogaster*: Shenoi et al., 2016). Some of these effects have been reported in *C. maculatus* (Katsuki & Lewis, 2015).

To measure behavioral traits, we allowed each experimental individual to mate with an unmanipulated individual in a standardized arena where behaviors could be observed, recorded and measured. Although we measured a suite of sex-specific mating behaviors, I will focus on male behaviors here: we measured antennation duration, antennation rate, and copulation duration. We measured the following morphological traits for both sexes: hind wing length as allocation to dispersal, and gonad area as allocation to reproduction. Both morphological traits were corrected for body size. As predicted, males reared under higher density developed larger wings. The changes in wing size we observed are consistent with those reported elsewhere (Utida 1972). Males reared under high density also developed smaller gonads, demonstrating a trade-off between dispersal and gonad size in this species. We also observed behavioral changes: males reared under higher density had increased antennation rates and shorter copulation durations (Gascoigne et al. 2022).

Since testis size can limit sperm production (Schärer et al., 2004; Schärer & Vizoso, 2007), which can lead to lower success in sperm competition (Parker, 1990; Parker & Pizzari, 2010; Perry et al., 2013), we then hypothesized that higher antennation rate could be a compensatory courtship behavior for reduced sperm competition ability in this species: if males with smaller gonads produced smaller ejaculates, a more intense antennation behavior would give them an advantage through female choice, if females indeed had a preference for these behavior. In a subsequent study, we tested the hypothesis that there would be a trade-off between antennation rate and ejaculate size in *C. maculatus* (Rice and Barbosa, *unpublished*).

To test this hypothesis, we reared individuals under two larval density environments, low and high, which we knew from our previous work would induce differences in testes size. Once adults emerged, we allowed the males to mate and video-recorded their copulations to measure antennation duration and rate. We then removed the transferred spermatophore from the mated females and measured the spermatophore size and sperm number by breaking down the spermatophore, diluting the contents and counting sperm cells with a hemocytometer. Sperm counts and spermatophore measurements were conducted so that the researcher was blind to the treatment of a given sample. As expected, we found that males reared under higher density had smaller testes and performed antennation at a faster rate. These males also had significantly fewer sperm per ejaculate (Figure 3a-b). However, we did not find any differences in the overall spermatophore size (Figure 3c). In summary, we found a trade-off between sperm number and antennation behavior between the two morphs: low-density males transfer more sperm and antennate at a lower rate, and vice-versa.

Conclusions and future directions

There remain many open questions about antennation behavior in *C. maculatus*, and whether it functions as courtship. Further studies are necessary to determine whether females express a preference for the intensity or the duration of this behavior through cryptic female choice. The presence of cryptic female choice in this species would open up important questions on the roles of courtship and female preferences in this species, given that the focus on sexual selection in bean beetles has largely been in sexual conflict (Crudgington & Siva-Jothy, 2000; Dougherty et al., 2017; Eady et al., 2006; Van Lieshout et al., 2014). Additionally, the proximate causes of trade-offs between dispersal and reproductive traits on this species, both morphological and behavioral, remain to be explored. We are currently studying the potential role of Juvenile Hormone in affecting these traits and have shown that changes in Juvenile Hormone titers during larval development induce differences in gonad size and mating behaviors in adults (Uwera Nalukwago and Barbosa, *unpublished*).

Most importantly, the studies described here exemplify how fruitful it can be to conduct detailed and systematic observations of copulatory behavior. The approach to behavioral studies Bill Eberhard used to train a generation of students has led to a multitude of discoveries, both in the field with understudied species such as the soldier fly and in the laboratory with model organism as the bean beetle. At a time where automated behavioral scoring becomes more and more common, it is important to remember the value of detailed behavioral observations: automated scoring may miss important details, not only about the actual behavior being scored but also on other behaviors that can lead to future questions.

Ethical note

The *C. maculatus* individuals used in this study come from a laboratory population maintained in Lake Forest College, Lake Forest, Illinois, USA. Lake Forest College does not require research performed in invertebrates to be reviewed by a regulatory body. Populations are housed in a 4-liter plastic container with a mesh top for ventilation and cowpeas are provided ad libitum. Containers are reared in an environmental chamber at 27C and 12:12 light cycle. Specimens were euthanized by freezing.

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Figure legends

Figure 1: (A) A male soldier fly, *Merosargus cingulatus*, guarding a territory. (B) A male bean beetle, *Callosobruchus maculatus*. Photos by F. Barbosa.

Figure 2: There is a significant effect of male-male competition treatment on (a) antennation duration (One-Way ANOVA, $F_{(2,55)}=3.83$, $p=0.02$) and (b) female clutch size (One-Way ANOVA, $F_{(2,55)}=5.80$, $p<0.01$). Asterisks indicate significance levels (*: $p<0.05$, **: $p<0.01$). Lower case letters show significant differences.

Figure 3: There is a significant effect of larval density on (a) antennation rate (T-test: $t_{54} = 7.91$, $p<0.01$) and (b) sperm number in ejaculate (T-test: $t_{54} = 40.49$, $p<0.001$), but no effect on (c) spermatophore size relative to male body weight (T-test: $t_{54} = 0.44$, $p=0.85$). Under higher density, males had higher antennation rate and higher sperm number per ejaculate. Asterisks indicate significance levels (*: $p<0.05$, ***: $p<0.001$).