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Mating Behavior Differences and the Cost of Mating in *Allonemobius fasciatus* and *A. socius*

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Abstract Close range prezygotic barriers are assumed to be present between sister taxa who have overlapping distributions. Here we report the results of studies designed to test the existence of prezygotic barriers between two closely related species, *A. fasciatus* and *A. socius*. We finely dissected the courtship and mating rituals and performed Monte Carlo analysis on lengths of time and number of occurrences of particular events in the courtship mating sequence. These detailed investigations of the courtship and mating behavior of conspecific and heterospecific pairs demonstrate that behavioral isolation is non-existent. We also measure the adult lifespan and number of progeny produced from singly and multiply mated males and females in conspecific and heterospecific trials. We found that cost of a heterospecific mating is asymmetric between the sexes with males paying a higher cost.

Keywords Behavioral isolation · *Allonemobius* · positive assortative mating · mating

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Introduction

Prezygotic isolation exists between many related species and the evolution of behavioral differences between species has long been considered an important component of speciation events (Alexander 1962; Martinez Wells and Henry 1992; Uzendosski and Verrell 1993; Eberhard 1994; Boake and Hoikkala 1995; Hoikkala and Welbergen 1995; Alexander et al. 1997; Seehausen et al. 1997; Ptacek 2000; Boake 2002; Coyne and Orr 2004). Fewer studies, however, have focused on mating preferences between divergent populations and closely related species leaving us with an unclear understanding of the role that prezygotic isolation plays in the initial onset of speciation. Studies investigating prezygotic isolation between divergent populations in a variety of taxa will enlighten our understanding of the role prezygotic isolation plays in speciation.

The frequent occurrence of prezygotic barriers between related, sympatric species may have one of two explanations. First, behavioral barriers may be under direct selection and critical to the emergence of new species, thereby, evolving early in the speciation process. Models of sexual selection and sexual conflict indicate that coevolution between the sexes can result in rapid behavioral divergence between allopatric populations and can even lead to splits in sympatric populations (Lande 1981; Turner and Burrows 1995; Takimoto et al. 2000; Gavrilets nd Waxman 2002). Second, behavioral isolation may evolve as a response to selection against costly mating interactions and gamete wastage. Here, we investigate the role that courtship may play in positive assortative mating between two closely related species of striped ground crickets as well as test for costly mating interactions between these two species.

Members of the ground cricket genus *Allonemobius* are small, ground-dwelling omnivores that inhabit short grassland areas of North America. Research on this genus is far reaching and includes the process of speciation (Reviewed in: Howard et al. 1998b), cytoplasmic incompatibility (Marshall 2004), sexual selection (Fedorka and Mousseau 2002c; Fedorka and Mousseau 2002b; Fedorka and Mousseau 2002a), sexual conflict (Fedorka and Mousseau 2004), temporal partitioning (Birge et al. 2007), and reproductive protein evolution (Braswell et al. 2006). One of the species pairs in this group, *A. fasciatus* and *A. socius*, represent one of the most intensively studied systems in evolutionary biology with regard to reproductive isolation (Coyne and Orr 2004).

Allonemobius fasciatus has a more northerly distribution in North America, whereas *A. socius* is found to the south. Where the two species occur together, in a zone of varying width that extends from New Jersey to at least as far west as Illinois. Roughly 5–8% of the contact zones consists of advanced backcrosses indicating that reproductive isolation is strong but incomplete (Britch et al. 2001). Trait differences responsible for reproductive isolation have been studied in the field and in the laboratory. As a result of this work, we know that *A. fasciatus* and *A. socius* are not isolated by male calling song differences (Doherty and Howard 1996). Early work also indicated that phenological differences and habitat utilization differences could not explain reproductive isolation (Howard et al. 1993). Similarly, there is no evidence of hybrid inviability, infertility, or reduction in hybrid fitness in natural populations or in a laboratory setting (Gregory and Howard 1993; Howard et al.

1993). However, evidence of assortative mating was found in population cages in which individuals of *A. fasciatus* were less abundant than individuals of *A. socius* (20% A. fasciatus, 80% A. socius, Howard et al. 1998a). When *A. socius* was less abundant, there was no assortative mating (Howard et al. 1998a). The only strong barriers to gene flow between *A. fasciatus* and *A. socius* that have been identified thus far are traits linked to post-mating, prezygotic isolation like conspecific sperm precedence and the ability of males to induce females to lay eggs (e.g., Howard and Gregory 1993; Howard et al. 1998a, b; Marshall 2007).

Because *Allonemobius* females are highly promiscuous (Howard et al. 1998b) and exhibit strong conspecific sperm precedence, heterospecific matings frequently result in few to no hybrid offspring. Females can mate with heterospecific males and still produce eggs fertilized only by conspecific males. If the cost of a heterospecific mating is low for females, then selection pressure for female discrimination between species should be weak in sympatric populations (Howard et al. 1998b; Marshall et al. 2002, see West-Eberhard 1983 for a more general discussion on this point). The situation is different for males of *Allonemobius* as they provide two types of nuptial gifts: a spermatophore and hemolymph which females feed upon from a specialized spur on the male's tibia during copulation (Fedorka and Mousseau 2002c). The nuptial feeding results in a loss of up to 10% of a male's body mass during a single mating (Fedorka and Mousseau 2002b). Thus, males in sympatric populations should be under strong selection pressure to avoid engaging in heterospecific matings that are energetically expensive and result in few to no offspring (Howard et al. 1998b; Marshall et al. 2002).

Despite the mate choice work that has been done to this point, in-depth comparisons of the mating sequence in *A. fasciatus* and *A. socius* have yet to be carried out. Here, we present the results from detailed investigations of the mating behavior of the two species that were designed to detect even slight differences in behavior that might contribute to reproductive isolation in the field. At the same time, we present data that provides further insight into the costs and benefits associated with mating for both males and females.

Materials and Methods

Crickets

To compare the mating sequence between *A. fasciatus* and *A. socius*, eggs of both species were obtained from two laboratory populations. The lab populations were created from 100 wild crickets obtained during the summer of 2000 from two sites in New Jersey: Lippincott Farm and mile marker 23–22 Hwy 50. Both populations are near, but just outside, the area of overlap between the two species. The populations were maintained in pure species cages and bred for one generation in the laboratory. Populations of *A. fasciatus* and *A. socius* were maintained in 28°C environmental chambers that had photoperiod regimes of (L: D/14:10). In spring of 2001, juveniles were reared in large Rubbermaid containers (53×38×23 cm). Fluker's Cricket Feed, water soaked cotton for humidity, and crumpled paper towels for refuge were provided ad libitum. Crickets were sorted by gender to ensure virginity and held in

single sex groups. Adults were identified on their eclosion date and segregated from the single sex juvenile populations.

Another collection from the same sites and the same year were used to document the costs and benefits of mating. Specifically, we measured lifespan, number of eggs layed and hatching success. Crickets were again supplied with Fluker's Cricket Feed and water soaked cotton for humidity were provided ad libitum before and after mating. They were maintained in rearing rooms at 28°C with a 14:10 h. light:dark cycle after mating. All crickets were 10–15 days post-eclosion when mated. Mating occurred in 1-pint glass jars on a moistened filter paper substrate. After completion of their treatment, females were maintained with oviposition dishes (Petri dishes with a mixture of sand/soil/vermiculite). After a 2 week period of time, the dishes were removed. Females in the lifespan experiment were maintained in environmentally controlled chambers until their natural deaths. The egg dishes were kept at 28°C for two additional weeks, then at room temperature for 1 week, and then moved to a 5°C refrigerator for 3.5 months. Upon removal from the refrigerator, each egg dish was kept at room temperature for 11 days and then moved to the 28°C rearing room until emergence was complete. Egg dishes were kept moist for one month and then allowed to dry naturally in an effort to stress eggs into hatching. Emergence was considered complete after no offspring appeared for 21 days. Emergence counts were conducted every other day.

Courtship Mating Sequence

Because flow diagrams rely on presence versus absence of traits, they are of restricted usefulness in this study. A priori, we know that these species are closely related and hybridize in nature, so we do not expect the complete absence of any behaviors. Therefore, we chose to measure lengths of time and/or number of occurrences of particular events in the courtship mating sequence. Here, we have indicated the position in the mating sequence of all measured attributes in the appendix with the number, in italics, assigned to it in the appendix. We have also provided a visual display of gross behavioral changes in Fig. 1. The complex mating ritual of Nemobiines was qualitatively described by Mays (1971). The ritual consists of an intricate stimulus-response chain that can last up to 2 h *(1)*. Once a male and a female are put in a mating arena, the male frequently begins stridulating with his forewings prior to physical contact with the female *(2, 3, 4, 5)*. Shortly after or during initial physical contact, the male will face and follow the female while stridulating and quickly jerking his body in a forward then backward motion *(6)*. This jerking motion continues throughout the mating ritual but the intensity and speed changes. After first physical contact, the male maintains a tactile presence throughout most of the mating sequence by attenating *(7)*, drumming *(8)*, and walking *(9)* on the female. Eventually, the male will expose his genitalia. Several minutes after genitalia exposure, the male will turn his back to the female and start singing and shaking from side to side, as well as forward and backward *(10, 11, 12)*. This is called the initiation dance. The female will then mount the male by walking onto his back *(13, 14)*. During this period of pseudocopulation, the female and male engage in genitalic contact *(15, 16)*. This first mounting is required for the production of the spermatophore, a sperm containing ampulla with an ejaculatory canal.

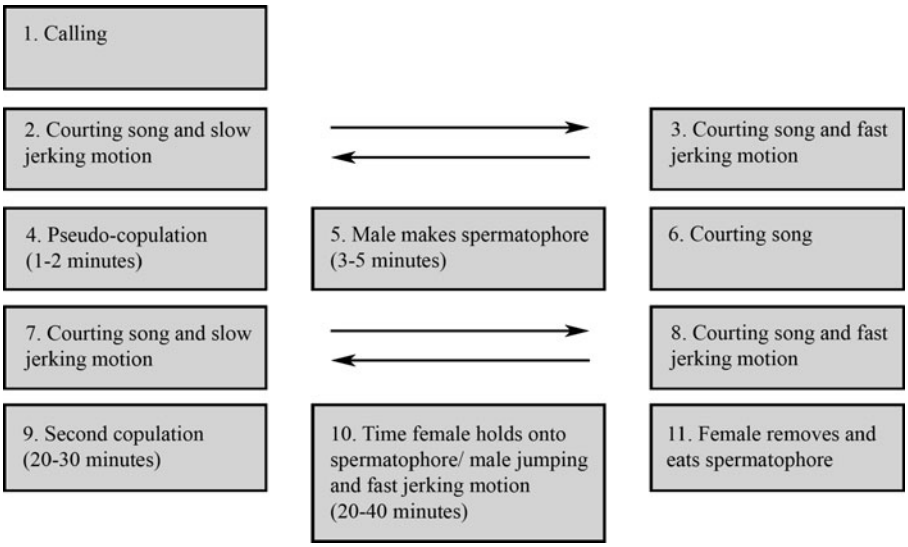

Fig. 1 A diagram of the mating ritual in *Allonemobius*. Arrows are used to indicate interchangeable behaviors.

Following the dismount by the female, the male continues courting the female while the spermatophore is produced *(17)*. During this time, the male stridulates, jerks his body *(18)*, and maintains close tactile contact with the female. As the spermatophore is exuded from the genital opening *(19)*, the male extends his metathoracic legs, i.e. the hind leg, lifting and then arching the posterior end of his abdomen toward the substrate.

The second phase of the courtship ritual is the copulatory step. Here, the mating sequence is characterized by two nuptial gifts. After the spermatophore is produced, a 10–30 min time interval ensues before copulation. During this time, the male stridulates, jerks *(20)*, and maintains close physical contact with the female. Then, the female mounts the male a second time *(21)*. This is the phase of mating in which the spermatophore is transferred from the male to the female *(22, 23, 24)*.

During copulation, the female feeds on a specialized tibial spur, one of which is located on each metathoracic leg of the male *(25)*. The tibial spurs exude a glandular substance, which is the first nuptial gift. Fedorka and Mousseau (2002b) were able to demonstrate that this glandular substance is, in fact, male hemolymph. Recent studies indicate that males may lose as much as 10% of their body weight during one of these feeding episodes (Fedorka and Mousseau 2002b).

To terminate copulation, the female and male walk off in different directions *(26, 27)*. After copulation, the male may commence a quick jumping and jerking dance *(28)*. In a few instances this dance was observed following unsuccessful matings. A mating is considered successful when the spermatophore is transferred from the male to the female. Shortly after copulation termination, the female will remove the spermatophore by rubbing it between her abdomen and the substrate in a backwards motion *(29)*.

Matings

All trials were performed under the same lighting and temperature conditions. In each trial, males were the focal sex, and each trial consisted of one virgin female mated to one virgin male, both individuals were 10–21 days post-eclosion to ensure sexual readiness.

Mating took place in a clear plastic box (17 cm×12 cm×6 cm) with soaked cotton to provide humidity. After a chamber was used it was rinsed out with water to remove any chemical stimulus. To allow females ample time to acclimate to the chamber, they were placed in the chamber at least 24 h before they were mated. If a male failed to commence courtship within 10 min of being placed in the chamber, he was replaced by a new male. Females could avoid courtship by kicking at the male.

The treatment groups were as follows: (A) *A. fasciatus* female mated to a conspecific male (n=17); (B) *A. fasciatus* female mated to a heterospecific male (n=24); (C) *A. socius* female mated to a conspecific male (n=12); (D) *A. socius* female mated to a heterospecific male (n=17). Treatments A and C provided the courtship sequence of conspecific pairings and allowed the identification of any differences in mating rituals between the two species. Heterospecific treatments B and D provided information on changes in action patterns associated with heterospecific pairings and serve to identify sequence elements that might play a role in behavioral isolation. All trials were videotaped so that they could be studied exhaustively and would be available for future reference. Continuous, discrete, and nominal data were collected. The total number of times discrete acts are performed is dependent on the total time the male and female are allowed to interact. To control for this potential difference, these count data were divided by the number of seconds that males and females were allowed to interact before statistical analyses were performed.

Monte Carlo procedures were used to evaluate differences between the four "cross-type" treatments above, as well as the treatment of successful or unsuccessful spermatophore transfer. Specifically, for each comparison, data from all treatments were randomly assigned (drawing without replacement) to each treatment. The original sample sizes per treatment were maintained for all randomly generated datasets. Test statistics were generated for all datasets. This procedure was repeated 1,000 times with the resulting distribution of test statistics being used to assess significance of the original dataset.

Videotaping

Each mating chamber was placed on a sheet of graph paper with 1 cm×1 cm squares for filming. All trials were video taped for future reference using a Panasonic WV-BP110 camera attached to a TESTRITE Instruments CS-3 copy-stand, a Panasonic AG-6040 time lapse video cassette recorder, and a Panasonic CT-2084Y color monitor. The mating sequence was videotaped from the time the male entered the chamber until 10 min after the spermatophore was knocked off the female or the male, depending on whether or not the mating was successful.

Discrimination and cost of reproduction in multiple matings in males: Courtship and copulation durations

In the set of experiments designed to understand the costs and benefits of mating in males and females, we mated a virgin male with two virgin females in one of the following combinations: 1) conspecific followed by conspecific; 2) heterospecific followed by conspecific. Each male had three hours to complete one successful spermatophore transfer. This initial mating period was followed by another mating session that began an hour after the first session ended. One hour was used in an attempt to challenge males; so, costs could be readily measured. As population densities are often quite high in nature, this is not an unrealistic recycle time. For each pair, we recorded the courtship duration (time from first contact to copulation commencement) and copulation duration. Using an analysis of variance, we tested whether courtship duration or copulation duration were different between conspecific and heterospecific females for *A. socius* and *A. fasciatus* males separately.

Effects of Single Versus Multiple Mating: Lifespan

To test for effects of single versus multiple matings on males, a virgin male was mated with a virgin female in one of the following combinations: 1) one single mating within 3 days; 2) three matings with the same female within 5 days. In both cases, only one successful spermatophore transfer per day was allowed. Females in the multiple-mating treatments that did not mate on the initial day were replaced the following day. We tested whether male lifespan decreased with multiple matings via an analysis of variance.

To test for effects of single versus multiple mating on females, a single virgin female was mated with a conspecific virgin male in one of the following combinations: 1) one single mating within three days, 2) three matings with the same male within five days, allowing only one successful spermatophore transfer per day. Thus, there were four treatment groups of females: two of *A. fasciatus* females and two of *A. socius* females. Females in the multiple-mating treatment that did not mate on the initial day were replaced the following day. Because species effects may be different, we tested whether female lifespan was affected by single versus multiple mating via an analysis of variance.

Effects of Conspecific Versus Heterospecific Mating on Females: Offspring Production

To determine whether offspring production varied between conspecific and heterospecific parings, a virgin female was mated with two virgin males in one of the following combinations: 1) conspecific followed by conspecific, 2) conspecific followed by heterospecific, 3) heterospecific followed by conspecific, and 4) heterospecific followed by heterospecific. Thus, there were eight groups of females, four for *A. fasciatus* and four for *A. socius*. Females were given a maximum of three days (four hours each day spent with a male) to achieve two successful spermatophore transfers. Only one spermatophore transfer was allowed per day.

For each mating event, we recorded the number of resulting offspring. We tested whether conspecific and heterospecific parings effected offspring production using an analysis of variance.

Effects of Single Versus Multiple Mating on Females: Offspring Production

To evaluate the effect of single versus multiple matings on offspring production and female lifespan, a single virgin female was mated with a conspecific virgin male in one of the following combinations: 1) one single mating within three days, 2) three matings with the same male within five days, allowing only one successful spermatophore transfer per day. Thus, there were four treatment groups of females: two of *A. fasciatus* females and two of *A. socius* females. Females in the multiple-mating treatment that did not mate on the initial day were replaced the following day. We tested whether offspring was affected by single versus multiple mating via an analysis of variance.

Results

Mating Sequence in Single Matings: Overall Comparisons

Because the treatments were unbalanced, Monte Carlo simulations were used for comparisons. We conducted multiple tests on the components of the mating system; therefore, a p-value of 0.05 would be inappropriate. We used a critical value of 0.002. This value was achieved using a strict Bonferroni adjustment (however, our results do not change even if a serial Bonferroni adjustment is used). In general, the mating sequences of the two species are very similar (Appendix). This seemed to be the case before and after copulation. Furthermore, males seemed to spend the same amounts of time calling and touching. While there were no significant differences, heterospecific males did tend to take longer in initiating the "first mount" (Appendix, #10 interaction $P=0.049$) and did attempt more first mounts (Appendix, #11 interaction $P=0.056$). It would be of interests to follow up on this pattern. In general, results suggest no evidence of species discrimination at any stage of the mating sequence during single mating events. This seems to be true whether the signal was tactile, acoustic, or visual.

Mating Sequence in Single Matings: Copulatory Comparisons

The critical measure during copulation is the time interval between the copulation mount (SM) and when the female dismounts the male (D); this is the amount of time *in copula*. Our Monte Carlo ANOVA did not reveal any significant effect (Appendix).

When females were mated twice, *A. socius* females did not spend more time *in copula* with *A. socius* males than with *A. fasciatus* males during the first ($F_{3,43}=0.04$, $p=0.9909$) or second matings ($F_{3,43}=0.44$, $p=0.7258$). Similar results were found with *A. fasciatus* females (first mating: $F_{3,41}=1.35$, $p=0.2720$; second mating: $F_{3,41}=2.47$, $p=0.0767$).

Mating Sequence in Single Matings: Post-Copulatory Comparisons

There were no significant male species, female species, or interaction effects for two post-copulation behaviors associated with mating trials that end with a successful spermatophore transfer, i.e., the number of post-copulation dances by the male (IZ) and the time interval between dismount (D) and when the female removes the spermatophore (KS) (Appendix). Once again, the data indicate that con- and heterospecific matings do not differ in these post-copulation behaviors.

Mate Discrimination and Cost of Reproduction in Multiple Matings: Courtship and Copulation Durations

To quantify any differences in the amount of time males spent in courtship with conspecific versus heterospecific females, we mated a virgin male with two virgin females in one of the following combinations: 1) conspecific followed by conspecific; 2) heterospecific followed by conspecific (Table 1). During the first courtship, neither *A. socius* or *A. fasciatus* males spent significantly different amounts of time with conspecific or heterospecific females ($F_{3,33}=0.274$, $p=0.8435$). Moreover during the second mating, neither *A. socius* or *A. fasciatus* males spent significantly different amounts of time courting the two female types ($F_{3,33}=1.643$, $p=0.1983$).

Similar results were obtained with regard to copulation (Table 2). Specifically, during the first mating, there was no difference across treatments ($F_{3,33}=0.347$, $p=0.7917$). Neither *A. socius* or *A. fasciatus* males spent significantly different amounts of time *in copula* with the different female types during the first mating (*A. fasciatus* mated to conspecifics vs. *A. fasciatus* mated to one heterospecific followed by a conspecific Fisher's PLSD=0.1539; *A. socius* mated to conspecifics vs. *A. socius* mated to one heterospecific followed by a conspecific Fisher's PLSD=0.8409). While there was a significant difference across mating

Table 1 Effects of Mating with Heterospecific Versus Conspecific Females: Courtship Duration. Note: Neither *A. socius* or *A. fasciatus* males spent significantly different amounts of time with conspecific or heterospecific females

Treatment	First Mating (mean±standard error in minutes)	Second Mating (mean±standard error in minutes)
A. fasciatus male X con female, con female ($n_1=10$, $n_2=10$)	49.7±9.1	40.4±2.5
A. fasciatus male X het female, con female ($n_1=13$, $n_2=13$)	44.0±7.2	50.8±9.1
A. socius male X con female, con female ($n_1=12$, $n_2=12$)	42.5±6.8	63.8±14.2
A. socius male X het female, con female ($n_1=12$, $n_2=12$)	39.0±9.5	51.8±8.5

Significant treatments are designated with asterisks

Table 2 Effects of Mating with Heterospecific Versus Conspecific Females: Copulation Duration. Note: *A. socius* males spent more time with the second mate than *A. fasciatus* males regardless of species identity

Treatment	First Mating (mean±standard error in minutes)	Second Mating (mean±standard error in minutes)
A. fasciatus male X con female, con female (n_1=12,, n_2=12)	17.2±3.4	10.3±2.8
A. fasciatus male X het female, con female (n_1=10, n_2=10)	18.9±2.2	15.5±2.2
A. socius male X con female, con female (n_1=11, n_2=11)	22.9±3.2	19.0*±2.7
A. socius male X het female, con female (n_1=12, n_2=12)	17.0±2.8	18.9*±1.8

$P<0.05$ are designated with asterisks

types for the second mating (F 5,49=14.569, $p<0.0001$), *A. socius* males spent more time with the second mate than *A. fasciatus* males did with conspecifics regardless of species identity (*A. fasciatus* mated to conspecifics vs. *A. socius* mated to two conspecifics Fisher's PLSD=0.0117; *A. fasciatus* mated to conspecifics vs. *A. socius* mated to a heterospecific and then a conspecific Fisher's PLSD=0.0114). Because these trials took place over the course of a day, a control mating group was established for the afternoon trials. The afternoon control group consisted of males mated singly to a conspecific. These data indicated that the significant difference in the overall analysis of variance for the second mating was largely due to a longer copulation times in the *A. fasciatus* afternoon matings rr (*A. fasciatus* mated to a conspecific vs. *A. fasciatus* mated to two conspecifics Fisher's PLSD<0.0001).

Effects of Single Versus Multiple Mating: Lifespan

Lifespan of males appeared to be negatively impacted by multiple mating (F 3,56=5.980, $p=0.0013$). *Allonemobius socius* males that mated only once lived 11.4 days longer than *A. socius* males that mated three times (one-mated: n_1=19, 53.526±2.381 days; thrice mated n_2=16, 42.125±1.938 days; Fisher's PLSD= 7.401; $p=0.0032$). Although not significant, *A. fasciatus* males that mated only once lived on average 2.5 days longer than *A. fasciatus* males that mated three times (once-mated: n_1=15, 41.200±3.046 days; thrice-mated: n_2=10, 38.700± 4.534 days; Fisher's PLSD=8.905; $p=0.5761$). Furthermore, singly mated *A. socius* males lived longer than both singly mated *A. fasciatus* males (Fisher's PLSD=7.532; $p=0.0018$) and multiply mated *A. fasciatus* males (Fisher's PLSD= 8.521; $p=0.0010$).

In contrast, lifespan of females appeared to be positively impacted by multiple mating (F 3,56=2.067, $p=0.1149$). There was a trend for *A. socius* females to enjoy increased lifespan with additional matings. *Allonemobius socius* females that mated

three times lived 9.279 days longer than those that mated only once (multi-mated: n=16, 57.188±2.530 days; single-mated: n=22, 47.909±3.47 days; Fisher's PLSD= 9.986; p=0.0680). *Allonemobius fasciatus* females did not give the same result. *A. fasciatus* females experienced similar life spans whether mated once or more than once (multi-mated: n=10, 45.100±2.755 days; single-mated: n=12, 45.083± 3.730 days; Fisher's PLSD=13.013; p=0.9980). Finally, multiply mated *A. socius* females tended to live longer than multiply mated *A. fasciatus* females (Fisher's PLSD=11.606; p=0.0412).

Effects of Conspecific Versus Heterospecific Mating: Offspring Production

Overall, *A. socius* females produced significantly different amounts of offspring across treatments (F 3,33=3.190, p=0.0363). There was a trend for *A. socius* females to produce more offspring in all of the treatments that involved at least one conspecific male than in the treatments in which females mated with two heterospecifics (Table 3). *A. socius* females mated with two conspecific males produced significantly more offspring than females mated with two heterospecific males (with two conspecifics: n=8, 54.9±10.2 offspring; with two heterospecifics: n=8, 6.3±16.6 offspring, Fisher's PLSD=41.532, p=0.0043).

Overall, *A. fasciatus* females produced significantly different amounts of offspring across treatments (F 3,31=7.430, p=0.0007). Specifically, *A. fasciatus* females produced more offspring in all of the treatments that involved at least one conspecific male than in the treatments in which females mated with two heterospecifics (Table 3). Moreover, *A. fasciatus* females produced the most offspring when mated to two conspecific males than the other three mating treatments (Table 3).

Effects of Single Versus Multiple Mating: Offspring Production

While the analysis of variance proved to be significant overall, this difference was do to the fact that *A. socius* produces more offspring than *A. fasciatus* (n=45, F3,41=6.256, p=0.0013). Of the matings that did produce offspring (15/18 of the multi-mated *A. socius*, 13/18 of the single-mated *A. socius*, 9/10 of the multi-mated *A. fasciatus*, and 8/14 of the single-mated *A. fasciatus*), multi-mated *A. socius* females showed an increase in hatchling production over their single-mated counterparts but it was not significant. (multi-mated: n=15, 82.133±10.940 offspring; single-mated: n=13, 63.692±11.138 offspring; Fisher's PLSD=26.994, p=0.1752). Similar results were found with *A. fasciatus* females. Of the *A. fasciatus* matings that produced hatchlings, the multi-mated *A. fasciatus* females showed an increase in offspring production over their single-mated counterparts, but it was not significant (multi-mated: n=9, 37.889±7.731 offspring; single-mated: n=8, 21.625±6.305 offspring; Fisher's PLSD=34.615; p=0.0.3482). Overall *A. socius* did produce significantly more offspring than *A. fasciatus* in both females mated multiply (Fisher's PLSD=30.037; p=0.0049) and females mated singly (Fisher's PLSD=32.011; p=0.0113).

Table 3 Effects of Heterospecific Versus Conspecific Mating on Female Offspring Production. Note: *A. fasciatus* females produced significantly more offspring in all of the treatments that involved at least one conspecific male than in the treatments in which females mated with two heterospecifics. Furthermore, a dosage effect is apparent. Females mated to two rather than one conspecific produced more offspring

Treatment	N	Offspring (mean±standard error)
1. *A. fasciatus* female X con male, con male	8	54.875±11.597
2. *A. fasciatus* female X con male, het male	8	26.875±7.654
3. *A. fasciatus* female X het male, con male	11	27.909±6.331
4. *A. fasciatus* female X het male, het male	8	2.375±1.558
5. *A. socius* female X con male, con male	10	91.400±17.205
6. *A. socius* female X con male, het male	11	65.273±10.084
7. *A. socius* female X het male, con male	8	57.000±16.952
8. *A. socius* female X het male, het male	8	28.875±11.340

Corresponding p-values for *A. fasciatus*

	1	2	3
1	–	–	–
2	0.0173*	–	–
3	0.0139*	0.9210	–
4	<0.0001*	0.0353*	0.0193*

Corresponding p-values for *A. socius*

	5	6	7
5	–	–	
6	0.1740	–	
7	0.1014	0.6818	
8	0.0043*	0.0778	0.2002

$P<0.05$ are designated with asterisks

Discussion

We compared courtship behavior in the two species by carefully observing mating behavior and measuring time intervals associated with components of this behavior in intraspecific and interspecific pairings. Differences in time intervals identify components of the signal—response system that have diverged between the two species and may play a role in reproductive isolation (Boake 2002). In addition, we compared the vigor with which males of the two species engaged in various behaviors, as females tend to mate with the most vigorous male. We also analyzed costs associated with both intra- and interspecific matings to determine: (1) whether multiple mating decreases the life span of males and females; (2) whether selection against hybridization exists; and (3) whether there are asymmetries between males and females in the costs associated with heterospecific matings.

All in all, *A. socius* and *A. fasciatus* are very similar in mating sequences and there are few significant differences. Not only do both species show the same

behaviors, the timing of the behaviors is similar. These findings are in basic agreement with the results of assortative mating studies in population cages (Howard et al. 1998a, b). In these experiments, the two species appeared to mate at random, except when *A. fasciatus* individuals were rare in the population. In this situation, females of *A. fasciatus* mated more frequently with conspecific males than expected based on the frequency of conspecific males in the population.

Spermatophore attachment time (element 27 in the Appendix) and time *in copula* (element 29 in the Appendix) did not vary significantly between groups. Moreover, we found no relationship between spermatophore attachment time after copulation and size of the nuptial gift (the amount of time spent chewing on the tibial spur of males by females). This result is in agreement with an intra-specific experiment performed on *A. socius* (Fedorka and Mousseau 2002a).

Based on the series of mate choice studies that have been carried out in the past (Gregory et al. 1998; Howard et al. 1998a; Howard et al. 1998b), as well as the results of the current behavioral work, the isolating potential of mating behavior seems to be quite low in the case of *A. fasciatus* and *A. socius*. In general, males and females of both species engage readily in heterospecific matings and these matings are generally successful (result in a spermatophore transfer). Thus, differences in mating behaviors cannot explain the strong reproductive isolation that exists between these two species in areas where they occur together.

The questions remain, does selection against hybridization exist and does mating entail a lifespan cost to males and females? The results of our studies indicate that mating with a heterospecific male does not cause a decline in offspring production for females, as long as they mate with a conspecific male (see Results). Moreover, if females mate with both heterospecific and conspecific males, they produce few, if any, hybrid offspring (Howard and Gregory 1993, Howard et al. 1998a; Gregory and Howard 1994). Finally, females appear to benefit from multiple matings. The more matings a female engages in, the longer she lives and the more eggs she lays (see Results). Taken together, these results indicate that heterospecific matings are not highly detrimental to females. Indeed, the cost of a heterospecific mating, in the presence of conspecifics, appears to be non-existent.

The situation is quite different for males. A male that mates with a heterospecific female has engaged in a costly behavior, as measured by weight loss (Fedorka and Mousseau 2002b) and impact on lifespan (see Results), and receives relative few, if any, offspring in return if the female mates with a conspecific male. Thus, males in sympatric populations should be under strong selection to discriminate against heterospecific females.

The enhanced lifespan of multiply-mated *A. socius* females is at odds with results reported by Fedorka and Mousseau (2002b), who found that females mated multiple times suffered a decline in lifespan compared to females mated only once. The disparity in results may be explained in a number of ways. First, the experimental protocols in the two studies were quite different. In our work, we controlled for male experience by mating females to virgin males each time. Fedorka and Mousseau (2002b) controlled for male experience by rotating males within the polyandrous treatment group. Since the quality of male ejaculates may change in relation to

mating frequency (Dewsbury 1982; Nakatsuru and Kramer 1982; Olsson et al. 1997; Engqvist and Reinhold 2006) studies that vary with regard to mating protocols may produce different results. Second, nutritional conditions varied between the studies. Crickets in our study were fed Fluker's Cricket Chow ad libitum while specimens in the Fedorka and Mousseau (2002b) study were reared on Purina cat chow before mating and carrots after matings began. Thus, differences in diet may account for the incongruent results. Indeed, the impact of multiple matings on female lifespan in *Drosophila* depends on the diet (Chippindale et al. 1993; Chapman and Partridge 1996; Piper et al. 2005).

The mating behaviors of *A. fasciatus* and *A. socius* are extremely similar and there is little indication that close range signals operating prior to insemination serve as a barrier to gene flow between them. This finding is consistent with the qualitative work conducted by Mays (1971), who found that close range courtship behaviors are similar across a variety of species of Nemobiinae suggesting that the mating sequence evolved early in the history of this genus and has evolved relatively slowly since then. These results resemble the findings of Phelan and Baker (1990) who reported relatively few mating pattern differences between 12 species of phycitine moths. Together, these findings demonstrate that speciation can occur prior to the evolution of behavioral barriers to gene flow. Ultimately, the lack of divergence in mating behaviors serves to underscore the remarkable rapidity with which post-mating, prezygotic barriers, such as conspecific sperm precedence and a male's ability to induce a female to lay eggs, have evolved between species in the *A. socius* complex—given that these species are estimated to have diverged from one another about 30,000 years ago (Marshall 2004; Marshall 2007).

The study of reproductive isolation is at the heart of studies of species formation (Howard and Berlocher 1998; Coyne and Orr 2004). Many studies of reproductive barriers between closely related species have been carried out, but very few have been exhaustive, exploring in detail the isolating potential of behavioral, ecological, gametic, and developmental differences between closely related species. The relative dearth of detailed studies examining reproductive isolation between pairs of closely related species means that while evolutionary biologists can catalog the diversity of isolating barriers that exist in nature, they still cannot determine whether some barriers arise earlier than others and hence play a more important role in the initial onset of reproductive isolation. Clearly, this gap in our knowledge must be filled if we hope to fully understand species formation. Here, we demonstrate that precopulatory isolation is not present in two incipient sister species who are separated by a postcopulatory but prezygotic mechanism of isolation.

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Appendix

Table 4 Mating Sequence Summary: Lengths of Time and, or Number of Occurrences of Particular Events in the Courtship Mating Sequence. Number Under 'Mating Sequence' Corresponds to Verbal Description in the 'Courtship Mating Sequence' of the Material and Methods

Mating sequence	Male effect *p*-value; Female effect *p*-value; Interaction effect *p*-value	F X F	F X S	S X F	S X S
1. The total time mating	*P*=0.052;	Mean=2981.1765	Mean=3187.5417	Mean=2677.0	Mean=3208.4706
	P=0.451;	SE=136.0343	SE=155.1584	SE=179.8735	SE=237.4626
	P=0.378	Median=2859.0	Median=3075.50	Median=2529.50	Median=3330.0
		IQR=802.0	IQR=1029.50	IQR=645.50	IQR=1397.0
		N=17	*N*=24	*N*=12	*N*=17
2. The time the male sang during the mating trial expressed as a proportion of total time mating	*P*=0.597;	Mean=0.3257	Mean=0.3987	Mean=0.2991	Mean=0.2838
	P=0.213;	SE=0.0401	SE=0.0469	SE=0.0649	SE=0.0656
	P=0.42	Median=0.3175	Median=0.3973	Median=0.2418	Median=0.2182
		IQR=0.1554	IQR=0.3449	IQR=0.3503	IQR=0.2634
		N=17	*N*=24	*N*=11	*N*=16
3. The time at which a male first began to sing a courtship song expressed as a proportion of the total time mating	*P*=0.425;	Mean=0.0461	Mean=0.0494	Mean=0.0535	Mean=0.0341
	P=0.685;	SE=0.0127	SE=0.0069	SE=0.0103	SE=0.0044
	P=0.222	Median=0.0227	Median=0.0394	Median=0.0486	Median=0.0361
		IQR=0.0356	IQR=0.0517	IQR=0.0600	IQR=0.0236
		N=17	*N*=24	*N*=11	*N*=17
4. The length of each singing period divided by the number of singing periods in each mating trial expressed as a proportion of the total time singing	*P*=0.286;	Mean=0.1050	Mean=0.1477	Mean=0.1113	Mean=0.1541
	P=0.894;	SE=0.0162	SE=0.0418	SE=0.0241	SE=0.0263
	P=0.999	Median=0.0797	Median=0.0773	Median=0.0781	Median=0.1321
		IQR=0.0642	IQR=0.1168	IQR=0.1470	IQR=0.1198
		N=16	*N*=24	*N*=11	*N*=12

5. The total number of times male sang during the mating trial divided by the total time mating	P=0.366; P=0.215; P=0.446	Mean=0.0050 SE=0.0005 Median=0.0053 IQR=0.0038 N=16	Mean=0.0050 SE=0.0007 Median=0.0044 IQR=0.0043 N=24	Mean=0.0047 SE=0.0008 Median=0.0051 IQR=0.0045 N=11	Mean=0.0034 SE=0.0009 Median=0.0023 IQR=0.0039 N=12
6. The total number of jerks performed by the male from beginning of the trial divided by the total time it took the female to mount the male for the first time	P=0.9; P=0.394; P=0.497	Mean=0.1321 SE=0.0224 Median=0.1123 IQR=0.0833 N=16	Mean=0.1464 SE=0.0198 Median=0.1323 IQR=0.1435 N=23	Mean=0.1274 SE=0.0365 Median=0.0684 IQR=0.1053 N=9	Mean=0.1074 SE=0.0202 Median=0.0960 IQR=0.1127 N=15
7. The total number of attenuation bouts the male performed on the female's body during the mating trial divided by the total time mating	P=0.84; P=0.312; P=0.025	Mean=0.0036 SE=0.0004 Median=0.0036 IQR=0.0014 N=12	Mean=0.0050 SE=0.0004 Median=0.0049 IQR=0.0023 N=21	Mean=0.0043 SE=0.0006 Median=0.0039 IQR=0.0008 N=9	Mean=0.0031 SE=0.0006 Median=0.0030 IQR=0.0034 N=11
8. The total number of bouts where the male drummed on the female with his palps performed during the mating trial divided by the total time mating	P=0.939; P=0.376; P=0.479	Mean=0.0040 SE=0.0008 Median=0.0028 IQR=0.0047 N=17	Mean=0.0046 SE=0.0006 Median=0.0048 IQR=0.0036 N=24	Mean=0.0055 SE=0.0014 Median=0.0048 IQR=0.0047 N=10	Mean=0.0048 SE=0.0011 Median=0.0045 IQR=0.0065 N=10
9. The total number of bouts where the male walked onto the female performed during the mating trial divided by the total time mating	P=0.408; P=0.14; P=0.308	Mean=0.0022 SE=0.0005 Median=0.0015 IQR=0.0017 N=17	Mean=0.0023 SE=0.0004 Median=0.0020 IQR=0.0027 N=23	Mean=0.0037 SE=0.0010 Median=0.0033 IQR=0.0046 N=11	Mean=0.0026 SE=0.0008 Median=0.0014 IQR=0.0028 N=13
10. The total time it took the male to initiate the "first mount" expressed as a proportion of the total time mating	P=0.647; P=0.961; P=0.049	Mean=0.1380 SE=0.0227 Median=0.1124	Mean=0.1799 SE=0.0241 Median=0.1411	Mean=0.1951 SE=0.0403 Median=0.1678	Mean=0.1260 SE=0.0222 Median=0.1064

Table 4 (continued)

Mating sequence	Male effect *p*-value; Female effect *p*-value; Interaction effect *p*-value	F X F	F X S	S X F	S X S
		IQR=0.0996	IQR=0.1556	IQR=0.2564	IQR=0.0933
		N=17	*N*=24	*N*=11	*N*=15
11. The number of times the male initiated the "first mount" divided by the total time mating	*P*=0.647;	Mean=0.0008	Mean=0.0012	Mean=0.0010	Mean=0.0004
	P=0.406;	SE=0.0002	SE=0.0003	SE=0.0003	SE=0.0001
	P=0.068	Median=0.0005	Median=0.0007	Median=0.0005	Median=0.0004
		IQR=0.0007	IQR=0.0009	IQR=0.0012	IQR=0.0003
		N=17	*N*=24	*N*=12	*N*=16
12. The time interval between the time the male began to sing and when he initiated of the "first mount" expressed as a proportion of the total time	*P*=0.97	Mean=0.0919	Mean=0.1305	Mean=0.1416	Mean=0.1048
	P=0.652;	SE=0.0218	SE=0.0253	SE=0.0354	SE=0.0249
	P=0.142	Median=0.0624	Median=0.0799	Median=0.1429	Median=0.0711
		IQR=0.0671	IQR=0.1642	IQR=0.2069	IQR=0.1183
		N=17	*N*=24	*N*=11	*N*=16
13. The time at which the "first mount" occurred expressed as a proportion of the total time mating	*P*=0.849;	Mean=0.1417	Mean=0.1680	Mean=0.1762	Mean=0.1416
	P=0.87;	SE=0.0226	SE=0.0198	SE=0.0407	SE=0.0264
	P=0.278	Median=0.1163	Median=0.1397	Median=0.1431	Median=0.1121
		IQR=0.0971	IQR=0.1580	IQR=0.1934	IQR=0.1469
		N=17	*N*=23	*N*=10	*N*=15
14. The number of times the male and female engaged in a "first mount" divided by the total time mating	*P*=0.691;	Mean=0.0005	Mean=0.0006	Mean=0.0005	Mean=0.0007
	P=0.646;	SE=0.0001	SE=0.0001	SE=0.0002	SE=0.0003
	P=0.819	Median=0.0004	Median=0.0004	Median=0.0004	Median=0.0003
		IQR=0.0002	IQR=0.0004	IQR=0.0004	IQR=0.0002
		N=17	*N*=24	*N*=12	*N*=17
15. The time interval between the time the male began to sing and when the "first mount"	*P*=0.82;	Mean=0.0956	Mean=0.1175	Mean=0.1190	Mean=0.1090
	P=0.762;	SE=0.0218	SE=0.0205	SE=0.0342	SE=0.0263

occurred expressed as a proportion of the total time mating	$P=0.507$	Median=0.0663 IQR=0.0672 $N=17$	Median=0.0772 IQR=0.1652 $N=23$	Median=0.0758 IQR=0.2011 $N=10$	Median=0.0738 IQR=0.1456 $N=15$
16. The time interval between the time the male initiated the "first mount" and when the "first mount" occurred expressed as a proportion of the total time mating	$P=0.056$; $P=0.501$; $P=0.605$	Mean=0.0037 SE=0.0005 Median=0.0034 IQR=0.0025 $N=17$	Mean=0.0030 SE=0.0004 Median=0.0027 IQR=0.0021 $N=23$	Mean=0.0032 SE=0.0008 Median=0.0029 IQR=0.0044 $N=10$	Mean=0.0019 SE=0.0004 Median=0.0014 IQR=0.0026 $N=16$
17. The time interval from the "first mount" to the production of the spermatophore expressed as a proportion of the total time mating	$P=0.4$; $P=0.658$; $P=0.78$	Mean=0.0696 SE=0.0045 Median=0.0660 IQR=0.0264 $N=17$	Mean=0.0562 SE=0.0030 Median=0.0572 IQR=0.0260 $N=22$	Mean=0.0660 SE=0.0069 Median=0.0613 IQR=0.0312 $N=7$	Mean=0.0551 SE=0.0032 Median=0.0554 IQR=0.0179 $N=14$
18. The total number of gyrations the male performed during the time interval from the "first mount" to the production of the spermatophore divided by the number of seconds in this time interval	$P=0.621$; $P=0.381$; $P=0.648$	Mean=0.2591 SE=0.0304 Median=0.2432 IQR=0.139 $N=16$	Mean=0.2968 SE=0.0279 Median=0.3010 IQR=0.2226 $N=22$	Mean=0.2422 SE=0.0421 Median=0.2199 IQR=0.2270 $N=7$	Mean=0.2436 SE=0.0492 Median=0.2131 IQR=0.2243 $N=15$
19. The time the male produced the spermatophore expressed as a proportion of the total time mating	$P=0.537$; $P=0.564$; $P=0.406$	Mean=0.2112 SE=0.0205 Median=0.1794 IQR=0.0963 $N=17$	Mean=0.2177 SE=0.0193 Median=0.1957 IQR=0.1429 $N=22$	Mean=0.2173 SE=0.0519 Median=0.1535 IQR=0.1401 $N=7$	Mean=0.1805 SE=0.0211 Median=0.1699 IQR=0.1134 $N=15$
20. The total number of gyrations the male performed during the time interval between the production of the spermatophore and copulation divided by the number of seconds in this time interval	$P=0.131$; $P=0.609$; $P=0.571$	Mean=0.3794 SE=0.0402 Median=0.3981 IQR=0.2080 $N=14$	Mean=0.4375 SE=0.0549 Median=0.4865 IQR=0.3362 $N=15$	Mean=0.4249 SE=0.0577 Median=0.3697 IQR=0.1766 $N=6$	Mean=0.3743 SE=0.0333 Median=0.3976 IQR=0.1536 $N=12$

Table 4 (continued)

Mating sequence	Male effect *p*-value; Female effect *p*-value; Interaction effect *p*-value	F X F	F X S	S X F	S X S
21. The time the male initiated copulation expressed as a proportion of the total time mating	*P*=0.238;	Mean=0.6236	Mean=0.5830	Mean=0.5718	Mean=0.5246
	P=0.137;	SE=0.0253	SE=0.0332	SE=0.0506	SE=0.0346
	P=0.934	Median=0.5851	Median=0.5895	Median=0.5874	Median=0.4594
		IQR=0.1667	IQR=0.2544	IQR=0.1660	IQR=0.2075
		N=15	*N*=16	*N*=6	*N*=12
22. The total number of times the male initiated a "second mount" during the trial divided by the total time mating	*P*=0.276;	Mean=0.0006	Mean=0.0004	Mean=0.0002	Mean=0.0002
	P=0.109;	SE=0.0002	SE=0.0001	SE=0.0001	SE=0.0000
	P=0.987	Median=0.0004	Median=0.0003	Median=0.0000	Median=0.0002
		IQR=0.0004	IQR=0.0005	IQR=0.0004	IQR=0.0003
		N=17	*N*=24	*N*=12	*N*=17
23. The time at which copulation occurred expressed as a proportion of the total time mating	*P*=0.186;	Mean=0.6260	Mean=0.5744	Mean=0.5769	Mean=0.5272
	P=0.183;	SE=0.0254	SE=0.0335	SE=0.0519	SE=0.0346
	P=0.975	Median=0.5874	Median=0.5429	Median=0.5897	Median=0.4623
		IQR=0.1651	IQR=0.2369	IQR=0.1833	IQR=0.2086
		N=15	*N*=15	*N*=6	*N*=12
24.The total number of "second mounts" the male attempted during the mating trial divided by the total time mating	*P*=0.257;	Mean=0.0003	Mean=0.0003	Mean=0.0002	Mean=0.0002
	P=0.875;	SE=0.0001	SE=0.0001	SE=0.0001	SE=0.0000
	P=0.161	Median=0.0003	Median=0.0003	Median=0.0000	Median=0.0002
		IQR=0.0001	IQR=0.0004	IQR=0.0004	IQR=0.0003
		N=17	*N*=24	*N*=12	*N*=17
25. Time when the female began feeding on tibial spur expressed as a proportion of the total time mating	*P*=0.116;	Mean=0.6049	Mean=0.5809	Mean=0.5810	Mean=0.4336
	P=0.107;	SE=0.0304	SE=0.0392	SE=0.0520	SE=0.0652
	P=0.277	Median=0.5869	Median=0.5929	Median=0.5964	Median=0.4600

		IQR=0.1323	IQR=0.2479	IQR=0.1802	IQR=0.1721
		N=14	*N*=12	*N*=6	*N*=12
26. The time when female dismounts the male expressed as a proportion of the total time mating	*P*=0.657; *P*=0.385; *P*=0.751	Mean=0.6816	Mean=0.6547	Mean=0.6994	Mean=0.6951
		SE=0.0249	SE=0.0307	SE=0.0374	SE=0.0329
		Median=0.7106	Median=0.6957	Median=0.7005	Median=0.7326
		IQR=0.1805	IQR=0.2351	IQR=0.1576	IQR=0.1371
		N=15	*N*=15	*N*=6	*N*=11
27. The time interval between the time copulation began and the time when the female dismounts expressed as a proportion of the total time mating	*P*=0.162; *P*=0.3; *P*=0.563	Mean=0.0557	Mean=0.0803	Mean=0.1225	Mean=0.1865
		SE=0.0164	SE=0.0233	SE=0.0357	SE=0.0383
		Median=0.0228	Median=0.0586	Median=0.1009	Median=0.1723
		IQR=0.0751	IQR=0.0756	IQR=0.1630	IQR=0.2310
		N=15	*N*=15	*N*=6	*N*=11
28. The total number of post-copulatory "dances" performed during the mating trial divided by the total time mating	*P*=0.425; *P*=0.879; *P*=0.797	Mean=0.0003	Mean=0.0002	Mean=0.0003	Mean=0.0002
		SE=0.0001	SE=0.0002	SE=0.0003	SE=0.0001
		Median=0.0000	Median=0.0000	Median=0.0000	Median=0.0000
		IQR=0.0004	IQR=0.0000	IQR=0.0003	IQR=0.0000
		N=17	*N*=23	*N*=11	*N*=17
29. Time at which the female knocked-off the spermatophore expressed as a proportion of the total time mating	*P*=0.683; *P*=0.537; *P*=0.7	Mean=0.7849	Mean=0.8061	Mean=0.8107	Mean=0.8121
		SE=0.0176	SE=0.0102	SE=0.0000	SE=0.0117
		Median=0.7901	Median=0.8106	Median=0.8107	Median=0.8202
		IQR=0.0389	IQR=0.0626	IQR=0.0000	IQR=0.0873
		N=13	*N*=20	*N*=1	*N*=14

Means, medians, standard errors (SE), intra-quartile ranges (IQR) and sample sizes (N) of traits measured in the mating sequence. *FF* represents an intraspecific cross between an *A. fasciatus* female and an *A. fasciatus* male. *FS* represents an interspecific cross between an *A. fasciatus* female and an *A. socius* male. *SF* represents the reciprocal interspecific cross to FS. Specifically, it is a cross between an *A. socius* female and an *A. fasciatus* male. *SS* represents the other intraspecific cross in this study. It is a cross between an *A. socius* female and an *A. socius* male

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