



The resilience of reproductive interference

Scott M. Villa¹ · Diane D. Han¹ · Malichai E. Jordan¹ · Nicole M. Gerardo¹

Received: 9 October 2020 / Accepted: 15 May 2021

© This is a U.S. government work and not under copyright protection in the U.S.; foreign copyright protection may apply 2021

Abstract

Mating with the wrong species is surprisingly common in nature. Interspecific mating can lead to reproductive interference, where wasted time, energy, nutrients, or gametes reduces the fitness of one or both of the interacting species. However, the ecological and evolutionary forces that maintain this seemingly maladaptive behavior remain poorly understood, in part because the natural complexity of heterospecific encounters is often not considered experimentally. The goal of this study was to directly test if the negative effects of heterospecific mating can be mitigated by conspecific mating. We used two closely related species of squash bug, *Anasa tristis* and *Anasa andresii*, which are known to readily mate with each other despite clear negative fitness consequences. We gave all females opportunities to mate with conspecific males before and after encountering heterospecific males. We found that *A. tristis* females can alleviate temporary bouts of heterospecific interference when given opportunities to mate with conspecifics. However, we found the opposite for *A. andresii* females. Mating with conspecifics did not shelter female *A. andresii* from the consequences of heterospecific mating. Our study reveals the complex dynamics of reproductive interference and highlights scenarios where mating with the wrong species can have either minimal or long-lasting effects on fitness. We emphasize the benefit of assessing reproductive interference using experiments that not only vary heterospecific encounter rates, but that also quantify lifetime measures of fitness. Our study adds to the growing body of research highlighting the importance of reproductive interference and sheds light on why this seemingly paradoxical behavior continues to persist.

Keywords Heterospecific mating · Squash bugs · *Anasa* · Hybridization

Introduction

Mating with the wrong species is rarely an adaptive reproductive strategy. Interspecific mating can waste time, energy, nutrients, or gametes and directly interferes with mating and reproducing with conspecifics. This so-called “reproductive interference” reduces the fitness of one or both of the interacting species (Gröning and Hochkirch 2008;

✉ Scott M. Villa
scott.villa@gmail.com

¹ Department of Biology, O. Wayne Rollins Research Center, Emory University, Atlanta, GA, USA

Burdfield-Steel and Shuker 2011). While in some cases heterospecific mating can result in viable hybrids (Lipshutz 2018), in most scenarios it is a reproductive dead end. However, despite these obvious fitness consequences, sexual interactions between species remain surprisingly common in nature (Hettyey and Pearman 2003; Dame and Petren 2006; Gröning and Hochkirch 2008; Valero et al. 2008; Kishi et al. 2009; Shuker and Burdfield-Steel 2017; Drury et al. 2019; Grether et al. 2020).

Reproductive interference can play an important role in shaping community structure and the evolutionary trajectories of populations (Kyogoku and Wheatcroft 2020). Sexual interactions between species can have consequences for population persistence (Liu et al. 2007; Hochkirch et al. 2007; Kishi et al. 2009) and species coexistence (Konuma and Chiba 2007; Thum 2007). For example, the short, intense breeding seasons of many frog species drive males to vigorously clasp and guard any object they perceive as a potential mate, including heterospecifics. This indiscriminate mating behavior used by one common species, *Rana dalmatina*, decreases the mating success and persistence of another, more threatened species, *Rana latastei* (Hettyey and Pearman 2003). Heterospecific mating can also influence habitat partitioning and competition (Kuno 1992; Gröning and Hochkirch 2008). McLain and Shure (1987), for example, found that aggressive heterospecific mating attempts by the seed bug *Neacoryphus bicrucis* structure community composition by chasing off heterospecific females, effectively preventing other insect species from co-colonizing host plants. However, despite the growing number of studies documenting reproductive interference and its consequences, the ecological and evolutionary forces that maintain this seemingly maladaptive behavior remain poorly understood.

Reproductive interference is traditionally investigated by comparing survival and reproductive performance among individuals subjected to continuous access to either conspecifics, heterospecifics, or a combination of both for the entire length of an experiment (Gröning and Hochkirch 2008; Shuker and Burdfield-Steel 2017). This approach has been key in determining not only which species display indiscriminate mating behavior, but also in revealing, through comparison of individuals exposed for the duration to either heterospecifics or conspecifics, the maximum possible effects reproductive interference can have on fitness (Gröning and Hochkirch 2008; Burdfield-Steel and Shuker 2011; Manzano-Winkler et al. 2017). However, in natural populations, organisms are rarely subjected to continuous exposure to heterospecifics. Instead, the opportunities for reproductive interference vary substantially over an organism's lifetime. The timing and length of heterospecific encounters depends on a wide variety of ecological factors (Drury et al. 2019) and is often driven by niche partitioning (Noriuki and Osawa 2016). Previous studies suggest that when encounter rates between ecologically similar species are low, conspecific mating may alleviate the costs of brief bouts of reproductive interference (Price 1997; Noriuki and Osawa 2016; Burdfield-Steel et al. 2015; Clemente et al. 2018). This may occur through a variety of pre- and post-copulatory mechanisms, including learning and avoidance (Svensson et al. 2010), sperm precedence (Price 1997; Howard et al. 1998; Reinhardt 2006), sperm competition (Lorch and Servodio 2007; Simmons 2014) and preferential sperm allocation via cryptic female choice (Wade et al. 1994; Tyler et al. 2013). For example, when female *Gryllus* field crickets are subjected to conspecific and heterospecific mating, they can preferentially store more sperm from conspecific males, which has a higher fertilization probability (Yeates et al. 2013). Thus, reproductive interference may persist in many systems because conspecific mating overrides the negative effects of mating with the wrong species.

Here we use two closely related species of squash bug, *Anasa tristis* and *Anasa andresii*, to directly test if conspecific mating can reduce the negative consequences of reproductive

interference. Both species are widespread agricultural pests of cucurbits and can feed, mate, and oviposit on the same plant species (Jones 1916; Beard 1940). *Anasa tristis* and *A. andresii* naturally interact in gardens and fields along a narrow contact zone in northern Florida and are known to show classic reproductive interference behavior. Hamel et al. (2015, 2018) showed that interspecific mating between these two species leads to substantial reproductive costs for females. However, despite these costs, males of both species readily engage in heterospecific mating. One possible reason that mating persists between these species is that the fitness consequences of heterospecific matings may be low in natural populations. Although both species overlap in the field, their generations are not entirely synchronous (Hamel et al. 2018). *Anasa tristis* tend to emerge from overwintering before *A. andresii* (Jones 1916; Beard 1940). Thus, interactions between species cycle between periods of low and high encounter rates as a season progresses. During periods when encounter rates are low, females may offset potential interference costs by mating with conspecifics and therefore weaken selection against this seemingly maladaptive behavior.

We conduct two parallel experiments designed to show why reproductive interference persists between these two species. The first experiment focuses on the survival and fecundity of *A. tristis* females, and the second experiment focuses on the survival and fecundity of *A. andresii* females. In both experiments, we allowed male–female and male–male interactions in not only conspecific and heterospecific contexts, but under varying sex ratios as well (i.e., one female with one male, or one female with two males). We give females opportunities to mate with conspecific males both before and after encountering heterospecific males. We hypothesize that when females are not continuously exposed to heterospecific males, interference will have negligible effects on their overall reproductive performance. By experimentally testing how encounter dynamics influence reproductive interference, we reduce the likelihood of over- or underestimating reproductive interference's effect in shaping species interactions (Gröning and Hochkirch 2008; Kyogoku and Nishida 2013; Kyogoku and Wheatcroft 2020).

Materials and methods

Squash bug collection and rearing

All squash bugs used in this study were lab-reared descendants of wild-caught populations from Gainesville, FL. Both *A. tristis* and *A. andresii* can be readily maintained in the laboratory for multiple generations.

Initially, immature bugs of each species were reared communally in 12×12×12 inch pop-up mesh insect cages. Rearing cages contained a single yellow summer squash (*Cucurbita pepo*) plant for food and were kept in walk-in environmental chambers (Environmental Specialties, Inc., Raleigh, NC, USA) set to a 14:10 h light/dark cycle at 26C and 50% relative humidity. To ensure that squash bugs were unmated prior to experiments, cages with developing juveniles were checked every 24–48 h for newly mature adults. Mature bugs were removed and placed in a new mesh cage containing other unmated adults of the same species and sex. These pools of unmated *A. tristis* and *A. andresii* adults were used for all subsequent experiments.

Lab observations suggest adults of both species are reproductively mature approximately 10 days after becoming adults. Moreover, adults that survive at least 10 days are considered “robust” and typically live up to three months in captivity. Thus, we only used squash bugs that were at least 10 days old to ensure that all individuals were reproductively active at the outset of the experiment and were likely to survive the length of the study.

Experimental design

Overview

We conducted two experiments. The first experiment focused on the reproductive performance of *A. tristis* females, and the second experiment focused on the reproductive performance of *A. andresii* females. Both experiments were conducted in three stages: (1) pre-exposure, (2) exposure, and (3) post-exposure (Fig. 1). Each stage lasted four weeks. Since squash bugs typically live 10–12 weeks in the wild (Beard 1940), the length of both experiments captures the lifetime reproductive performance of squash bugs. Initially, unmated females were individually placed in a 12 × 12 × 12 inch mesh cage containing a single yellow summer squash plant, which provided food and a substrate on which to mate and lay eggs. Females were randomly assigned to one of three treatments: heterospecific (*A. tristis* n = 10 females, *A. andresii* n = 5 females), conspecific (*A. tristis* n = 10 females, *A. andresii* n = 5 females) or single (*A. tristis* n = 10 females, *A. andresii* n = 5 females). All females were paired with one conspecific male, and, in the heterospecific treatment, during

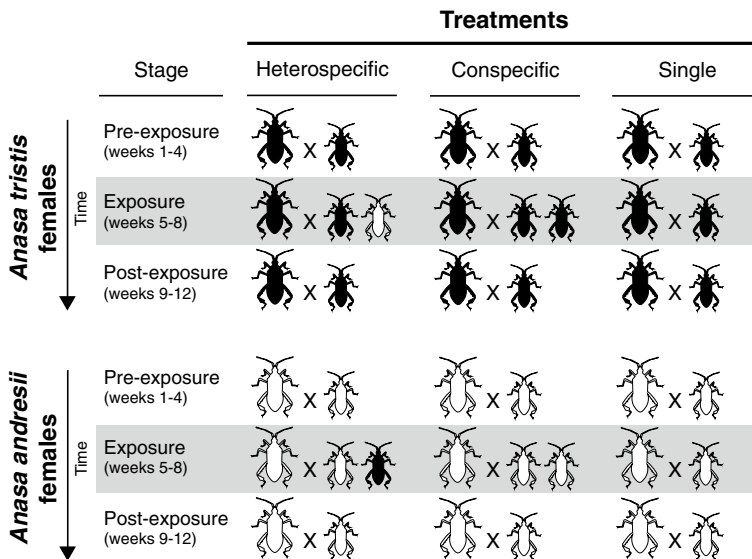


Fig. 1 Summary of two experiments designed to test the influence of a window of reproductive interference on the fecundity of *Anasa tristis* females (top) and *Anasa andresii* females (bottom). Black bugs represent *A. tristis* and white bugs represent *A. andresii*. In both species, the larger bugs indicate females and the smaller indicate males. Each experiment has three treatments and was conducted in three stages. The gray bar represents the “Exposure” period of each experiment, in which we manipulated the number and species of males allowed to mate with females

the exposure stage, females were exposed to a second, heterospecific male. In the conspecific treatment, during the exposure stage, they were exposed to a second conspecific male, and in the single treatment a second male was never introduced (Fig. 1).

Details

The experiment began with the “pre-exposure” stage, where a single, conspecific male was added to each cage. Thus, all cages at this time contained two squash bugs: one female and one conspecific male (Fig. 1). All males were labeled with a unique number drawn on the pronotum using a non-toxic silver pen. These labels do not influence male survival or mating behavior. Conspecific pairs were left to mate and reproduce for four weeks. This no-choice period insured that each female was given a reproductive “head start” and allowed unrestricted mating with a conspecific male. Throughout the pre-exposure stage, each cage was checked 2–3 times per week for eggs and adult survival. Eggs are typically glued directly to the squash plants and can easily be removed without harming the bugs or damaging the plants. Eggs were collected and left to hatch in $7 \times 7 \times 3$ mm plastic boxes, allowing us to determine the total number of eggs and offspring produced by each female during the pre-exposure stage of the experiment.

Immediately following the pre-exposure period, we conducted the “exposure” stage. During this four-week period, we manipulated the number and species of males that were in each cage (Fig. 1). The females assigned to the “heterospecific” treatment were each exposed to a second, heterospecific male. Thus, these cages now contained three squash bugs: one female, one conspecific male and one heterospecific male. The females assigned to the “conspecific” treatment were each exposed to a second, conspecific male. Thus, these cages also contained three squash bugs: one female and two conspecific males. The final females, assigned to the “single” treatment, were not exposed to an additional male. Thus, these cages simply contained the original mating pair of adults from the pre-exposure stage.

All males that were added to cages during the exposure stage were individually marked as described above. Again, each cage was checked 2–3 times per week for adult survival and eggs, which were collected and left to hatch in a $7 \times 7 \times 3$ mm plastic box. From each box, we could determine the total number of eggs and offspring produced by each female during the exposure stage of the experiment.

For the final “post-exposure” stage of the experiment, we removed all of the males that were introduced to the cages during the exposure period. Thus, during this stage all cages were back to containing only the original pairs from the pre-exposure stage (Fig. 1). This design insured, for the last four weeks of the experiment, that females once again had uninterrupted access to a conspecific male without the presence of a competing male, allowing us to test for any delayed or recovery effects on female reproduction after the exposure stage. Again, each cage was checked 2–3 times per week for eggs and adult survival. Eggs were collected and left to hatch in a $7 \times 7 \times 3$ mm plastic box, allowing us to determine the total number of eggs and offspring produced by each female during the last four weeks of the experiment.

Throughout both experiments, we tracked female survival. If a female died, the trial ended. On the rare occasion that a male died, he was immediately replaced with a male of the same species. Over the course of both experiments, four *A. andresii* males and three *A. tristis* males died and were replaced.

Data analysis

All analyses were conducted in R v3.3.3 (R Core Team 2016) using RStudio v1.0.136 (RStudio 2015). We used a series of models to test for changes in reproduction throughout the experimental stages within each of the three treatments. We chose this approach over larger, full factorial models that simultaneously compared measures among stages and treatments due to the lower sample sizes of the *A. andresii* experiment. For consistency, and to facilitate comparisons between species, we analyzed both experiments similarly. The numbers of eggs laid and the numbers of offspring produced within each treatment were normally distributed (Shapiro-Wilks normality test; $P > 0.05$). Thus, we used a series of linear mixed models (LMMs) to explore the influence of intraspecific competition and interspecific interference on female reproductive performance among experimental stages (i.e., pre-exposure vs. exposure vs. post-exposure). For each treatment, we predicted changes in the numbers of eggs laid and offspring produced over the course of the experiment by modeling the experimental stage as a fixed effect and individual cage as a random effect. The random effect was included to account for the repeated measures of each female over the three stages. The intercept of each LMM was set to the value of the pre-exposure stage (i.e., the beginning of the experiment). We also used a series of mixed models to explore changes in egg fertility rates (i.e., proportion of eggs that successfully hatched) across stages. Because eggs were scored as “hatched” or “not hatched”, we used generalized linear mixed models (GLMMs) assuming a binomial distribution to analyze changes in fertility rates. For each GLMM, experimental stage was modeled as a fixed effect and individual cage as a random effect. The random effect was included to account for the repeated measures of each female over the three stages. The intercept of each GLMM was set to the value of the pre-exposure stage (i.e., the beginning of the experiment).

We ran additional models to compare the lifetime reproductive success among the treatments (i.e., heterospecific vs. conspecific vs. single). Lifetime reproductive success is defined as the overall eggs, egg fertility, and offspring produced while the female was alive in the experiment. Lifetime numbers of eggs and offspring were each obtained by simply adding together the numbers produced from each stage of the experiment. Both of these lifetime measures were normally distributed within species (Shapiro-Wilks normality test; $P > 0.05$), so we opted to use linear models for comparisons among treatments. LMs were modeled with treatment as a fixed effect. Lifetime egg fertility was calculated as the total number of eggs hatched out of all the eggs laid over the female's life. The lifetime egg fertility was modeled for each species with a generalized linear model (GLM) assuming a binomial distribution. GLMs were modeled with treatment as a fixed effect. All models were fit in R using the ‘lme4’ library package (Bates et al. 2015; R Core Team 2016). Degrees of freedom and resulting p-values were estimated with the Satterwhite approximation using the lmerTest library (Kuznetsova et al. 2016). Post hoc comparisons between treatments were performed using Tukey's honestly significant difference tests (HSD) using the R library package ‘emmeans’.

Finally, we analyzed survival of *A. tristis* and *A. andresii* females as a step function using two Kaplan–Meier survival analyses with the ‘survival’ package in R (Therneau and Grambsch 2000). We analyzed differences in survival between treatments using the log-rank test with post hoc Pairwise t-tests.

Results

Reproductive performance of *Anasa tristis* females

We collected a total of 19,316 eggs from 30 *Anasa tristis* females over the course of the 12-week experiment. We tracked: (1) the numbers of eggs laid, (2) egg fertility and (3) total offspring produced by each female during each stage of the experiment (Fig. 2; Table S1).

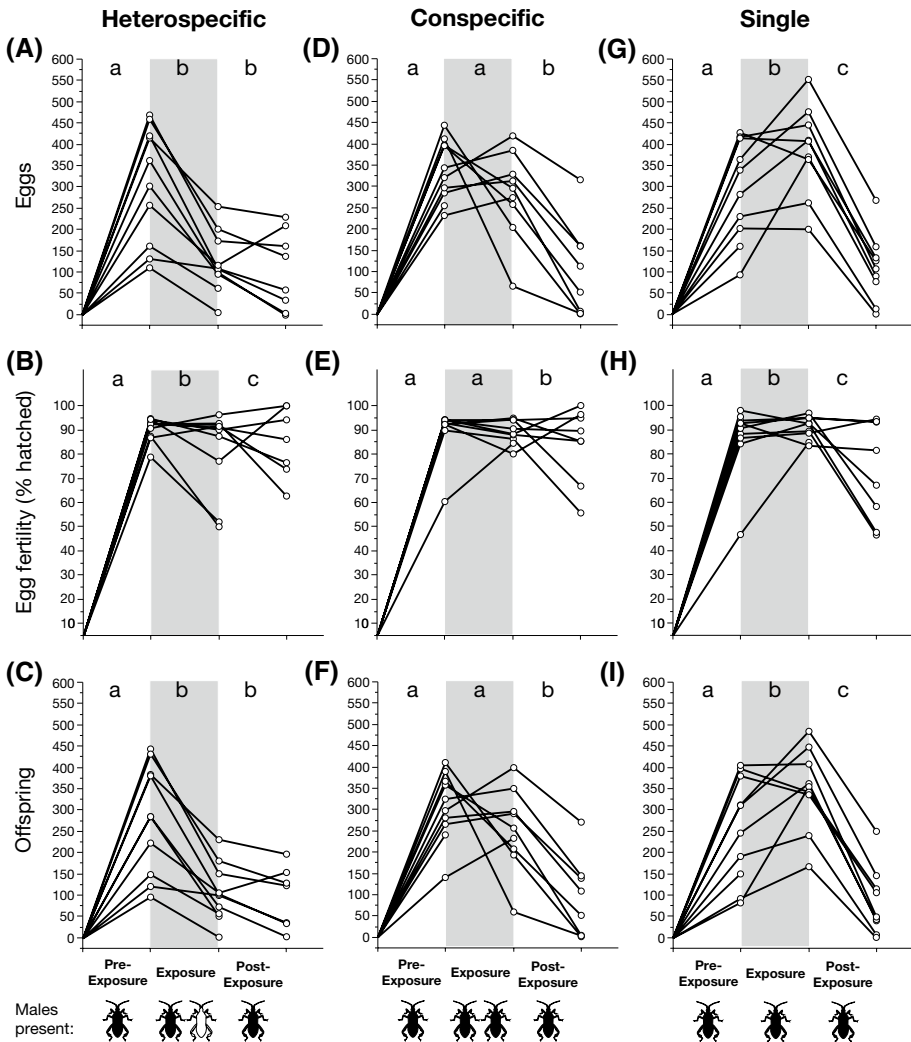


Fig. 2 Reproductive performance of *A. tristis* females. Each line tracks an individual female's egg laying (A, D, G), egg hatching success (B, E, H), and offspring production (C, F, I). Bugs on the x-axis indicate the number and species of males that were allowed to mate with *A. tristis* females during each stage of the experiment. Shaded region highlights the “Exposure” periods, in which the mating scenario differed between treatments. Black bugs = *Anasa tristis* males; white bugs = *Anasa andresii* males. Different letters in each panel indicate significant differences ($P < 0.05$) between stages

Female *A. tristis* in the heterospecific treatment laid significantly fewer eggs during both the exposure (LMM with Tukey HSD post hoc tests, $P < 0.0001$) and post-exposure ($P < 0.0001$) stages compared to the initial pre-exposure stage (Fig. 2A; Table S3A). There was no significant difference in the number of eggs laid between the exposure and post-exposure stages. There were significant differences in fertility rates among these females in all three experimental stages (Fig. 2B; Table S3B; GLMM with Tukey HSD post hoc tests, $P < 0.001$ for all comparisons). Fertility rates were highest during the pre-exposure stage and lowest in the exposure stage (Table S1). Females had significantly fewer number of offspring during both the exposure ($P < 0.0001$) and post-exposure ($P < 0.0001$) stages compared to the pre-exposure stage (Fig. 2C; Table S3C). There was no significant difference in the offspring produced between the exposure and post-exposure stages.

For *A. tristis* females in the conspecific treatment, there was no significant difference in the numbers of eggs laid, fertility rates or offspring produced between the pre-exposure and exposure stages (Fig. 2D–F; Table S4). However, all three reproductive measures were significantly lower during the post-exposure stage than in the previous two stages ($P < 0.004$ for all comparisons; Fig. 2D–F; Table S4).

Finally, for *A. tristis* females in the single treatment, there were significant differences in the numbers of eggs laid, fertility rates or offspring produced among all three stages of the experiment ($P < 0.04$ for all comparisons; Fig. 2G–I; Table S5). Specifically, all three reproductive measures peaked during the exposure stage and were the lowest during the post-exposure stage (Table S1). Note, however, that the significant increase in egg fertility in the exposure stage is driven by the large increase for a single female (Fig. 2H). When this female is removed, there is no longer difference in egg fertility between the pre-exposure and exposure stages ($P = 0.85$).

We also compared lifetime measures of fitness among treatments (i.e., heterospecific vs. conspecific vs. single). Lifetime numbers of eggs laid, egg fertility, and offspring produced were obtained by combining the values produced in the three experimental stages. This provided the total reproductive performance of each female over the course of her life. There were no significant differences for lifetime numbers of eggs laid or offspring produced (Table S6A,C; LMs with Tukey HSD post hoc tests, $P > 0.19$ for all comparisons) among the three treatments. The females in the conspecific treatment had a significantly higher lifetime egg fertility than those in either the heterospecific (Table S6B; $P = 0.03$) or conspecific treatments ($P = 0.01$).

Reproductive performance of *Anasa andresii*

We collected a total of 4616 eggs from 15 *Anasa andresii* females over the course of the 12-week experiment. We tracked: (1) the numbers of eggs laid, (2) egg fertility and (3) total offspring produced of each female during each stage of the experiment (Fig. 3; Table S2).

Female *A. andresii* in the heterospecific treatment had significantly lower numbers of eggs laid, egg fertility and offspring produced in the exposure and post-exposure stages than in the initial pre-exposure stage (Fig. 3A–C; Table S7; $P < 0.008$ for all comparisons). There were no significant differences in any reproductive measures between the exposure and post-exposure stages (Fig. 3A–C; Table S7).

For *A. andresii* females in the conspecific treatment, there were no significant differences in the number of eggs laid (Fig. 3D; Table S8A) or offspring produced (Fig. 3F; Table S8C) among any stage of the experiment. However, females did have significantly lower fertility rates during both the exposure (Fig. 3E; Table S8B; $P < 0.0001$) and

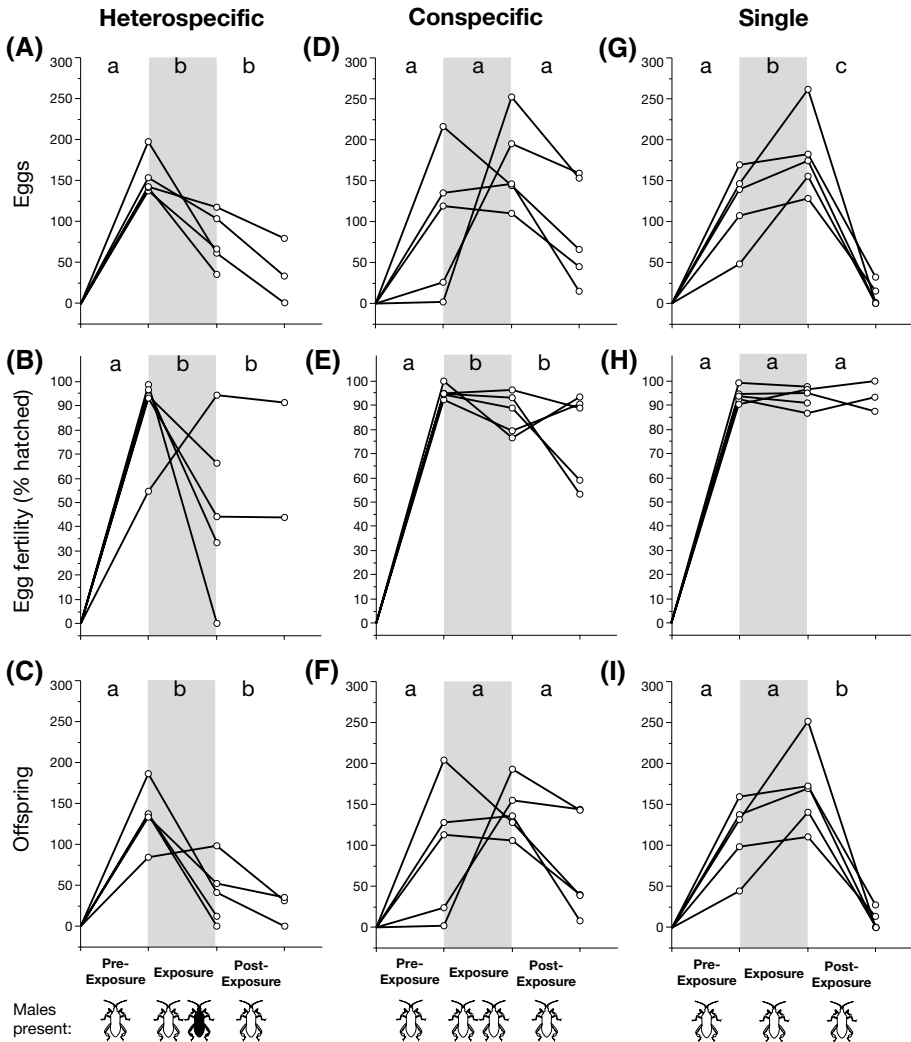


Fig. 3 Reproductive performance of *A. andresii* females. Each line tracks an individual female’s egg laying (A, D, G), egg hatching success (B, E, H), and offspring produced (C, F, I). Bugs on the x-axis indicate the number and species of males that were allowed to mate with *A. andresii* females during each stage of the experiment. Shaded region highlights the “Exposure” periods, in which the mating scenario differed between treatments. Black bugs=*Anasa tristis* males; white bugs=*Anasa andresii* males. Different letters in each panel indicate significant differences ($P < 0.05$) between stages

post-exposure ($P = 0.0003$) stages compared to the initial pre-exposure stage. There was no significant difference in fertility between the exposure and post-exposure stages (Fig. 3E; Table S8B).

Finally, the *A. andresii* females in the single treatment laid significantly different numbers of eggs in all three stages of the experiment (Fig. 3G; Table S9A; $P < 0.05$ for all comparisons). Egg production peaked in the exposure stage and was the lowest in the post-exposure stage. There were no significant differences in egg hatching rates among the three

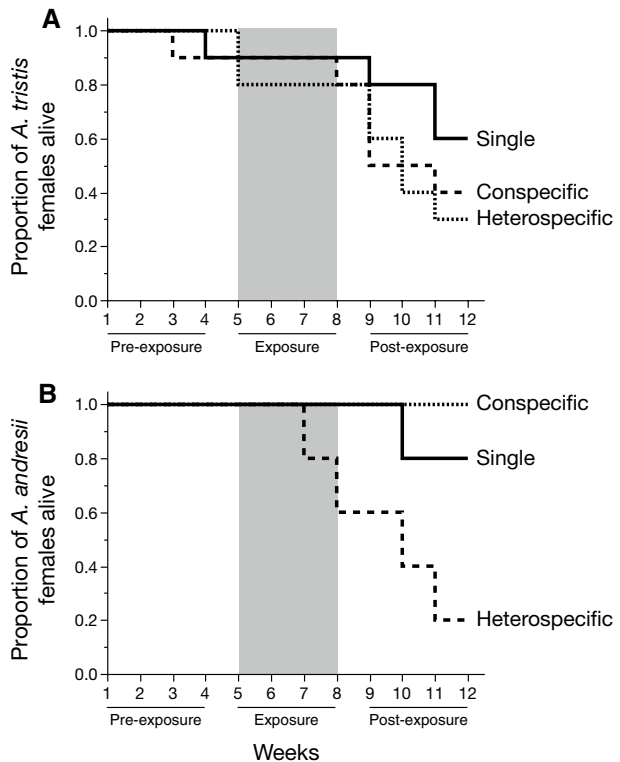
experimental stages (Fig. 3H; Table S9B). The number of offspring produced between the pre-exposure and exposure stages was marginally significantly different (Fig. 3I; Table S9C; $P=0.07$). However, females produced significantly fewer offspring during the post-exposure stage than both the pre-exposure ($P=0.001$) and exposure ($P<0.0001$) stages.

Similar to the first experiment, we compared lifetime measures of fitness among treatments. There were no significant differences in the lifetime number of eggs laid among the three treatments (S10A; $P>0.12$ for all comparisons). However, the overall fertility rates of *A. andresii* females were significantly different among all three treatments (Table S10B; $P<0.0001$ for all comparisons). Lifetime fertility was highest for females in the single treatment and lowest for females in the heterospecific treatment. The overall number of offspring produced by *A. andresii* females in the heterospecific treatment was significantly lower than those in either the conspecific ($P=0.02$) or single ($P=0.05$) treatments (Table S10C). There were no significant differences in the number of offspring produced between females in the conspecific and single treatments ($P=0.87$).

Survival of *A. tristis* and *A. andresii* females

We tracked the survival of both females of both species in our experiments (Fig. 4). Survival of *A. tristis* females did not vary among treatments over the course of 12 weeks (Fig. 4A; Kaplan–Meier Survival, log-rank test, $\chi^2=2.01$, $df=2$, $P=0.37$). However, survival of

Fig. 4 The survival of (A) *Anasa tristis* females and (B) *Anasa andresii* females exposed to different numbers and species of males during the “Exposure” period (shaded region). See Fig. 1 for details on which males were present during each experimental stage



A. andresii females varied significantly among treatments (Fig. 4B; Kaplan–Meier Survival, log-rank test, $\chi^2=8.62$, $df=2$, $P=0.01$). Specifically, pairwise post hoc tests show that females in the heterospecific treatment died at faster rates than females in either the conspecific ($P=0.01$) or control ($P=0.04$) treatments. There was no difference in survival between females in the conspecific or control ($P=0.40$).

Discussion

The overall goal of this study was to test if the negative effects of heterospecific mating can be mitigated by conspecific mating. We designed two experiments to mimic more realistic encounter rates with heterospecifics, which can vary periodically over an individual's lifetime. Both experiments were conducted in three stages that collectively covered the typical lifespan of *Anasa* squash bugs (Beard 1940). However, we found that female reproductive output naturally decreases after about eight weeks. In both experiments, female reproduction in our control groups (i.e., single treatments) sharply declined during the post-exposure stage (Figs. 2G, I, 3G, I). This significant reduction in fitness was consistent across all replicates of both experiments and happened in the absence of any addition of males. These patterns suggest that reproductive data from the post-exposure stage are more likely driven by age-related effects rather than any experimental manipulation. Our data are consistent with previous results describing decreases in fecundity over time in natural populations of *Anasa* (Nechols 1987; Bonjour et al. 1993). Thus, our conclusions primarily focus on the pre-exposure and exposure stages of the experiments. However, we stress that reproductive output during these older, less fecund stages still contributes to an organism's overall fitness and can help buffer the effects of reproductive interference.

Our first experiment focused on the reproductive performance of *A. tristis* females (Fig. 1). All females in this experiment successfully mated and produced offspring (Fig. 2; Table S1). However, despite an initial reproductive "head start," the subsequent addition of a heterospecific male still caused a significant reduction in all measures of female fecundity (Fig. 2A–C; Table S3). Female *A. tristis* simultaneously housed with both *A. tristis* and *A. andresii* males laid 60% fewer eggs and produced 62% fewer offspring than when they were previously housed with only a single conspecific male. This reduction in fitness was consistent across all 10 replicates and demonstrates clear signs of reproductive interference. These data are similar to previous studies showing the negative effects of heterospecific mating between male *A. andresii* and female *A. tristis* (Hamel et al. 2015, 2018). Importantly, in the cages that did not receive a second male during the exposure period (i.e., the single treatment), fecundity actually increased (Fig. 2G, I). Thus, the lower reproductive performance of females exposed to *A. andresii* males was due to reproductive interference and not merely natural reduction in fecundity as squash bugs age. Interestingly, when females were exposed to a second conspecific male, the effect on female reproduction was much more variable. While, overall the data indicate no difference in reproduction between the pre-exposure and exposure stages in the conspecific treatment (Table S4), some females experienced sharp declines in eggs and offspring while others had these measures increase when a second male was added (Fig. 2D, F). These data, combined with the higher success of females in the single treatment, suggest that in some scenarios there is a potential reproductive cost of polyandrous mating and male-male competition. Further studies should investigate these possibilities in *Anasa*.

It remains unclear how male *A. andresii* were able to reduce female *A. tristis* reproduction. It is possible that during the 4-week exposure period, females just ran out of sperm and were prevented from re-mating with the conspecific male. However, this scenario is unlikely given that a single mating can provide female squash bugs with enough sperm to fertilize eggs throughout her lifetime (Beard 1940). Instead, interference is likely due to pre-zygotic harassment and increased mate guarding behavior. Previous studies show that male harassment and subsequent denial of oviposition opportunities is a common reproductive interference strategy (Gomez-Llano et al. 2018). When male *A. andresii* were to added cages, females likely spent more time and energy trying to avoid heterospecific mating and less time laying eggs. In fact, during the exposure stage we observed females were more often on the mesh walls of the cages and not on the plants themselves. Males can also reduce egg laying by remaining *in copula* for prolonged periods of time. For example, Andrews et al. (1982) showed that heterospecific courtship behavior between two tick species severely reduced mating between male and female conspecifics. Future studies should quantify squash bug interference behavior to determine how mating and guarding behavior changes in response to the presence of heterospecific competitors.

Interestingly, even though *A. tristis* females suffered an immediate reduction in fecundity when exposed to *A. andresii* males, the effect was not large enough to significantly reduce their survival (Fig. 4A) or lifetime reproductive performance relative to those that were not exposed to heterospecific males (Table S6AC). We should note that lifetime fertility rate was highest in the conspecific treatment; specifically, females exposed to two conspecific males had lifetime fertility rates roughly 2% higher than those in the heterospecific or single treatments (Table S1, S6B). This slight but significant increase may indicate a benefit of polyandrous mating in this species. Regardless, our data suggest that sufficient reproduction with conspecifics, particularly before encountering a heterospecific, can moderate a temporary bout of interference. In other words, if female *A. tristis* can avoid *A. andresii* long enough to sufficiently mate and reproduce with other *A. tristis*, then the reproductive consequences of interference are actually relatively low. This scenario seems plausible for many *A. tristis* females in the wild because, although both species overlap in the field, their generations are not entirely synchronous (Hamel et al. 2018). *Anasa tristis* tend to emerge from overwintering before *A. andresii*, and therefore females have plenty of opportunity to mate with conspecifics in the absence of *A. andresii*. Our data suggest that selection against heterospecific mating should be relatively weak in natural populations and likely explain why female *A. tristis* have not evolved heterospecific avoidance mechanisms.

Here we stress the importance of conducting experiments that not only vary encounter rates with heterospecifics but also quantify lifetime measures of reproductive success. If *A. tristis* females were continuously housed with *A. andresii* males for the entire 12-weeks, they would have suffered a massive reduction in lifetime fecundity (Hamel et al. 2018), leading to the conclusion that interference should be strongly selected against and avoided at all costs. This scenario oversimplifies the interactions between these two species and assumes females are incapable of avoiding *A. andresii* males. Instead, our experiment built in periods of heterospecific avoidance. Moreover, while the data clearly show the negative effects of heterospecific mating for *A. tristis* females, the magnitude of this effect appear lessened when combined with the mating success before and after heterospecific exposure. Thus, assessing lifetime measures of reproductive performance was critical to reveal how conspecific mating might alleviate the temporary consequences of reproductive interference for *A. tristis* females.

Our second experiment focused on the reproductive performance of *A. andresii* females. Once again, the addition of a heterospecific male in the exposure stage caused

an immediate reduction in female fitness (Fig. 3A–C; Table S7). However, unlike the first experiment, this temporary bout of interference was severe enough to significantly influence lifetime measures of fitness. Female *A. andresii* exposed to heterospecific males produced significantly fewer offspring over their lifetimes than females exposed only to conspecific males (Table S10C). This reduction in total offspring was due, in part, to a more severe reduction in lifetime fertility rates (Fig. 3B). The addition of male *A. tristis* to cages caused mean lifetime egg hatching rates to drop by nearly 40% (Table S2). Thus, unlike the first experiment, reproductive interference here might involve post-copulatory mechanisms. Hamel et al. (2018) showed that eggs produced by mating between these species are mostly infertile. The drop in egg hatching rates suggests that *A. tristis* were not only successful at inseminating *A. andresii* females, but that their sperm potentially outcompete or remove *A. andresii* sperm (Simmons 2014). Alternatively, heterospecific matings may directly reduce female fertility (Howard et al. 1998; Reinhardt 2006; Lorch and Servedio 2007; Simmons 2014). For example, Nasci et al. (1989) showed that female *Aedes aegypti* are rendered sterile after mating with invasive *Ades albopictus*. Further assessments of sperm competition and post-copulatory fertilization dynamics in squash bugs are needed.

Additionally, male *A. tristis* significantly reduced female *A. andresii* survival (Fig. 4B). Only one of the five *A. andresii* females exposed to *A. tristis* males survived the entire 12-week experiment. These data suggest that male *A. tristis*, which are much larger than male *A. andresii*, cause physical harm when mating with *A. andresii* females. Males initiate mating by forcibly grasping the female and positioning the distal tip of their abdomens to meet the distal tip of the female's abdomen. Females can reject mounting attempts by shaking their bodies or kicking the males with their hind legs. In many cases, prolonged copulation attempts can result in serious injury (Schraft 2017). Once a male successfully mounts a female, he internally latches onto the female's genitalia using aedeagus hooks. While these hooks likely co-evolved as a "lock and key" mechanism with female genitalia to keep pairs *in copula* while they move and feed, they may also inadvertently injure females. Adaptations that prevent males from being dislodged while mating are widespread among insects (Kokko et al. 2014; Moore 2014) and may indeed be a major contributor to the consequences of reproductive interference. Curiously, most of the *A. andresii* female deaths occurred during the post-exposure period, when the heterospecific males had been removed (Fig. 4B). Future work should examine females immediately after heterospecific copulation to assess injuries sustained during mating. Regardless of how *A. tristis* cause harm, this experiment shows that interference can have both immediate and long-term effects on *A. andresii* health and fitness.

Unlike the first experiment, mating with conspecifics did not shelter female *A. andresii* from the consequences of reproductive interference. These results add to the growing evidence that interference can remain a problem despite successful conspecific mating (Clemente et al. 2018; Kyogoku and Nishida 2013). Our data are particularly surprising given that females were only temporarily exposed to heterospecific males and had continuous access to conspecific mates throughout the experiment. While we stress caution in interpreting the interference results from this experiment given the lower sample size, the effects of reproductive interference on the *A. andresii* females included in the study are clear and consistent. Across all three reproductive measures, four of the five females showed the same, negative patterns when exposed to heterospecific males. Moreover, all four of these females died before the end of the experiment. The reductions in both fecundity and survival strongly demonstrate that heterospecific mating can be much worse for female *A. andresii* than for female *A. tristis*.

The resilience of interference in this experiment suggests that female *A. andresii* should avoid mating with *A. tristis* males at all costs. Interestingly, *A. andresii* females have not evolved mechanisms to reject *A. tristis* copulating attempts. This lack of defense could be due to the relatively narrow overlap in range between the two species. If *A. andresii* and *A. tristis* only come in contact in northern Florida, selection may not be widespread enough for this species as a whole to evolve mate rejection adaptations. Alternatively, *A. tristis* males may simply be too large for *A. andresii* females to reject them. A more effective strategy may be for *A. andresii* females to simply avoid areas with *A. tristis* males altogether (Svensson et al. 2010). Here, avoidance was challenging for females given the limited space in our cages. However, in the wild, *A. andresii* may limit encounters with hetero-specific males by avoiding *A. tristis* infested plants. Avoidance behavior may explain why the two squash bug species often partition into different plant microhabitats in the field. Hamel et al. (2015) noted that although both species feed, mate, and oviposit on the same *Cucurbita* spp., *A. tristis* typically occur on substrates and plant stems near the soil surface, whereas *A. andresii* are often observed on vertical plant surfaces (e.g., vining, trellised plants) above the soil. Indeed, many theoretical studies have hypothesized that reproductive interference can be a stronger driver of microhabitat partitioning than resource competition (McLain and Shure 1987; Noriyuki and Osawa 2016; Drury et al. 2019; Grether et al. 2020). Quantifying female choice between plants with and without *A. tristis* males would be useful for determining if female *A. andresii* actively avoid *A. tristis* altogether.

The negative effects of interference between *A. tristis* and *A. andresii* were asymmetrical. We show that *A. tristis* is the superior competitor and imposes much stronger effects on *A. andresii* than it receives. Our data suggest that there should be weak selection for *A. tristis* females to avoid heterospecific mating but strong selection for heterospecific avoidance by *A. andresii* females. Interestingly, these assumptions are consistent with the direction of heterospecific mating most commonly observed in the field. Hamel et al. (2015) noted that mating between these species are typically observed in one direction: male *A. andresii* copulating with female *A. tristis*. The rare occurrence of male *A. tristis* copulating with female *A. andresii* may reflect the more severe consequences of this pairing reported here. Previous theoretical and empirical work show that asymmetry is common in reproductive interference (Fujimoto et al. 1996; Takafuji et al. 1997; Wirtz 1999; Deering and Scriber 2002; Marshall et al. 2006; Hochkirch et al. 2007; Liu et al. 2007) and should eventually lead to the exclusion of the competitively inferior species (Hochkirch et al. 2007; Kyogoku and Wheatcroft 2020). It is possible that *A. tristis* will eventually eliminate *A. andresii* from gardens and fields where the two species are found in sympatry. However, microhabitat partitioning may ultimately allow both species to co-exist (Gröning and Hochkirch 2008; Kyogoku and Wheatcroft 2020). Future studies should track the long-term dynamics of these two species in the wild to determine the effects of reproductive interference on community ecology and composition.

In summary, we emphasize the benefit of assessing reproductive interference using experiments that not only vary heterospecific encounter rates, but that also quantify lifetime measures of fitness. Most empirical studies test for interference by comparing the survival and reproductive performance of individuals that have constant access to either conspecifics, heterospecifics, or a combination of the two throughout a relatively short period of time (Gröning and Hochkirch 2008). We took a complementary approach and varied the contact between conspecific and heterospecific mates over an individual's entire reproductive lifetime. This approach allowed us to not only identify scenarios that might mitigate the negative effects of reproductive interference, but also reveal how even temporary bouts of interference can have lasting fitness consequences. By experimentally demonstrating

how fragile and resilient reproductive interference can actually be, we reduce the likelihood of over- or underestimating its influence on species interactions (Gröning and Hochkirch 2008; Kyogoku and Nishida 2013; Kyogoku and Wheatcroft 2020). Our study adds to the growing body of literature highlighting the importance of reproductive interference and sheds light on why this seemingly paradoxical behavior continues to persist.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10682-021-10120-1>.

Acknowledgements We thank N. Lindo, V. Wu for help talking care of the squash bugs, and E. Edwards for help rearing plants. We also thank J. Hamel, J. de Roode, S. Mendiola, K. Stoy, and the rest of the GerDer group for discussion and other assistance. Funding for this work was supported by a National Institutes of Health K12 Grant (GM00680) as well as a USDA-NIFA Grant (2019-67013-29371) to NMG. SMV was also supported by Emory University, an Institutional Research and Academic Career Development Award (IRACDA), and the Fellowships in Research and Science Teaching (FIRST) postdoctoral program. The authors declare no conflicts of interest.

References

- Andrews RH, Petney TN, Bull CM (1982) Reproductive interference between three parapatric species of reptile tick. *Oecologia* 52:281–286
- Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. *J Stat Softw* 67(1):1–48. <https://doi.org/10.18637/jss.v067.i01>
- Beard RL (1940) The biology of *Anasa tristis* DeGeer with particular reference to the tachinid parasite, *Trichopoda pennipes*. *Fabr Conn Agric Exp Stn Bull* 440:597–679
- Bonjour EL, Fargo WS, Al-Obaidi AA, Payton ME (1993) Host effects on reproduction and adult longevity of squash bugs (Heteroptera: Coreidae). *Environ Entomol* 22:1344–1348
- Burdfield-Steel ER, Auty S, Shuker DM (2015) Do the benefits of polyandry scale with outbreeding? *Behav Ecol* 26:1423–1431
- Burdfield-Steel ER, Shuker DM (2011) Reproductive interference. *Curr Biol* 21:R450–R451
- Clemente SH, Santos I, Ponce R, Rodrigues LR, Varela SAM, Magalhães S (2018) Despite reproductive interference, the net outcome of reproductive interactions among spider mite species is not necessarily costly. *Behav Ecol* 29:321–327
- Dame EA, Petren K (2006) Behavioural mechanisms of invasion and displacement in Pacific island geckos (*Hemidactylus*). *Anim Behav* 71:1165–1173
- Deering MD, Scriber JM (2002) Field bioassays show heterospecific mating preference asymmetry between hybridizing North American *Papilio* butterfly species (Lepidoptera: Papilionidae). *J Ethol* 20:25–33
- Drury JP, Anderson CN, Cabezas Castillo MB, Fisher J, McEachin S, Grether GF (2019) A general explanation for the persistence of reproductive isolation. *Am Nat*. <https://doi.org/10.1086/704102>
- Fujimoto H, Hiramatsu T, Takafuji A (1996) Reproductive interference between *Panonychus mori* and *P. citri* (Acari: Tetranychidae) in peach orchards. *Appl Entomol Zool* 31:59–65
- Gomez-Llano MA, Bensch HM, Svensson EI (2018) Sexual conflict and ecology: species composition and male density interact to reduce male mating harassment and increase female survival. *Evolution* 72:906–915
- Grether GF, Drury JP, Okamoto KW, McEachin S, Anderson CN (2020) Predicting evolutionary responses to interspecific interference in the wild. *Ecol Lett* 23:221–230
- Gröning J, Hochkirch A (2008) Reproductive interference between animal species. *Quart Rev Biol* 83:257–282
- Hamel JA, Eskeland EE, Lehmann TK, Stover PL (2018) Reproductive costs for hybridizing female *Anasa tristis* (Hemiptera: Coreidae), but no evidence of selection against interspecific mating. *J Insect Sci* 18:1–7
- Hamel JA, Nease SA, Miller CW (2015) Male mate choice and female receptivity lead to reproductive interference. *Behav Ecol Sociobiol* 69:951–956
- Hettley A, Pearman PB (2003) Social environment and reproductive interference affect reproductive success in the frog *Rana latastei*. *Behav Ecol* 14:294–300

- Hochkirch A, Gröning J, Bücker A (2007) Sympatry with the devil: reproductive interference could hamper species coexistence. *J Anim Ecol* 76:633–642
- Howard DJ, Gregory PG, Chu J, Cain ML (1998) Conspecific sperm precedence is an effective barrier to hybridization between closely related species. *Evolution* 52:511–516
- Jones T (1916) Notes on *Anasa andresii* Guer. *J Econ Entomol* 9:431–434
- Kishi S, Nishida T, Tsubaki Y (2009) Reproductive interference determines persistence and exclusion in species interactions. *J Anim Ecol* 78:1043–1049
- Kokko H, Klug H, Jennions MD (2014) Mating systems. In: Shuker DM, Simmons LW (eds) *The evolution of insect mating systems*. Oxford University Press, Oxford, UK, pp 42–58
- Konuma J, Chiba S (2007) Ecological character displacement caused by reproductive interference. *J Theory Biol* 247:354–364
- Kuno E (1992) Competitive exclusion through reproductive interference. *Res Popul Ecol* 34:275–284
- Kuznetsova A, Brockhoff PB, Haubo R, Christensen B (2016) lmerTest Package: tests in linear mixed effects models. R package version 2.0–30. <https://CRAN.Rproject.org/package=lmerTest>
- Kyogoku D, Nishida T (2013) The mechanism of the fecundity reduction in *Callosobruchus maculatus* caused by *Callosobruchus chinensis* males. *Popul Ecol* 55:87–93
- Kyogoku D, Wheatcroft D (2020) Heterospecific mating interactions as an interface between ecology and evolution. *J Evol Biol* 33:1330–1344
- Lipshutz SE (2018) Interspecific competition, hybridization, and reproductive isolation in secondary contact: missing perspectives on males and females. *Curr Zool* 64:75–88
- Liu S, De Barro PJ, Xu J, Luan J, Zang L, Ruan Y, Wan F (2007) Asymmetric mating interactions drive widespread invasion and displacement in a whitefly. *Science* 318:1769–1772
- Lorch PD, Servedio MR (2007) The evolution of conspecific gamete precedence and its effect on reinforcement. *J Evol Biol* 20:937–949
- Manzano-Winkler B, Hish AJ, Aarons EK, Noor MAF (2017) Reproductive interference by male *Drosophila subobscura* on female *D. persimilis*: a laboratory experiment. *Ecol Evol* 7:2268–2272
- Marshall VT, Schwartz JJ, Gerhardt HC (2006) Effects of heterospecific call overlap on the phonotactic behaviour of grey treefrogs. *Anim Behav* 72:449–459
- McLain DK, Shure DJ (1987) Pseudocompetition: interspecific displacement of insect species through misdirected courtship. *Oikos* 49:291–296
- Moore P (2014) Reproductive physiology and behavior. In: Shuker DM, Simmons LW (eds) *The evolution of insect mating systems*. Oxford University Press, Oxford, UK, pp 78–91
- Nasci RS, Hare SG, Willis FS (1989) Interspecific mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and laboratory. *J Am Mosq Control Assoc* 5:416–421
- Nechols JR (1987) Voltinism, seasonal reproduction, and diapause in the squash bug (Heteroptera: Coreidae). *Kans Environ Entomol* 16:269–273
- Noriyuki S, Osawa N (2016) Reproductive interference and niche partitioning in Aphidophagous insects. *Psyche* 2016:4751280
- Price CS (1997) Conspecific sperm precedence in *Drosophila*. *Nature* 388:663–666
- R Core Team (2016) R: a language environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. <https://www.R-project.org/>
- Reinhardt K (2006) Sperm numbers vary between inter- and intra-population matings of the grasshopper *Chorthippus parallelus*. *Biol Lett* 2:239–241
- RStudio: Integrated Development for R (2015) RStudio, Inc, Boston, MA. <http://www.rstudio.com>
- Schraft HA (2017) Mobility cost of copulation is independent of female:male size ratio in *Anasa tristis*. *Southwest Nat* 62:162–165
- Shuker DM, Burdfield-Steel ER (2017) Reproductive interference in insects. *Ecol Entomol* 42:65–75
- Simmons LW (2014) Sperm competition. In: Shuker DM, Simmons LW (eds) *The evolution of insect mating systems*. Oxford University Press, Oxford, UK, pp 181–203
- Svensson EI, Eroukhmanoff F, Karlsson K, Runemark A, Brodin A (2010) A role for learning in population divergence of mate preferences. *Evolution* 64:3101–3113
- Takafuji A, Kuno E, Fujimoto H (1997) Reproductive interference and its consequences for the competitive interactions between two closely related *Panonychus* spider mites. *Exp Appl Acarol* 21:379–391
- Therneau TM, Grambsch PM (2000) *Modeling survival data: extending the Cox model*. Springer, New York, New York, USA
- Thum RA (2007) Reproductive interference, priority effects and the maintenance of parapatry in *Skistodiaptomus* copepods. *Oikos* 116:759–768
- Tyler F, Harrison XA, Bretman A, Veen T, Rodríguez-Muñoz R, Tregenza T (2013) Multiple post-mating barriers to hybridization in field crickets. *Mol Ecol* 22:1640–1649

- Valero A, Garcia CM, Magurran AE (2008) Heterospecific harassment of native endangered fishes by invasive guppies in Mexico. *Biol Lett* 4:149–152
- Wade MJ, Patterson H, Chang NW, Johnson NA (1994) Postcopulatory, prezygotic isolation in flour beetles. *Heredity* 72:163–167
- Wirtz P (1999) Mother species-father species: unidirectional hybridization in animals with female choice. *Anim Behav* 58:1–12
- Yeates SE, Diamond SE, Einum S, Emerson BC, Holt WV, Gage MJ (2013) Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. *Evolution* 67:3523–3536

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.