EXPERIMENTAL INFECTION WITH A NATURALLY OCCURRING PROTOZOAN PARASITE REDUCES MONARCH BUTTERFLY (*DANAUS PLEXIPPUS*) MATING SUCCESS

Tolulope S. Babalola, **Jacobus C. de Roode, and Scott M. Villa**

Department of Biology, O. Wayne Rollins Research Center, Emory University, Atlanta, Georgia 30322.

Correspondence should be sent to Scott M. Villa at: *scott.villa@gmail.com*

ABSTRACT

Parasitic infection is known to drive sexual selection in persuasive mating systems, where parasites influence the secondary sexual characteristics that underlie mate choice. However, comparatively little is known about their effects on animals that use coercive mating behavior. We use a tractable system consisting of monarch butterflies and their naturally occurring parasite *Ophryocystis elektroscirrha* to test how parasites influence host mating dynamics when males force females to copulate. Monarchs were placed in mating cages where all, half, or no individuals were experimentally infected with *O. elektroscirrha.* We found that parasites reduce a male's mating success such that infected males were not only less likely to copulate but obtained fewer lifetime copulations as well. This reduction in mating success was due primarily to the fact that infected males attempt to mate significantly less than uninfected males. However, we found that *O. elektroscirrha* did not influence male mate choice. Males chose to mate with both infected and uninfected females at similar rates, regardless of their infection status.

Overall, our data highlight how mating dynamics in coercive systems are particularly vulnerable to parasites.

KEY WORDS

Sexual selection, Mate choice, Coercive behavior, *Ophryocystis elektroscirrha*

Parasites can be important drivers of sexual selection and mate choice within species (Hamilton and Zuk, 1982; Read, 1988; Møller, 1990). Most studies on parasite-mediated sexual selection have focused on persuasive mating systems, where parasites influence the secondary sexual characteristics that underlie mate choice (Arnold and Duvall, 1994; Andersson and Simmons, 2006). These traits, which typically evolve in males, are thought to be honest signals of fitness where their expression indicates a degree of parasite resistance and/or current levels of infection (Hamilton and Zuk, 1982). For example, Stephenson et al. (2020) found that male guppies (*Poecilia reticulata*) with more symmetrical and larger areas of ornamental coloration are more resistant to parasite infection and are consequently preferred by females when accepting mates. In addition to their influence on morphological traits, parasites can also influence sexual behavior. Macedo et al. (2012) found that parasitized male blue-black grassquits (*Volatinia jacarina*) displayed to females less than unparasitized males. As a result, females preferentially chose to mate with healthy males that displayed more. By relying on secondary sexual characteristics to choose mates, females can ensure that the males they produce offspring with are either parasite-free or able to resist and/or tolerate parasites (Read, 1988; Beltran-Bech and Richard, 2014).

While a majority of parasite-mediated sexual selection has focused on female choice, in some systems males bypass female preferences and have instead evolved coercive mating tactics. In these scenarios, males dictate sexual encounters by physically forcing or harassing females into mating (Kokko, 2005; Andersson and Simmons, 2006). Forced copulation has evolved in a variety of animals, including insects (Arnqvist and Rowe, 1995), reptiles (Shine et al., 2004), and fish (Plath et al., 2007). Sexual selection in coercive systems is driven primarily by a combination of male-male competition and male choice (Goater et al., 1993; Able, 1996; Bisazza et al., 2000; Kokko, 2005; Hoysak and Godin, 2007). Parasites can mediate forced mating dynamics by directly or indirectly (i.e., through male-male competition) reducing a male's ability to subdue females. Moreover, parasites may also influence mating dynamics by influencing a female's ability to resist males. For example, Deaton (2009) reported that infected female western mosquitofish (*Gambusia affinis*) resisted coercive males less often than uninfected females did. However, despite evidence that coercive systems may be especially vulnerable to parasitic influence, relatively little is known about how parasites affect forced copulatory dynamics.

Monarch butterflies (*Danaus plexippus*) provide a tractable system for understanding how parasites mediate sexual selection in coercive mating systems. Unlike most Lepidoptera, male monarchs forego the chemical or visual courtship that is typical of butterflies and moths. Instead, many studies have found that males either pounce on perched females or grab them midflight to take them to the ground and force them into copulation (Leong, 1995; Falco 1998; Solensky 2004; Solensky and Oberhauser, 2004).

Since there seems to be no evidence of pre- or postcopulatory female choice (Hill et al., 1976; Solensky, 2004; Mongue et al., 2015), sexual selection in monarchs is likely driven by intense male-male competition and some degree of male choice. Males presumably exercise choice by selecting which females to pursue. Females, in turn, counter this choice with varying degrees of resistance. The resulting struggle can vary wildly in duration and intensity and may result in injuries to both males and females (Brower et al., 2007). This intense physicality presumably favors strong, healthy males that have the energy and stamina to subdue resisting females. Indeed, the frequency of mating success between individual male monarchs is highly variable, and previous studies found that only 20–40% of attempts end in copulation (Frey, 1997; Solensky and Oberhauser, 2004). Thus, parasites may be especially influential on sexual selection in monarchs by determining a male's ability to compete for females and obtain copulations.

Monarchs are commonly infected with the virulent protozoan parasite *Ophryocystis elektroscirrha*. Transmission is most often vertical (McLaughlin and Myers, 1970), where spores attached to the surface of the female's abdomen fall on eggs and/or milkweed surfaces during oviposition. Spores can also be indirectly transmitted paternally when infected males transfer spores to females during copulation or extended bouts of contact (Altizer et al., 2004). Upon hatching, caterpillars ingest the spores by feeding on infected egg casings or milkweed leaves. Once ingested, the spores become active and penetrate the intestinal wall, enter the hypoderm, and reproduce asexually throughout larval development. *Ophryocystis elektroscirrha* then sexually reproduces in the pupal stage and forms new, dormant spores that lace the abdomens of newly eclosed

adult butterflies (Leong et al., 1995; Altizer and Oberhauser, 1999). Previous studies found that *O. elektroscirrha* infections have severe negative effects on the body size, lifespan, fecundity, and flight ability of adult monarchs (de Roode et al., 2008). *Ophryocystis elektroscirrha* also appears to reduce monarch mating success. Altizer and Oberhauser (1999) report that *O. elektroscirrha* infections reduced the number of times males, but not females, mated. de Roode et al. (2008) reported that higher parasite loads reduced female mating success, in part because *O. elektroscirrha* reduces lifespan. However, while *O. elektroscirrha* appears to reduce mating success, it remains largely unclear if this effect is due simply to monarchs having reduced lifespans (and therefore fewer mating opportunities) or because *O. elektroscirrha* influences sexual selection and mate choice within this system.

Here we conduct a series of mate choice trials to assess the effects of *O. elektroscirrha* on monarch mating behavior. Specifically, we manipulate the number of infected and uninfected monarchs in cages to decouple the effects of *O. elektroscirrha* on male-male competition, male mate choice, and female acceptance. This study highlights how parasites may drive sexual selection and mating dynamics in a coercive mating system.

MATERIALS AND METHODS

Monarchs

All monarchs used in this study were descendants of wild-caught, eastern North American migratory monarchs from St. Marks, Florida. Five unique pairs (1 m, 1 f) of unrelated monarchs were mated in July of 2021 to create 5 distinct lineages. Once a

female mated, she was individually placed in a 38 cm (diameter) x 60 cm (height) mesh cage (Carolina Biological Supply Company, Burlington, North Carolina) containing a single potted *Asclepias incarnata* (swamp milkweed) to lay eggs. Females were provided 10% honey solution ad libitum and laid eggs for up to 3 days. Once eggs hatched, the first instar larvae were allowed to feed on the oviposition plant. Upon development to the second instar, a total of 200 larvae (40 from each lineage) were collected for experimental use.

Experimental inoculations

Second instar larvae from each lineage were randomly split into 2 groups: infected and uninfected. Larvae in the infected group were experimentally inoculated with spores from a single parasite clone following methods described in de Roode et al. (2008). Specifically, each of these larvae was individually placed in a 100-mm plastic Petri dish and fed a 0.5 cm^2 leaf disk of *A. incarnata* manually laced with 10–20 parasite spores. Larvae in the uninfected group were fed leaf disks that did not contain parasite spores. After the disks were consumed, caterpillars were individually placed on a new potted *A. incarnata* plant that was surrounded by a clear plastic tube (13 cm diameter x 57 cm height) with a netted covering to mature. All larvae were reared in a greenhouse under summer light and temperature conditions (range: 23.5–39.6 C).

Upon pupation, each chrysalis was monitored for 2–3 days before adult eclosion for visual signs of parasite infection (de Roode et al., 2008). All pupae were given a parasite score ranging from 0 to 5, where zero indicates no sign of spore development and 5 indicates severe spore development throughout the monarch's body. All scores of zero

were considered “uninfected” and scores greater than one were considered “infected.” Following eclosion from pupae, the size of each adult was obtained from forewing lengths and each monarch was assigned a unique ID number that indicated its sex, lineage, and infection status. Adults were then individually placed in glassine envelopes for up to 10 days in an incubator set to 14 C to slow metabolism and reduce stress. Once all pupae eclosed, monarchs were placed in mating trials.

Mating trials

A series of mating trials were conducted in July of 2021 designed to test the influence of parasitism on monarch mating performance. All mating trials consisted of 4 monarchs (2 m, 2 f) placed in a 30 cm (diameter) x 30 cm (height) cylindrical mesh popup insect cage. All cages were kept in walk-in environmental chambers (Environmental Specialties, Inc., Raleigh, North Carolina) set to a 14:10 hr light/dark cycle at 26 C and 50% relative humidity.

Before the start of the experiment, the effects of size and genetic background in each cage were controlled for by making sure that within each sex, the 2 monarchs were of the same size and lineage. Importantly, potential effects of inbreeding on mate choice were eliminated by making sure that males and females in each cage were from different lineages. Additionally, the 4 monarchs within each cage were given a unique dot on the dorsal and ventral side of either their right or left hindwing using a non–toxic black permanent marker. These markings provided a minimally invasive way to distinguish individuals within cages. Care was taken so that different hindwings were marked within sexes (i.e., if 1 male had a dot on the right hindwing, the other male was given a dot on

154 the left hindwing). Wing marking was randomized for infected and uninfected
155 individuals.
156 Mating trials were of 3 types: all-uninfected, mixed infection, and all-infected
157 (Fig. 1A–C). In the all-uninfected trials, both males and both females were parasite free.
158 Hence, all the mating activity within these trials was between uninfected males and
159 uninfected females (uM/uF). In mixed infection trials, 1 male and 1 female were infected,
160 and the other male and female were uninfected. Thus, in these trials, both infected and
161 uninfected males could choose to mate with either infected or uninfected females,
162 creating four possible mating combinations (uM/uF, uM/iF, iM/uF, iM/iF). In the all-
163 infected trials, all 4 monarchs were parasitized. Mating within these trials could only
164 involve infected males copulating with infected females (iM/iF).
165 Mating trials lasted approximately 5 days, during which monarchs were provided
166 10% honey water ad libitum for food. All cages were spot-checked for mating once every
167 evening. Butterflies were allowed to mate as many times as they could during the 5-day
168 experiment. Additionally, 2 all-infected, 6 mixed infection, and 2 all-uninfected cages
169 were filmed continuously for the entire experiment using high–definition Night Owl®
170 AHD10-841-B cameras (Night Owl Security Products, Naples, Florida). Cameras were
171 equipped with infrared bulbs to film in complete darkness. All cameras were hung
172 approximately 30 cm above a cage and provided a clear recording 24 hr per day. These
173 filmed cages allowed us to quantify finer-scale mating behavior beyond the evening spot-
174 checks, which quantified the individuals that were in copula each day.

Commented [A1]: When figure is located inside parentheses, then it should be abbreviated. When it isn't then it should be spelled out.

If a monarch died during the experiment, the dead individual, as well as their same-sex counterpart, were removed and replaced with individuals of the same sex and infection status. For example, if the infected male died in a mixed infection cage, the living, uninfected male in that cage was also removed and replaced. This ensured that both males had equal exposure to the females and that mating performance was not based on the time a male spent in the trial.

Quantification of mating behavior

Monarch mating behavior was broken down into 2 stages: the attempt stage and the copulatory stage. The attempt stage is defined as the precopulatory coercive behavior between males and females (Solensky, 2004). Attempts begin when males pounce on females to physically coerce them into mating. Pouncing is easily distinguished from inadvertent contact as the monarchs fly around the cage. Successful attempts end when the pair achieves copulation. An attempt is unsuccessful when the male either gives up or the female escapes the male's grasp. The attempt stage could only be quantified in the subset of cages that were filmed. Observers watched video recordings and scored which 2 butterflies were involved in each attempt as well as the total number, success rate (number of attempts that end in copulation out of total attempts tried), and the length of all attempts that occurred in each cage. Mating attempts were recorded up to day 5 after monarchs were placed into cages.

Multiple performance measures were quantified during the copulatory stage. Copulation begins as soon as the male latches onto the distal tip of the female's abdomen with his genitalic claspers (Solensky, 2004; Brower et al., 2007). Immediately following

attachment, the pair positions themselves into a stereotypical lepidopteran mating posture where males and females face away from each other while the tips of their abdomens remain joined (Cannon, 2020). Copulations end as soon as the pair separates. Copulations were assessed using both spot-checking and video recordings. Specifically, each cage was inspected once each evening between 19:00–20:00 hr to record which butterflies successfully mated. Monarchs only mate once per day, with peak mating activity starting around 16:00 and ending around 19:00 hr (Oberhauser, 1988). If pairs successfully mate, they will be in copula by approximately 19:00 hr and no additional mating activity happens at night. Pairs that are in copula after 19:00 hr will mate through the evening and typically break up between 02:00–06:00 hr the following morning (Svärd and Wiklund, 1988). Thus, 1 evening check right before the lights turn off (20:00 hr) is sufficient to quantify all mating events in the experiment. Additionally, in the cages that were filmed, observers could watch video recordings to quantify the length of all copulations. Since mating typically lasts into the next morning, copulations were recorded up to day 6 after monarchs were placed into cages.

Statistical analysis

Analyses focused on male copulation performance. These data come from mating observations from all cages in the experiment. Specifically, the factors that influenced a male's probability of mating were tested. Male reproductive status was designated as "mated" if they were observed in copula at least once, and "unmated" if there were never observed in copula. A generalized linear mixed-effects model (GLMM) with binomial distribution and logit link function was used to model male reproductive status as a

function of male infection type (uninfected vs. infected) while including trial number as a random effect (to account for multiple males per cage). Male mate preference was tested to observe if males chose to mate with uninfected females first. Male preference was tested by restricting the analysis to the first mating observed for each male in the mixed infection cages (i.e., the trials where males had a "choice" between infected and uninfected females). In this analysis, the proportion of uninfected and infected females involved in the first copulations of both types of males was tested against a random 50-50 mate preference using a Chi-squared test with $\alpha = 0.05$.

Factors that influenced total matings per male over 5 days were also investigated. The copulation totals for each male were determined by daily spot-checks. Some males never mated during the experiment, and these zero totals were included in the analyses. A GLMM with Poisson distribution was used to model total copulations per male as a function of infection status (uninfected vs. infected) while including trial number as a random effect (to account for multiple males in each cage). A Poisson GLMM with the same fixed and random effect structure was used to model total copulations per male as a function of cage type (i.e., all-uninfected vs. mixed infection vs. all-infected). Pairwise post hoc comparisons among treatments were performed using Tukey's honestly significant difference tests (HSD). Female infection status was tested to determine if it influenced copulation success for both uninfected and infected males. This analysis was restricted to cages where males could mate with both female infection types (i.e., only mixed infection trials). A Poisson GLMM was used to model copulations achieved as a function of female infection type (infected vs. uninfected), male infection type (infected

vs. uninfected), and their interaction. Male ID and trial number were included as random effects.

Additional models were run focusing on mating attempt performance. Mating attempt data come from the subset of trials that were filmed continuously. Factors that influenced the number of times males attempted to mate throughout the experiment were investigated. Analyses of attempt totals used the same 3 models as those to investigate copulation totals described above (i.e., Poisson GLMMs). Some males never attempted to mate, and these zero totals were included in these analyses.

Factors that influenced the likelihood that a given attempt ended in copulation were analyzed. These attempt success rates were determined from the subset of cages that were filmed. The attempt success rate is a 2-column variable that column binds (using the command 'cbind') successful attempts and unsuccessful attempts by each male. Two binomial GLMMs were used to test how success rates are a function of male infection type and cage type. In both models, the trial number was again included as a random effect. Additionally, female infection status was tested to determine if it influenced attempt success rates for both uninfected and infected males. This analysis was restricted to cages where males could attempt copulation with both female infection types (i.e., only mixed infection trials). Specifically, success rates achieved as a function of female infection type (infected vs. uninfected), male infection type (infected vs. uninfected), and their interaction were modeled. Male ID and trial number were included as random effects.

Two more series of models testing the factors that influence how long both attempts and copulations lasted were included. This required quantifying the stop and start times for each of these behaviors, which was done in the subset of cages that were filmed. Both aspects of mating performance were modeled in the same way as the number of attempts described above but had male ID nested within-trial number as random effects to account for the repeated measures of each male throughout the experiment. Before analysis, attempt times and copulation times were log-transformed to achieve normality. Linear mixed-effects models (LMMs) were used instead of GLMMs to analyze attempt and copulation time data.

All LMMs and GLMMs were conducted in R v3.3.3 (R Core Team, 2016) with the 'lme4' package v.1.1e12 (Bates et al., 2014). The intercept for all models was set to the performance of uninfected males. The distribution that best fit the data for each model described above was determined using the 'fitdisplus' package v.1.1e12 (Delignette-Muller and Dutang, 2015).

RESULTS

Experimental inoculations

Rearing and inoculation of monarchs were both successful. In the control group, 85% (85/100) of caterpillars fed leaf disks without parasites developed into pupae. Of these, 0% (0/85) showed signs of parasite infection. All but two of these pupae eclosed into healthy adult monarchs, leaving a total of 83 (36 m, 47 f) uninfected monarchs to use for mating trials. In the inoculated group, 88% (88/100) of caterpillars fed leaf disks containing parasite spores developed into pupae. Of these, 93% (82/88) developed a

parasite infection with a mean (± se) parasite score of 3.40 ± 0.07 out of 5. However, 19% (16/83) of infected pupae eclosed with wing deformities, leaving a total of 66 (35 m, 31 f) infected monarchs to use for mating trials.

Mating trials

After controlling for size, genetic background, and inbreeding, there were 96 usable monarchs (48 m, 48 f) to create 24 mating trials. Each trial consisted of 2 males and 2 females and included 6 all-uninfected trials, 12 mixed infection trials, and 6 all-infected trials (Fig. 1A–C). Of these, 10 trials (2 all-uninfected, 6 mixed infection, and 2 all-infected) were filmed continuously for the 5-day experiment.

Parasitism significantly reduced survival of infected monarchs compared to uninfected monarchs (Likelihood ratio test; n = 96, df = 1, $\chi^2 = 16.68$, $P < 0.0001$). Throughout the experiment, 23% (11/48) of the infected monarchs died and needed to be replaced (7 m, 4 f). In contrast, 0% (0/48) of the uninfected monarchs died during the experiment.

In addition to survival, parasite infection also influenced male mating behavior. Infected males were significantly less likely to achieve copulation than uninfected males (Fig. 1D; Table I). This analysis came from tracking copulations for all 48 males (2 per cage) across the experiment. Of the 24 uninfected males, 17 mated at least once during the experiment. In contrast, only 5 of the 24 infected males were able to achieve copulation at least once over 5 days. Interestingly, when given a choice, uninfected males tended to mate with uninfected females first and the infected males that achieved copulation tended to do so with infected females first (Fig. 1D; middle 2 mosaic plots).

However, neither of these tendencies significantly deviated from random mate choice (Likelihood ratio test; $P > 0.05$ for both uninfected and infected males).

Parasite infection also influenced the total number of copulations males achieved throughout the experiment. For each of the 48 males, the total number of times they copulated throughout the experiment was determined. Copulation totals ranged from 0 to 4 and infected individuals were observed in copula significantly less than uninfected males (Fig. 1E; Table IIa). Across the 24 trials, uninfected males mated an average (± se) of 1.67 ± 0.31 times while infected males mated 0.25 ± 0.11 times. This relationship was also consistent when comparing among cage types. There was significantly more copulation in all-uninfected trials than in the all-infected trials (Table IIb). Males in the all-uninfected cages mated 1.41 ± 0.34 times while those in the all–infected cages mated 0.25 ± 0.18 times. Those in the mixed–infection trials fell in between these 2 cage types, mating an average of 1.08 ± 0.32 times over 5 days. When the analysis was restricted to the 12 mixed infection trials where uninfected and infected males were in direct competition with each other, uninfected males again significantly outperformed infected males (Fig. 1E; Table IIc). Specifically, uninfected males mated 1.92 ± 0.53 times while infected males mated 0.25 ± 0.13 times. However, neither type of male showed a copulation bias toward uninfected or infected females (Fig. 1E; Table IIc).

To understand why infected males achieved fewer copulations, mating attempt behavior in the subset of 10 cages that were filmed was analyzed. For each of the 20 males filmed, the total number of times they attempted to mate throughout the experiment was determined. Mating attempts ranged from 0 to 23 and infected individuals attempted

to mate significantly less than uninfected males (Fig. 2A; Table IIIa). Across the trials that were filmed, uninfected males tried to mate an average of 11.60 ± 2.40 times while infected males tried to mate 5.00 ± 1.53 times. This relationship was also consistent when comparing among cage types. There were significantly more mating attempts in all-uninfected trials than in the all-infected trials (Table IIIb). Males in the all-uninfected cages attempted 13.50 ± 3.43 times while those in the all-infected cages attempted only 1.50 ± 0.65 times. Those in the mixed infection trials fell in between these two cage types, attempting to mate an average of 8.83 ± 1.96 times. When the analysis was restricted to cases when uninfected and infected males were in direct competition with each other (i.e., mixed infection cages), uninfected males attempted to mate at similar rates as infected males (Fig. 2A; Table IIIc). Specifically, within the mixed infection trials that were filmed, uninfected males attempted 10.33 ± 3.44 times while infected males attempted 7.33 ± 2.04 times. Neither type of male showed an attempt bias toward uninfected or infected females (Fig. 2A; Table IIIc).

Success rates of these mating attempts were compared; this analysis involved the 10 filmed trials. Two of the 20 males that were filmed never attempted to mate. Thus, we quantified attempt rates among 18 males. We found that parasite infection did not affect the probability that a given mating attempt ended in copulation. Attempts from infected individuals were just as likely to succeed as those from uninfected males (Fig. 2B; Table IVa). Similarly, there was no significant difference in attempt success rate among the 3 cage types (Table IVb). When the analyses were restricted to the filmed mixed infection

trials, attempt success rates were not influenced by male infection status, female infection status, or their interaction (Table IVc).

Finally, the films were used to assess how long attempts and copulations lasted. Again, this analysis included the 18 of the 20 males in filmed cages that tried to mate. The lengths of 166 attempts from these males throughout the experiment were quantified. None of the factors tested influenced how long attempts lasted (Fig. 3A; Table V). Lengths of copulations were also quantified. Only 13 of the 18 males that attempted to mate successfully achieved copulation. Lengths of 28 copulations from these males were quantified. None of the factors tested influenced how long males stayed in copula (Fig. 3B; Table VI).

DISCUSSION

Our study demonstrates the direct, immediate effects parasites can have on mating dynamics within coercive mating systems. As expected, we found that male monarchs experimentally infected with *O. elektroscirrha* suffered a reduction in mating performance. The effect of *O. elektroscirrha* was most pronounced on male copulation success. Among uninfected males, 70% successfully copulated during the experiment. In contrast, only 20% of infected males were ever observed in copula (Fig. 1D). We also found a similar disparity in lifetime copulations where uninfected males copulated significantly more often than infected males (Fig. 1E). These results are especially telling given that monarchs in our study were confined to cages and expended much less energy tracking down females than they would in the wild. The consequences of O. elektroscirrha are likely even more exaggerated in natural populations, where males need

371 to patrol tree canopies and find females to pounce on or grab during flight (Leong, 1995;
372 Falco, 1998; Solensky, 2004; Solensky and Oberhauser, 2004). Moreover, the reduction
373 in copulation success among infected males was apparent in both the presence and
374 absence of uninfected males (Fig. 1E). Thus, *O. elektroscirrha* does not just simply
375 reduce a male's ability to compete with healthy males, but likely has inherent negative
376 effects on male behavior as well.
377 Importantly, even though our monarchs were confined to small cages, we
378 observed similar mating dynamics described from both wild and captive populations (Hill
379 et al., 1976; Pliske, 1975; Frey et al., 1998; Frey, 1999; Solensky, 2004; Solensky and
380 Oberhauser, 2004; Brower et al., 2007). Previous studies examining monarchs in
381 overwintering populations suggest that matings initiated with aerial captures are quite
382 infrequent. Instead, males in these populations are often observed initiating mating
383 attempts by pouncing on a stationary female (Leong, 1995; Falco, 1998; Solensky, 2004;
384 Solensky and Oberhauser, 2004). Conversely, males in summer breeding populations
385 typically initiate mating by grabbing females' out of the air and taking them to the
386 ground. While opportunities for aerial takedowns in our cages were extremely limited,
387 the summer breeding males used here could and did initiate attempts by pouncing on
388 females perching on the sides of the cages or feeding. Thus, it appears that summer
389 breeding males can shift approach tactics when needed. If males did engage in mating by
390 the pouncing method, they frequently took females to the ground. During the ground
391 "wrestling" phase, we observed females deploying the typical battery of resistance
392 behaviors reported from wild populations (Frey, 1999; Solensky, 2004; Brower et al.,

2007). Moreover, the coercive attempts across our experiment lasted an average of 2.52 min (n = 223). This attempt effort was nearly identical to the 2.20 min (n = 273) average attempt observed in wild populations (Solensky, 2004). Thus, the smaller confines of cages did not encourage males to be more persistent when trying to subdue females. The cage environment also did not put females at a disadvantage. Unlike females in wild populations, those in our experiment could not fly away once they were able to escape the male's attempt. However, this did not translate into unusually high mating success rates. In our experiment, only 17% of all attempts resulted in copulation. These success rates are very similar to those reported from wild populations (Van Hook, 1993; Frey, 1999; Oberhauser and Frey, 1999; Solensky, 2004; Solensky and Oberhauser, 2004). Taken together, the small cages used in our experiments did not appear to significantly influence overall monarch mating dynamics.

The reduction in copulation success by infected males appears to stem, in part, from decreases in mating effort. We found that infected males made significantly fewer mating attempts than uninfected males (Fig. 2A). These results make sense given the physical nature of monarch mating behavior. Males infected with *O. elektroscirrha* presumably have less energy to allocate to wrestling females into copulation. Indeed, previous studies have shown that parasitized monarchs have significantly lower flight endurance (Bradley and Altizer, 2005). Similar endurance-related pathology is likely influencing how many times monarchs choose to mate. However, this discrepancy was largely driven by differential mating efforts between males in the all-uninfected trials and those in the all-infected trials (Fig. 2A). When all 4 monarchs in a cage were infected, we

surprisingly observed very little activity in general, let alone mating attempts. Both males and females in these cages almost exclusively fed or stayed perched on the side of the cage. Curiously, the negative effects of *O. elektroscirrha* on mating attempts were not apparent in the mixed-infection cages (Fig. 2A). Both infected and uninfected males displayed a similar number of attempts when housed together. So why did infected males in the mixed infection cages try to mate while those in the all-infected cages forwent chances to mate? One possibility is that mating in monarchs is generally related to overall activity. If some individuals in the cage are agitated or flying around, this may induce mating behavior. Thus, the more active the population, the higher the likelihood of mating. Alternatively, it is possible that males directly adjust their mating effort relative to their immediate competition. Previous studies have shown that the quality of competitors can induce male sexual promiscuity and increase male-male competition. For example, male guppies (*Poecilia reticulata*) increase aggression and courtship behavior when surrounded by more mature males (Price and Rodd, 2006). In mixed infection cages, the curiously high attempt rates of infected males may simply be a response to counter the mating activity of the healthy male competitor. It would be interesting in future studies to swap out the uninfected male for an infected one midway through the experiment to test if male mating effort is relative to the effort of direct competitors.

Importantly, the analysis of copulations and mating attempts only included surviving monarchs. Survival is a critical component of fitness, as dead individuals cannot attempt to mate or achieve copulation. Indeed, one of the most direct ways parasites influence the mating dynamics of their host is to reduce survival and therefore

437 the probability of mating (de Roode et al., 2008). Many studies have shown the negative
438 effects of parasite infection on host fitness in monarchs, including shortened adult
439 lifespan (Altizer and Oberhauser, 1999, de Roode et al., 2008). We too found a
440 significant effect, with 25% of the infected starting population dying and needing to be
441 replaced before mating could take place. If we take survival into account, the effect of *O.*
442 *elektroscirrha* on mating behavior becomes more pronounced. When we include the
443 zeroes for copulation and attempt totals of the monarchs that died during the experiment,
444 we see that infected males in the mixed infection cages do, in fact, attempt to mate
445 significantly less than uninfected males (GLMM; $z = -2.30$, $P = 0.022$). Thus, when
446 survival and performance are considered together, it becomes clear that in both the
447 presence and absence of uninfected competitors, infected monarchs achieved
448 significantly fewer copulations due to reduced mating attempts. These data complement
449 previous studies of this system that have found significant negative effects of infection on
450 host fitness (Altizer and Oberhauser, 1999, de Roode et al., 2008, Bradley and Altizer,
451 2005).

452 We also show that *O. elektroscirrha* does not influence assortative mating in
453 monarchs. In general, males mated at similar rates to both infected and uninfected
454 females, regardless of their infection status. These data are consistent with previous
455 studies showing a lack of avoidance of infected individuals. For example, milkweed leaf
456 beetles (*Labidomera clivicolliss*) that are infected by a sexually transmitted mite show no
457 evidence of avoidance of infected mates, resulting in a high prevalence of the parasite
458 (Abbott and Dill, 2003). However, our results were particularly unexpected since

parasites that influence host endurance should be especially important in coercive mating systems. The negative effects of *O. elektroscirrha* on both male coercion and female rejection capability should have resulted in a variety of assortative mating scenarios. First, we would have expected that infected males should only be able to mate with infected females, who are not as capable of resisting copulations as healthy females. Second, we would have predicted that healthy males should preferentially mate with healthy females to reduce the probability of infection in the offspring. Alternatively, healthy males could force infected females to mate first given their reduced rejection ability. But we found no evidence for any of these assortative mating scenarios in our data. We also found no evidence that *O. elektroscirrha* influences behavior within a coercive bout. The infection status of males or females did not influence how long attempts lasted or how long pairs stayed *in copula*. Together, these data emphasize that: 1) monarchs cannot sense if a potential mate is infected, an ability possessed by a variety of insects (Wittman and Fedorka, 2015), and 2) when infected monarchs do muster the effort to coerce a female, they can be just as effective at obtaining copulations as healthy males.

Finally, and possibly most surprising, infected females were just as capable of rejecting male advances as healthy females. For example, only 21% of mating attempts by healthy males toward infected females ended in copulation. This rate is similar to those reported for mating success in general among wild populations (Solensky and Oberhauser, 2004). These data indicate monarchs may have more complex mating dynamics and that females may drive more selection in this coercive system than

previously realized. Future studies should investigate how the female ability to tolerate infection may mask the effects of *O. elektroscirrha* on mate choice in this system.

Overall, our study aimed to tease apart the complex interactions between hosts and parasites in coercive mating systems. We show that the negative effect of *O. elektroscirrha* on monarch mating success is driven, in part, by its influence on mating effort. Such parasites can be particularly influential on mating in systems that deploy coercive tactics, where physicality is the primary mode of mating success. These data align well with previous studies showing this parasite's effects on both survival and endurance-related pathology. Moreover, our data suggest that when *O. elektroscirrha* prevalence is especially high in both males and females, mating activity, in general, shuts down. These results have strong implications for non-migratory monarch populations, which do not benefit from the yearly culling of parasitized individuals (Altizer et al., 2000; Bartel et al., 2011; Freedman et al., 2020). Resident populations of monarchs can have 30–60% higher *O. elektroscirrha* prevalence than migratory populations (Satterfield et al., 2015; Majewska et al., 2019). This study highlights potential community-level influences of parasite prevalence on monarch mating dynamics and provides additional evidence for the threat that *O. elektroscirrha* may pose to the persistence of this iconic species.

ACKNOWLEDGMENTS

We thank Ella Zhao, Andrew Pahnke, Joselyne Chavez, and Erik Edwards for help growing plants and raising monarchs. SMV was supported by Emory University, an Institutional Research and Academic Career Development Award (IRACDA), and the

503 Fellowships in Research and Science Teaching (FIRST) postdoctoral program. This work
504 was supported by both a National Institutes of Health K12 grant (GM00680), National
505 Science Foundation grant (IOS–1922720) to JdR, and the Summer Undergraduate
506 Research Experience (SURE) program at Emory.

507 **LITERATURE CITED**

508 Abbot, P., and L. M. Dill. 2001. Sexually transmitted parasites and sexual selection in the
509 milkweed leaf beetle, *Labidomera clivicollis*. Oikos 92: 91–100.
510 Able, D. J. 1996. The contagion indicator hypothesis for parasite-mediated sexual
511 selection. Proceedings of the National Academy of Sciences of the United States of
512 America 93: 2229–2233.
513 Altizer, S. M., and K. S. Oberhauser. 1999. Effects of the protozoan parasite
514 *Ophryocystis elektroscirrha* on the fitness of monarch butterflies (*Danaus plexippus*).
515 Journal of Invertebrate Pathology 74: 76–88.
516 Altizer, S. M., K. S. Oberhauser, and L. P. Brower. 2000. Associations between host
517 migration and the prevalence of a protozoan parasite in natural populations of adult
518 monarch butterflies. Ecological Entomology 25: 125–139.
519 Altizer, S. M., K. S. Oberhauser, and K. A. Geurts. 2004. Transmission of the protozoan
520 parasite, *Ophryocystis elektroscirrha*, in monarch butterfly populations: Implications for
521 prevalence and population–level impacts. *In* The Monarch Butterfly: Biology and
522 Conservation, K. S. Oberhauser and M. J. Solensky (eds.). Cornell University Press,
523 Ithaca, New York, p. 203–218.

524 Andersson, M., and L.W. Simmons. 2006. Sexual selection and mate choice. Trends in
525 Ecology & Evolution 21: 296–302.
526 Arnqvist, G., and L. Rowe. 1995. Sexual conflict and arms races between the sexes: A
527 morphological adaptation for control of mating in a female insect. Proceedings of the
528 Royal Society of London. Series B: Biological Sciences 261: 123–127.
529 Arnold, S. J., and D. Duvall. 1994. Animal mating systems: A synthesis based on
530 selection theory. American Naturalist 143: 317–348.
531 Bartel, R. A., K. S. Oberhauser, J. C. de Roode, and S. M. Altizer. 2011. Monarch
532 butterfly migration and parasite transmission in eastern North America. Ecology 92: 342–
533 351.
534 Bates, D., and M. Maechler. 2010. Matrix: Sparse and dense matrix classes and methods.
535 R package version 0.999375-43. Available at: http://cran. r-project. org/package=Matrix.
536 Accessed 20 September 2021.
537 Beltran-Bech, S., and F.-J. Richard. 2014. Impact of infection on mate choice. Animal
538 Behaviour 90: 159–170.
539 Bisazza, A., S. Manfredi, and A. Pilastro. 2000. Sexual competition, coercive mating and
540 mate assessment in the one–sided livebearer, *Jenynsia multidentata*: Are they predictive
541 of sexual dimorphism? Ethology 106: 961–978.
542 Bradley, C. A., and S. Altizer. 2005. Parasites hinder monarch butterfly flight:
543 Implications for disease spread in migratory hosts. Ecology Letters 8: 290–300.
544 Brower, L. P., K. S. Oberhauser, M. Boppré, A. V. Brower, and R. Vane-Wright. 2007.
545 Monarch sex: Ancient rites, or recent wrongs. Antenna 31: 12–18.

546 Cannon, R. J. 2020. Courtship and Mating in Butterflies. CABI, Boston, Massachusetts,
547 375 p.
548 Deaton, R., 2009. Effects of a parasitic nematode on male mate choice in a livebearing
549 fish with a coercive mating system (western mosquitofish, *Gambusia affinis*).
550 Behavioural Processes 80: 1–6.
551 de Roode, J. C., A. J. Yates, and S. Altizer. 2008. Virulence-transmission trade-offs and
552 population divergence in virulence in a naturally occurring butterfly parasite. Proceedings
553 of the National Academy of Sciences of the United States of America 105: 7489–7494.
554 Delignette-Muller, M. L., and C. Dutang. 2015. fitdistrplus: An R package for fitting
555 distributions. Journal of Statistical Software 64: 1–34.
556 Falco, L. R. 1998. Variation in male courtship behaviors of the monarch butterfly
557 (*Danaus plexippus* L.) at central California overwintering sites. Master's thesis,
558 California Polytechnic State University, San Luis Obispo.
559 Freedman, M. G., H. Dingle, S. Y. Strauss, and S. R. Ramírez. 2020. Two centuries of
560 monarch butterfly collections reveal contrasting effects of range expansion and migration
561 loss on wing traits. Proceedings of the National Academy of Sciences of the United
562 States of America 117: 28887–28893.
563 Frey, D. 1999. Resistance to mating by female monarch butterflies. *In* 1997 North
564 American Conference on the Monarch Butterfly, Montreal, Canada. Commission for
565 Environmental Cooperation, 1999, p. 79–87.

566 Frey, D., K. L. H. Leong, E. Peffer, R. Smidt, and K. Oberhauser. 1998. Mating patterns
567 of overwintering monarch butterflies (*Danaus plexippus* L.) in California. Journal of the
568 Lepidopterists' Society 52: 84–97.
569 Goater, C. P., R. D. Semlitsch, and M. V. Bernasconi. 1993. Effects of body size and
570 parasite infection on the locomotory performance of juvenile toads, *Bufo bufo*. Oikos 66:
571 129–136.
572 Hamilton, W. D., and M. Zuk. 1982. Heritable true fitness and bright birds: A role for
573 parasites? Science 218: 384–387.
574 Hill Jr., H. F., A. M. Wenner, and P. H. Wells. 1976. Reproductive behavior in an
575 overwintering aggregation of monarch butterflies. American Midland Naturalist 95: 10–
576 19.
577 Hoysak, D. J., and J. G. J. Godin. 2007. Repeatability of male mate choice in the
578 mosquitofish, *Gambusia holbrooki*. Ethology 113: 1007–1018.
579 Kokko, H. 2005. Treat 'em mean, Keep 'em (sometimes) keen: Evolution of female
580 preferences for dominant and coercive males. Evolutionary Ecology 19: 123–135.
581 Leong, K. L. H. 1995. Initiation of mating activity at the tree canopy level among
582 overwintering monarch butterflies in California. Pan-Pacific Entomology 71: 66–68.
583 Macedo, R. H., L. Manica, and R. I. Dias. 2012. Conspicuous sexual signals in a socially
584 monogamous passerine: The case of Neotropical Blue-black Grassquits. Journal of
585 Ornithology 153: 15–22.
586 Majewska, A. A., D. A. Satterfield, R. B. Harrison, S. Altizer, and J. Hepinstall-
587 Cymerman. 2019. Urbanization predicts infection risk by a protozoan parasite in non-

588 migratory populations of monarch butterflies from the southern coastal U.S. and Hawaii.
589 Landscape Ecology 34: 649–661.
590 McLaughlin, R. E., and J. Myers. 1970. *Ophryocystis elektroscirrha* sp. n., a
591 neogregarine pathogen of the monarch butterfly *Danaus plexippus* (L.) and the Florida
592 queen butterfly *D. gilippus berenice* Cramer 1. Journal of Protozoology 17: 300–305.
593 Møller, A. P. 1990. Parasites and sexual selection: Current status of the Hamilton and
594 Zuk hypothesis. Journal of Evolutionary Biology 3: 319–328.
595 Mongue, A. J., M. Z. Ahmed, M. V. Tsai, and J. C. de Roode. 2015. Testing for cryptic
596 female choice in monarch butterflies. Behavioral Ecology 26: 386–395.
597 Oberhauser, K. S. 1988. Male monarch butterfly spermatophore mass and mating
598 strategies. Animal Behavior 36: 1384–1388.
599 Oberhauser, K., and D. Frey 1999. Coercive mating by overwintering male monarch
600 butterflies. *In* 1997 North American Conference on the Monarch Butterfly, Montreal,
601 Canada. Commission for Environmental Cooperation, 1999, p. 67–78.
602 Plath, M., A. M. Makowicz, I. Schlupp, and M. Tobler. 2007. Sexual harassment in live-
603 bearing fishes (Poeciliidae): Comparing courting and noncourting species. Behavioral
604 Ecology 18: 680–688.
605 Pliske, T. E. 1975. Courtship behavior of the monarch butterfly, *Danaus plexippus* L.
606 Annals of the Entomological Society of America 68: 143–151.
607 Price, A. C., and F. Helen Rodd. 2006. The effect of social environment on male-male
608 competition in guppies (*Poecilia reticulata*). Ethology 112: 22–32.

R Core Team. 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Read, A. F. 1988. Sexual selection and the role of parasites. Trends in Ecology & Evolution 3: 97–102.

Satterfield, D. A., J. C. Maerz, and S. Altizer. 2015. Loss of migratory behaviour increases infection risk for a butterfly host. Proceedings of the Royal Society B: Biological Sciences 282: 20141734. doi:10.1098/rspb.2014.1734.

Solensky, M. J. 2004. The effect of behavior and ecology on male mating success in overwintering monarch butterflies (*Danaus plexippus*). Journal of Insect Behavior 17: 723–743.

Solensky, M. J., and K. S. Oberhauser. 2004. Behavioral and genetic components of male mating success in monarchs. *In* The Monarch Butterfly: Biology and Conservation, Cornell University Press, Ithaca, New York, p. 61–68.

Shine, R., B. Phillips, T. Langkilde, D. I. Lutterschmidt, H. Waye, and R. T. Mason, 2004. Mechanisms and consequences of sexual conflict in garter snakes (*Thamnophis sirtalis*, Colubridae). Behavioral Ecology 15: 654–660.

Stephenson, J. F., M. Stevens, J. Troscianko, and J. Jokela. 2020. The size, symmetry, and color saturation of a male guppy's ornaments forecast his resistance to parasites. American Naturalist 196: 597–608.

Svärd, L., and C. Wiklund. 1988. Fecundity, egg weight and longevity in relation to multiple matings in females of the monarch butterfly. Behavioral Ecology and Sociobiology 23: 39–43.

631 Van Hook T. 1993. Non-random mating in monarch butterflies overwintering in Mexico.
632 *In* Biology and Conservation of the Monarch Butterfly. Natural History Museum of Los
633 Angeles County, Science Series 38, Los Angeles, California, p. 49–60.
634 Wittman, T., and K. M. Fedorka. 2015. Male mate choice for unparasitized females in
635 *Drosophila melanogaster*. Journal of Insect Behavior 28: 37–43.
636
637 **Figure 1.** Influence of parasite infection on monarch mating performance. Mating trials
638 (n = 24) consisted of 4 monarchs placed in 1 of 3 types of cages. (**A**) All-uninfected cages
639 (n = 6) contained 2 uninfected females (uF) and 2 uninfected males (uM). (**B**) Mixed
640 infection cages (n = 12) contained 1 uninfected female (uF), 1 infected female (iF), 1
641 uninfected male (uM), and 1 infected male (iM). (**C**) All-infected cages (n = 6) contained
642 2 infected females (iF) and 2 infected males (iM). (**D**) The proportion of males that
643 achieved at least 1 copulation and if so, the infection status of the female they mated with
644 first. (**E**) The mean number of copulations achieved by uninfected and infected males
645 over the 5-day experiment.
646 **Figure 2.** Mating attempt performance in the subset of cages (n = 10) that were filmed
647 continuously. (**A**) Mean number and (**B**) success rate of mating attempts by uninfected
648 (uM) and infected (iM) males over the 5-day experiment. In the all-uninfected trials,
649 uninfected males (uM) could only attempt to mate with uninfected females (uF). In mixed
650 infection trials, males could attempt to mate with either uninfected (uF) or infected (iF)
651 females. In the all-infected trials, infected males (iM) could only attempt to mate with
652 infected females (iF).

Figure 3: Influence of parasite infection on the mean (**A**) attempt and (**B**) copulation lengths observed in the subset of cages (n = 10) that were filmed continuously over the 5-day experiment. In the all-uninfected trials, uninfected males (uM) could only mate with uninfected females (uF). In mixed infection trials, males could mate with either uninfected (uF) or infected (iF) females. In the all-infected trials, infected males (iM) could only mate with infected females (iF).

Table I. Summary of GLMM model results comparing the mating probability of infected and uninfected male monarchs.

Influence of parasite infection on the probability of mating at least once GLMM with 48 observations from 48 males in 24 cages				
Random effects	Variance	Std. Dev.		
Trial	<0.001	<0.001		
Fixed effects	Estimate	Std. Error	*z* value	*P*
Male type: Uninfected (I)*	0.89	0.45	1.98	0.05
Male type: Infected	-2.22	0.67	-3.30	<0.0001

*The intercept (I) is set to uninfected males.

Table II. Summary of generalized linear mixed effects model (GLMM) model results comparing the number of copulations achieved by infected and uninfected male monarchs.

a. Influence of parasite infection on the number of copulations. GLMM with 48 observations from 48 males in 24 cages

Random effects	Variance	Std. Dev.		
Trial	0.22	0.48		
Fixed effects	**Estimate**	**Std. Error**	***z* value**	***P***
Male type: Uninfected (I)*	0.49	0.20	2.51	0.01
Male type: Infected	-1.93	0.45	-4.24	<0.0001

b. Influence of cage type on the number of copulations. GLMM with 48 observations from 48 males in 24 cages

Random effects	Variance	Std. Dev.			
Trial	0.19	0.44			
Fixed effects	**Estimate**	**Std. Error**	***z* value**	***P***	**Group †**
Cage type: All uninfected (I)	0.33	0.30	1.09	0.28	a
Cage type: Mixed infection	-0.28	0.38	-0.74	0.46	ab
Cage type: All infected	-1.72	0.68	-2.55	0.01	b

c. Influence of female infection on male copulation choice (mixed infection trials). GLMM with 48 observations from 24 males in 12 cages

Random effects	Variance	Std. Dev.		
Male ID	0.00	0.00		
Trial	0.38	0.62		
Fixed effects	**Estimate**	**Std. Error**	***z* value**	***P***
Male choice: Uninfected females (I)	-0.37	0.38	-0.97	0.33
Male choice: Infected females	0.44	0.43	1.03	0.30
Male type: Infected	-1.50	0.78	-1.92	0.05
Male choice x Male type	-1.14	1.30	-0.88	0.38

* (I) indicates the intercept for all models.
† Group indicates significant differences among cage types using Tukey HSD post hoc tests. Different letters indicate significance for $P \leq 0.05$.

Table III. Summary of generalized linear mixed effects model (GLMM) model results comparing the number of mating attempts performed by infected and uninfected male monarchs.

a. Influence of parasite infection on the number of male mating attempts. GLMM with 20 observations from 20 males in 10 cages.

Random effects	Variance	Std. Dev.		
Trial	0.97	0.99		
Fixed effects	Estimate	Std. Error	*z* value	*P*
Male type: Uninfected (I)*	1.92	0.34	5.63	<0.0001
Male type: Infected	-0.41	0.20	-2.08	0.04

b. Influence of cage type on the number male mating attempts. GLMM with 20 observations from 20 males in 10 cages.

Random effects	Variance	Std. Dev.			
Trial	0.57	0.76			
Fixed effects	Estimate	Std. Error	*z* value	*P*	Group †
Cage type: All uninfected (I)	2.52	0.56	4.55	<0.0001	a
Cage type: Mixed infection	-0.61	0.65	-0.93	0.35	ab
Cage type: All infected	-2.21	0.88	-2.50	0.01	b

c. Influence of female infection on male attempt choice (mixed infection trials). GLMM with 24 observations from 12 males in 6 cages.

Random effects	Variance	Std. Dev.		
Male ID	0.00	0.00		
Trial	0.91	0.95		
Fixed effects	Estimate	Std. Error	*z* value	*P*
Male choice: Uninfected females (I)	1.09	0.45	2.42	0.02
Male choice: Infected females	0.46	0.26	1.76	0.08
Male type: Infected	-0.04	0.29	-0.15	0.88
Male choice x Male type	-0.55	0.40	-1.38	0.17

* (I) indicates the intercept for all models.

† Group indicates significant differences among cage types using Tukey HSD post hoc tests. Different letters indicate significance for $P \leq 0.05$.

Table IV. Summary of generalized linear mixed effects model (GLMM) results comparing the attempt success rates of infected and uninfected male monarchs.

a. Influence of parasite infection on the success rate of mating attempts. GLMM with 18 observations from 18 males in 10 cages.

Random effects	Variance	Std. Dev.		
Trial	0.48	0.69		
Fixed effects	Estimate	Std. Error	*z* value	*P*
Male type: Uninfected (I)*	-1.13	0.36	-3.12	<0.01
Male type: Infected	-0.87	0.53	-1.65	0.10

b. Influence of cage type on the success rate of mating attempts. GLMM with 18 observations from 18 males in 10 cages.

Random effects	Variance	Std. Dev.			
Trial	0.08	0.28			
Fixed effects	Estimate	Std. Error	*z* value	*P*	Group†
Cage type: All uninfected (I)	-2.07	0.48	-4.30	<0.0001	a
Cage type: Mixed infection	0.60	0.56	1.07	0.28	a
Cage type: All infected	2.10	0.98	2.15	0.03	a

c. Influence of female infection on male attempt success (mixed infection trials). GLMM with 21 observations from 11 males in 6 cages.

Random effects	Variance	Std. Dev.		
Male ID	0.00	0.00		
Trial	0.28	0.53		
Fixed effects	Estimate	Std. Error	*z* value	*P*
Male choice: Uninfected females (I)	-1.09	0.57	-1.91	0.06
Male choice: Infected females	0.27	0.64	0.43	0.67
Male type: Infected	-1.30	0.91	-1.42	0.16
Male choice x Male type	-0.63	1.44	-0.44	0.66

* (I) indicates the intercept for all models.
† Group indicates significant differences among cage types using Tukey HSD post hoc tests. Different letters indicate significance for $P \leq 0.05$.

Table V. Summary of linear mixed effects model (LMM) results comparing the length of mating attempts performed by infected and uninfected male monarchs.

a. Influence of parasite infection on the length (min) of male mating attempts. LMM with 166 observations from 18 males in 10 cages.

Random effects	Variance	Std. Dev.		
Male ID: Trial	0.13	0.36		
Fixed effects	**Estimate**	**Std. Error**	***t* value**	***P***
Male type: Uninfected (I)*	3.80	0.19	19.51	<0.0001
Male type: Infected	0.02	0.33	0.05	0.97

b. Influence of cage type on the length (min) of male mating attempts. LMM with 166 observations from 18 males in 10 cages.

Random effects	Variance	Std. Dev.			
Male ID: Trial	0.05	0.23			
Fixed effects	**Estimate**	**Std. Error**	***t value***	***P***	**Group†**
Cage type: All uninfected (I)	3.34	0.24	13.76	<0.0001	a
Cage type: Mixed infection	0.71	0.30	2.41	0.03	a
Cage type: All infected	0.44	0.690	0.64	0.52	a

c. Influence of female infection on male attempt length (mixed infection trials). LMM with 106 observations from 11 males in 6 cages.

Random effects	Variance	Std. Dev.		
Male ID: Trial	0.00	0.00		
Fixed effects	**Estimate**	**Std. Error**	***t* value**	***P***
Male choice: Uninfected females (I)	4.16	0.24	17.25	<0.001
Male choice: Infected females	0.14	0.39	0.37	0.71
Male type: Infected	-0.11	0.40	-0.26	0.80
Male choice x Male type	-0.51	0.59	-0.86	0.40

* (I) indicates the intercept for all models.

† Group indicates significant differences among cage types using Tukey HSD post hoc tests. Different letters indicate significance for $P \leq 0.05$.

Table VI. Summary of linear mixed effects model (LMM) results comparing the length of copulations by infected and uninfected male monarchs.

a. Influence of parasite infection on the length (hr) of copulations. LMM with 28 observations from 13 males in 10 cages.

Random effects	Variance	Std. Dev.		
Male ID: Trial	0.00	0.00		
Fixed effects	Estimate	Std. Error	*t* value	*P*
Male type: Uninfected (I)*	10.09	0.23	38.39	<0.0001
Male type: Infected	0.56	0.57	0.98	0.33

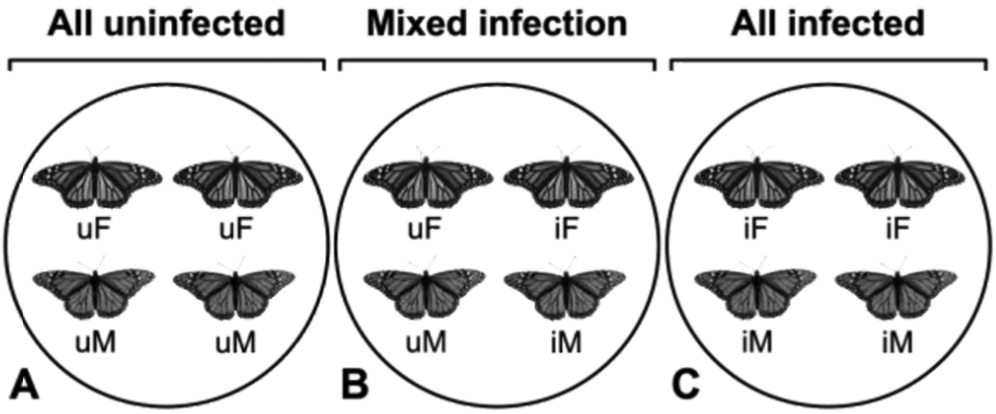
b. Influence of cage type on the length (hr) of copulations. LMM with 28 observations from 13 males in 10 cages.

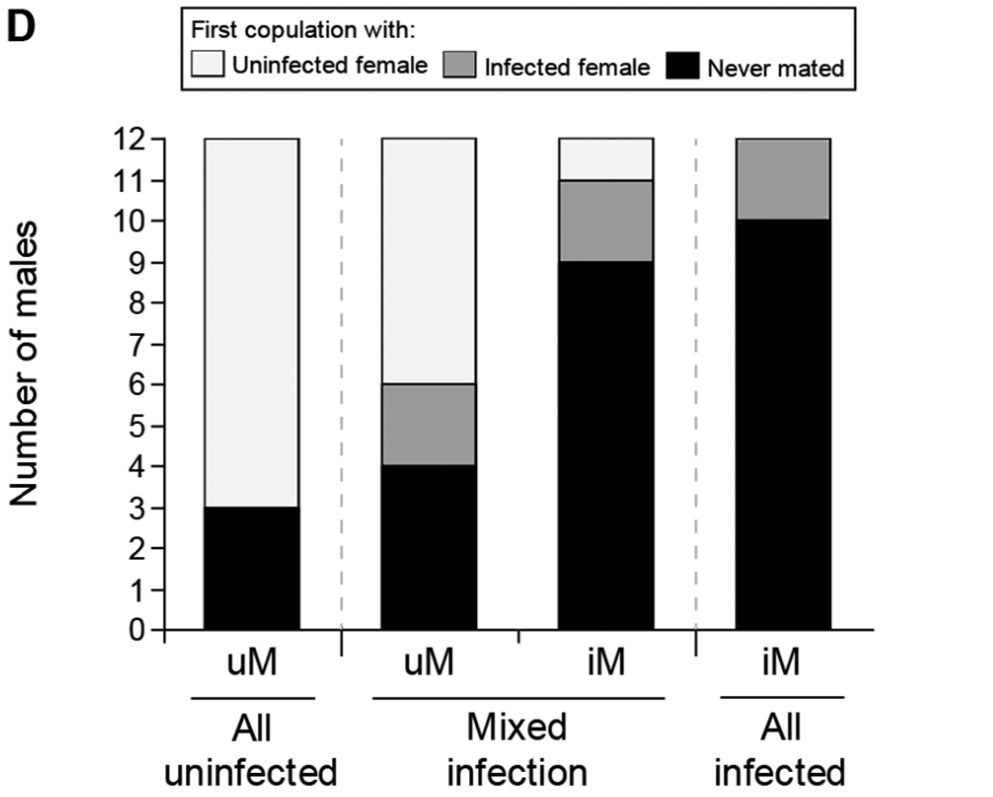
Random effects	Variance	Std. Dev.			
Male ID: Trial	0.00	0.00			
Fixed effects	Estimate	Std. Error	*t* value	*P*	Group†
Cage type: All uninfected (I)	9.72	0.50	19.43	<0.0001	a
Cage type: Mixed infection	0.59	0.57	1.03	0.31	a
Cage type: All infected	0.85	0.86	0.99	0.33	a

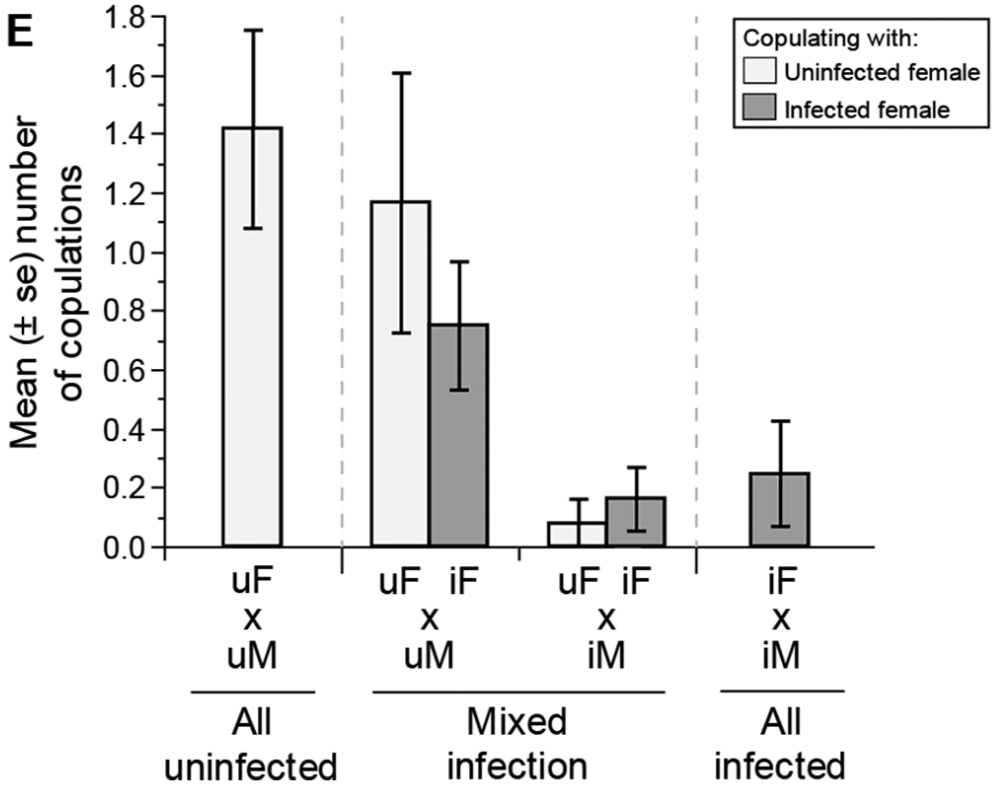
c. Influence of female infection on male copulation length (mixed infection trials). LMM with 19 observations from 8 males in 6 cages.

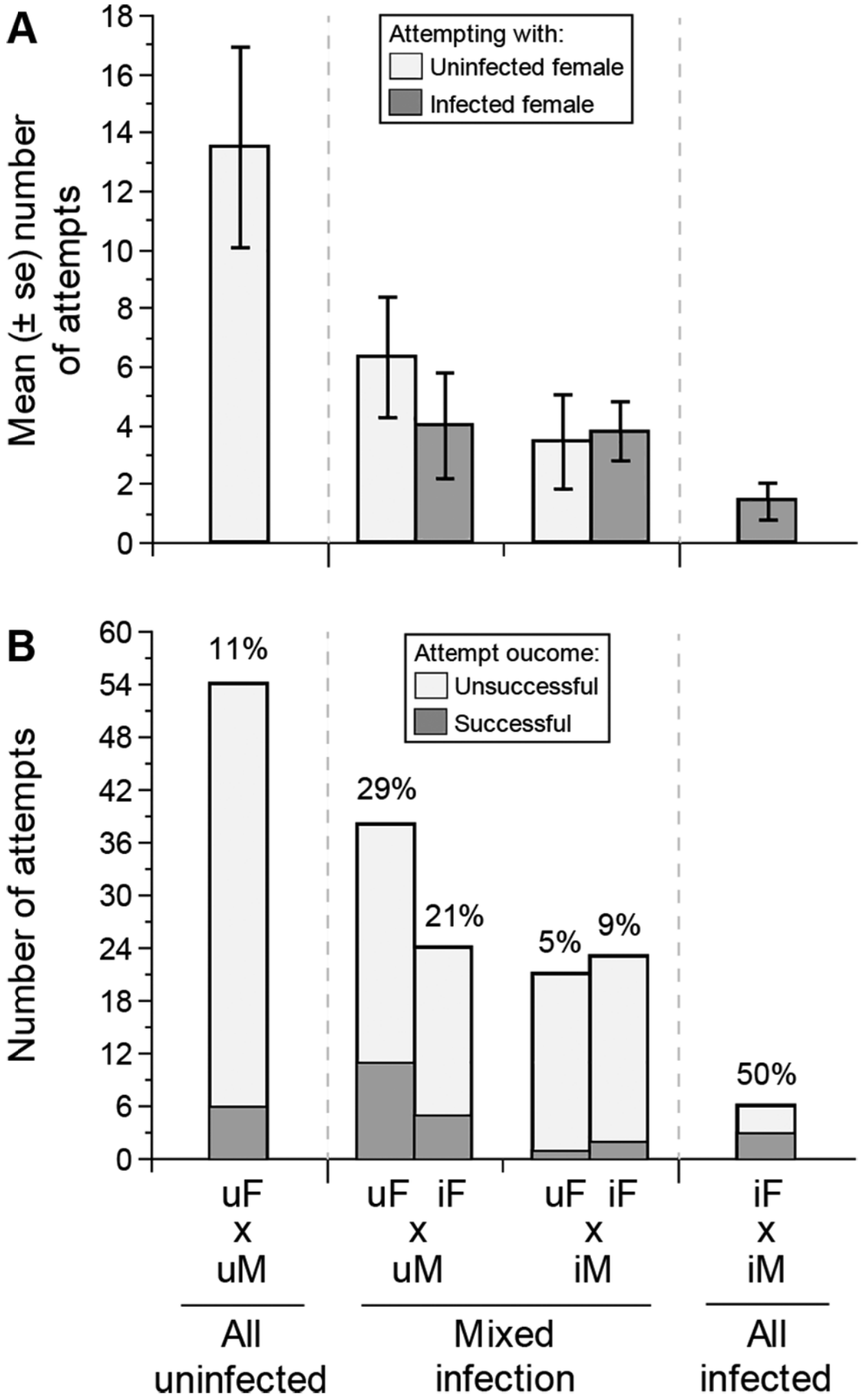
Random effects	Variance	Std. Dev.		
Male ID: Trial	0.00	0.00		
Fixed effects	Estimate	Std. Error	*t* value	*P*
Male choice: Uninfected females (I)	10.39	0.25	41.64	<0.001
Male choice: Infected females	-0.53	0.45	-1.18	0.25
Male type: Infected	0.50	0.45	0.58	0.57
Male choice x Male type	0.28	1.11	0.25	0.81

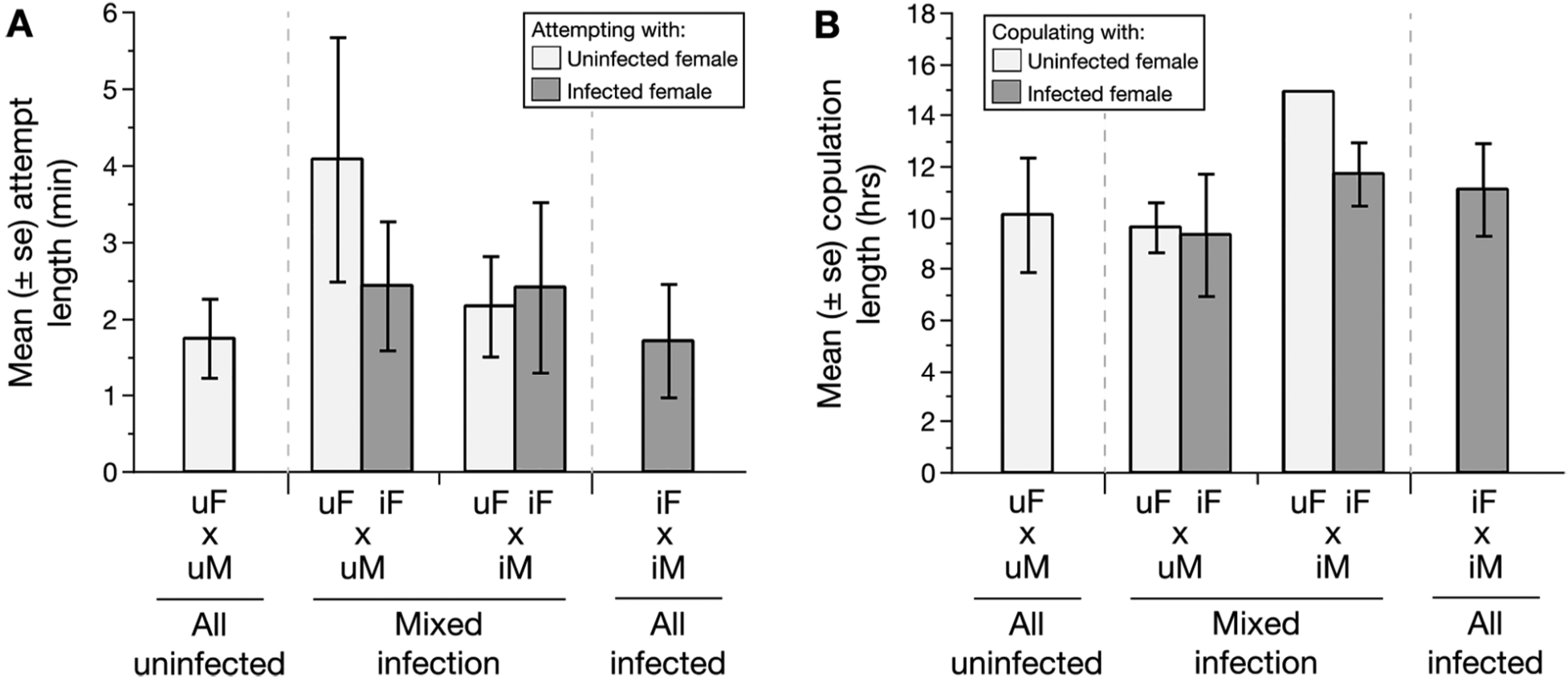
* (I) indicates the intercept for all models.
† Group indicates significant differences among cage types using Tukey HSD post hoc tests. Different letters indicate significance for $P \leq 0.05$.


All uninfected
Mixed infection
All infected
uF
uF
uM
uM
A
uF
iF
uM
iM
B
iF
iF
iM
iM
C


D
First copulation with:
Uninfected female
Infected female
Never mated
Number of males
0
1
2
3
4
5
6
7
8
9
10
11
12
uM
uM
iM
iM
All uninfected
Mixed infection
All infected


E
Copulating with:
Uninfected female
Infected female
Mean (± se) number of copulations
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
uF x uM
uF iF x uM
uF iF x iM
iF x iM
All uninfected
Mixed infection
All infected


A
Mean (± se) number of attempts
Attempting with:
Uninfected female
Infected female
B
Number of attempts
Attempt oucome:
Unsuccessful
Successful
11%
29%
21%
5%
9%
50%
uF x uM
uF iF x uM
uF iF x iM
iF x iM
All uninfected
Mixed infection
All infected


A
Mean (± se) attempt length (min)
Attempting with:
Uninfected female
Infected female
0
1
2
3
4
5
6
uF x uM
uF iF x uM
uF iF x iM
iF x iM
All uninfected
Mixed infection
All infected
B
Mean (± se) copulation length (hrs)
Copulating with:
Uninfected female
Infected female
0
2
4
6
8
10
12
14
16
18
uF x uM
uF iF x uM
uF iF x iM
iF x iM
All uninfected
Mixed infection
All infected