



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

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KEY WORDS ABSTRACT

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Mate choice
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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.

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, 1999; 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, 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 whether 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) × 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 cm² 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 × 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 0 were considered “uninfected” and scores greater than 1 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 was 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) × 30 cm (height) cylindrical mesh pupal 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

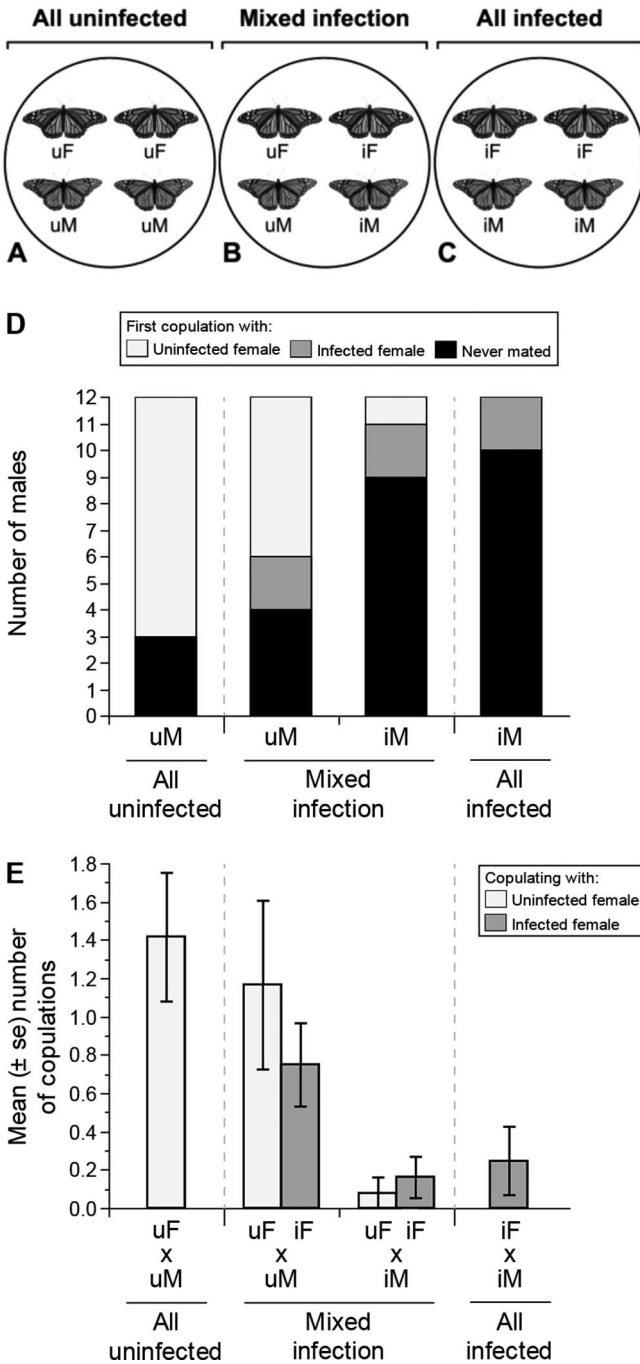


Figure 1. Influence of parasite infection on monarch mating performance. Mating trials ($n = 24$) consisted of 4 monarchs placed in 1 of 3 types of cages. (A) All-uninfected cages ($n = 6$) contained 2 uninfected females (uF) and 2 uninfected males (uM). (B) Mixed infection cages ($n = 12$) contained 1 uninfected female (uF), 1 infected female (iF), 1 uninfected male (uM), and 1 infected male (iM). (C) All-infected cages ($n = 6$) contained 2 infected females (iF) and 2 infected males (iM). (D) The proportion of males that achieved at least 1 copulation and, if so, the infection status of the female they mated with first. (E) The mean number of copulations achieved by uninfected and infected males over the 5-day experiment.

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 the left hindwing). Wing marking was randomized for infected and uninfected individuals.

Mating trials were of 3 types: all-uninfected, mixed infection, and all-infected (Fig. 1A–C). In the all-uninfected trials, both males and both females were parasite free. Hence, all the mating activity within these trials was between uninfected males and uninfected females (uM/uF). In mixed infection trials, 1 male and 1 female were infected, and the other male and female were uninfected. Thus, in these trials, both infected and uninfected males could choose to mate with either infected or uninfected females, creating 4 possible mating combinations (uM/uF, uM/iF, iM/uF, iM/iF). In the all-infected trials, all 4 monarchs were parasitized. Mating within these trials could only involve infected males copulating with infected females (iM/iF).

Mating trials lasted approximately 5 days, during which monarchs were provided 10% honey water ad libitum for food. All cages were spot-checked for mating once every evening. Butterflies were allowed to mate as many times as they could during the 5-day experiment. Additionally, 2 all-infected, 6 mixed infection, and 2 all-uninfected cages were filmed continuously for the entire experiment using high-definition Night Owl® AHD10-841-B cameras (Night Owl Security Products, Naples, Florida). Cameras were equipped with infrared bulbs to film in complete darkness. All cameras were hung approximately 30 cm above a cage and provided a clear recording 24 hr per day. These filmed cages allowed us to quantify finer-scale mating behavior beyond the evening spot-checks, which quantified the individuals that were in copula each day.

If a monarch died during the experiment, the dead individual and 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 genitalia

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 and 20:00 hr to record which butterflies successfully mated. Monarchs only mate once per day, with peak mating activity starting around 16:00 hr 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 and 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 whether 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 whether 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 whether 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., 2015). 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 "fitdistr" 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 2 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%

Table I. Summary of generalized linear mixed effects model (GLMM) 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.						
	Variance	SD	Estimate	SE	<i>z</i>	<i>P</i>
Random effects						
Trial	<0.001	<0.001				
Fixed effects						
Male type:			0.89	0.45	1.98	0.05
Uninfected (I)*						
Male type:			-2.22	0.67	-3.30	<0.0001
Infected						

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

(82/88) developed a parasite infection with a mean (\pm 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 frequently than uninfected males (Fig. 1E; Table IIa). Across the 24 trials, uninfected males mated an average (\pm 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 frequently 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 2 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).

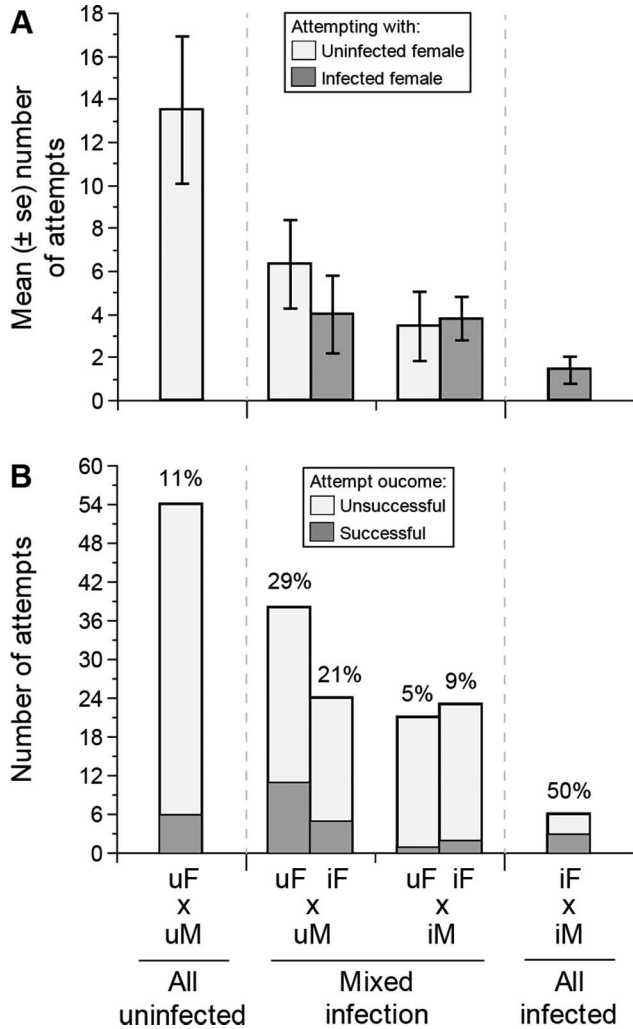


Figure 2. Mating attempt performance in the subset of cages (n = 10) that were filmed continuously. (A) Mean number and (B) success rate of mating attempts by uninfected (uM) and infected (iM) males over the 5-day experiment. In the all-uninfected trials, uninfected males (uM) could only attempt to mate with uninfected females (uF). In mixed infection trials, males could attempt to mate with either uninfected (uF) or infected (iF) females. In the all-infected trials, infected males (iM) could only attempt to mate with infected females (iF).

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. elektroscirra* suffered a reduction in mating performance. The effect of *O. elektroscirra* 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. elektroscirra* are likely even more exagger-

ated in natural populations, where males need to patrol tree canopies and find females to pounce on or grab during flight (Leong, 1995; Falco, 1998; Solensky, 2004; Solensky and Oberhauser, 2004). Moreover, the reduction in copulation success among infected males was apparent in both the presence and absence of uninfected males (Fig. 1E). Thus, *O. elektroscirra* does not just simply reduce a male’s ability to compete with healthy males, but likely has inherent negative effects on male behavior as well.

Importantly, even though our monarchs were confined to small cages, we observed similar mating dynamics described from both wild and captive populations (Pliske, 1975; Hill et al., 1976; Frey et al., 1998; Frey, 1999; Solensky, 2004; Solensky and Oberhauser, 2004; Brower et al., 2007). Previous studies examining monarchs in overwintering populations suggest that matings initiated with aerial captures are quite infrequent. Instead, males in these populations are often observed initiating mating attempts by pouncing on a stationary female (Leong, 1995; Falco, 1998; Solensky, 2004; Solensky and Oberhauser, 2004). Conversely, males in summer breeding populations typically initiate mating by grabbing females out of the air and taking them to the ground. While opportunities for aerial takedowns in our cages were extremely limited, the summer breeding males used here could and did initiate attempts by pouncing on females perching on the sides of the cages or feeding. Thus, it appears that summer breeding males can shift approach tactics when needed. If males did engage in mating by the pouncing method, they frequently took females to the ground. During the ground “wrestling” phase, we observed females deploying the typical battery of resistance 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. elektroscirra* 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 likely influences the number of 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 were surprised to observe

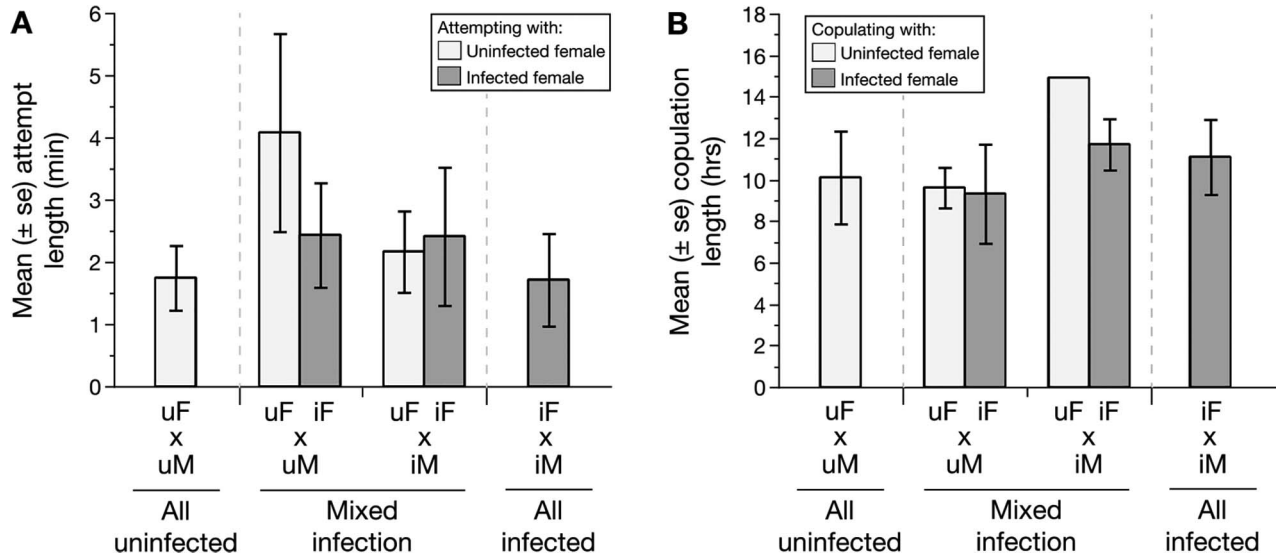


Figure 3. Influence of parasite infection on the mean (A) attempt and (B) copulation lengths observed in the subset of cages (n = 10) that were filmed continuously over the 5-day experiment. In the all-uninfected trials, uninfected males (uM) could only mate with uninfected females (uF). In mixed infection 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 II. Summary of generalized linear mixed effects model (GLMM) model results comparing the number of copulations achieved by infected and uninfected male monarchs.

	Variance	SD	Estimate	SE	z	P
a. Influence of parasite infection on the number of copulations. GLMM with 48 observations from 48 males in 24 cages.						
Random effects						
Trial	0.22	0.48				
Fixed effects						
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						
Trial	0.19	0.44				
Fixed effects						
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						
Male ID	0.00	0.00				
Trial	0.38	0.62				
Fixed effects						
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 × Male type			-1.14	1.30	-0.88	0.38

* (I) indicates the intercept for all models.

† Indicates significant group differences among cage types using Tukey HSD post hoc tests. Different superscripted letters indicate significance for $P < 0.05$.

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.

	Variance	SD	Estimate	SE	<i>z</i>	<i>P</i>
a. Influence of parasite infection on the number of male mating attempts. GLMM with 20 observations from 20 males in 10 cages.						
Random effects						
Trial	0.97	0.99				
Fixed effects						
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						
Trial	0.57	0.76				
Fixed effects						
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						
Male ID	0.00	0.00				
Trial	0.91	0.95				
Fixed effects						
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 × Male type			-0.55	0.40	-1.38	0.17

* (I) indicates the intercept for all models.

† Indicates significant group 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.

	Variance	SD	Estimate	SE	<i>z</i>	<i>P</i>
a. Influence of parasite infection on the success rate of mating attempts. GLMM with 18 observations from 18 males in 10 cages.						
Random effects						
Trial	0.48	0.69				
Fixed effects						
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						
Trial	0.08	0.28				
Fixed effects						
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						
Male ID	0.00	0.00				
Trial	0.28	0.53				
Fixed effects						
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 × Male type			-0.63	1.44	-0.44	0.66

* (I) indicates the intercept for all models.

† Indicates significant group 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.

	Variance	SD	Estimate	SE	<i>t</i>	<i>P</i>
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						
Male ID: Trial	0.13	0.36				
Fixed effects						
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						
Male ID: Trial	0.05	0.23				
Fixed effects						
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						
Male ID: Trial	0.00	0.00				
Fixed effects						
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 × Male type			-0.51	0.59	-0.86	0.40

* (I) indicates the intercept for all models.

† Indicates significant group 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.

	Variance	SD	Estimate	SE	<i>t</i>	<i>P</i>
a. Influence of parasite infection on the length (hr) of copulations. LMM with 28 observations from 13 males in 10 cages.						
Random effects						
Male ID: Trial	0.00	0.00				
Fixed effects						
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						
Male ID: Trial	0.00	0.00				
Fixed effects						
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						
Male ID: Trial	0.00	0.00				
Fixed effects						
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 × Male type			0.28	1.11	0.25	0.81

* (I) indicates the intercept for all models.

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

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. elektroscirra* 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 whether male mating effort is relative to the effort of direct competitors.

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

We also show that *O. elektroscirra* does not influence assortative mating in monarchs. In general, males mated at similar rates to both infected and uninfected females, regardless of their infection status. These data are consistent with previous studies showing a lack of avoidance of infected individuals. For example, milkweed leaf beetles (*Labidomera clivicollis*) that are infected by a sexually transmitted mite show no evidence of avoidance of infected mates, resulting in a high prevalence of the parasite (Abbot 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. elektroscirra* 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. elektroscirra* 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 whether 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. elektroscirra* 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. elektroscirra* 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. elektroscirra* 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. elektroscirra* 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. elektroscirra* may pose to the persistence of this iconic species.

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