Lack of inbreeding avoidance during mate selection in migratory monarch butterflies

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Abstract

Inbreeding is generally thought to have negative consequences for organismal health. However, despite the potential fitness effects, it remains surprisingly common among wild populations. In many cases, the complex factors that underlie mating dynamics make predicting whether individuals should or do avoid inbreeding quite challenging. One reason inbreeding may persist among species is that the likelihood of encountering relatives can be rare. Thus, even if inbreeding has severe consequences, selection to avoid mating with kin will be weak in species that are highly dispersed. Here we investigated if migratory monarch butterflies (*Danaus plexippus*), which are famous for their dispersal ability, actively avoid inbreeding. We found that neither female nor male monarchs choose mates based on relatedness. These results support the hypothesis that movement ecology can mask the deleterious effects of inbreeding and relax selection for active inbreeding avoidance behaviors. Overall, our data add to the growing list of studies showing that inbreeding avoidance is not the behavioral "default" for most species. We also highlight the implications that inbreeding may have on the declining populations of this iconic butterfly.

Keywords: mate choice, coercive mating, *Lepidoptera*, body size

1. Introduction

Inbreeding is an important phenomenon that influences the health of wild and captive populations. In general, the negative consequences of mating and reproducing with related individuals are well known (Crnokrak and Roff 1999; Charlesworth and Willis 2009; Frankham 2010; Hedrick and Garcia-Dorado 2016). Inbreeding increases the likelihood that individuals are homozygous for deleterious or lethal recessive alleles, which can reduce individual fitness (Keller and Waller 2002; Hedrick and Garcia-Dorado 2016). This so-called "inbreeding depression" can reduce the evolutionary potential for species to adapt to changing environments and increase the risk of extinction (Hedrick and Kalinowski 2000; Keller and Waller 2002; Reed et al. 2003; Frankham 2010; Reid and Keller 2010).

Animals have evolved numerous ways to reduce the likelihood of mating with related individuals (Pusey and Wolf 1996; Blouin and Blouin 1988; Szulkin et al. 2013). Two common avoidance strategies are sex-biased natal dispersal and mate choice. Sex-biased natal dispersal is a passive strategy to avoid inbreeding, which uses physical separation of related individuals to reduce contacts with kin (Pusey 1987; Handley and Perrin 2007). In contrast, mate choice is an active inbreeding avoidance strategy where organisms distinguish between related and unrelated individuals to avoid inbreeding-related fitness costs (Andersson and Simmons 2006; Jones and Ratterman 2009). Kin recognition and mating avoidance have been reported in many groups of animals including mammals (Milinski 2006), birds (Bonadonna and Sanz-Aguilar 2012), fishes (Fitzpatrick and Evans 2014) and insects (Cannon 2020). Active and passive avoidance mechanisms can work both independently and synergistically to play critical roles in determining species persistence. However, in many cases, the complex factors that underlie species distributions and mating dynamics make predicting whether individuals should or do avoid inbreeding challenging (Kokko and Ots 2006; Pemberton 2008; Szulkin et al. 2013; de Boer et al. 2021; Pike et al. 2021).

Curiously, despite the negative consequences of inbreeding, recent meta-analyses have found weak evidence for general inbreeding avoidance across species (de Boer et al. 2021; Pike et al. 2021). While some species, like long-tailed tits (*Aegithalos caudatus*), actively avoid kin (Leedale et al. 2018), mating in other species, such as yellow-bellied toads (*Bombina variegate*), is not influenced by relatedness (Cayuela et al. 2017). One potential reason that inbreeding avoidance is not the behavioral "default" for most species is that the risk of sexually interacting with kin is rare. Pike et al. (2021) highlight two criteria that need to be met for inbreeding avoidance to evolve: 1) inbreeding needs to reduce fitness, and 2) the risk of interacting with a related sexual partner is relatively high. The former criterion is typically the focus of studies that presume inbreeding should be avoided. However, an organism's mobility and resulting probability of actually encountering relatives is often overlooked. Thus, the general influence of inbreeding on mating behavior among systems remains unclear.

Here we examine active inbreeding avoidance in monarch butterflies (*Danaus plexippus*), a species famous for its mobility. Currently, very little is known about whether and how monarchs avoid inbreeding. Previous studies have shown that inbreeding depression in monarchs can be severe. Mongue et al. (2016) found that just a single round of full-sibling inbreeding can reduce egg viability by 26% and offspring lifespan by roughly 10%. The authors report similar drops in fitness after a second round of inbreeding as well. However, despite these immediate consequences of inbreeding, monarchs are unlikely to interact with close kin in the wild.

Monarchs are well known for their annual migration cycles (Gustafsson et al. 2015; Reppert and de Roode 2018), where individuals in eastern North America can undergo a > 4000 km transcontinental journey from the eastern United States and southern Canada to overwintering grounds in central Mexico (Urquhart and Urquhart 1978; Brower 1995). Monarchs found west of the Rocky Mountains migrate shorter distances to overwinter along the coast of California (Nagano et al. 1993; James et. al 2018), but still regularly travel up to 800 km from breeding grounds. This extreme movement ecology reduces the likelihood that monarchs encounter close relatives and should presumably weaken selection to evolve inbreeding avoidance mechanisms.

We conduct two captive mate-choice experiments, one designed to test female inbreeding avoidance and the other designed to test male inbreeding avoidance. Caged mating experiments have been critical for revealing the dynamics of mate choice in not only monarchs (Mongue et al. 2015), but many other butterfly species (Canon 2020), including the model *Bicyclus anynana* (Saccheri et al. 1996; Robertson et al. 2020). We hypothesize that despite the extreme costs of inbreeding, monarchs should not have mechanisms to actively avoiding mating with kin. Ultimately, we aim to test how dispersal ecology masks the negative effects of inbreeding and relaxes selection for active inbreeding avoidance in this iconic species.

2. Methods

2.1 Monarch rearing

All monarchs used in this study were descendants of wild-caught, eastern North American migratory monarchs from Florida, Ohio, and Georgia. Monarchs were reared in two batches. To generate the first batch, we mated four unique females to four unique males to create four distinct lineages, each consisting of full siblings. Up to 200 offspring from each mating pair were raised in a greenhouse at Emory University in Atlanta, GA under summer light and temperature conditions (range: 23.5-39.6°C), during May and June of 2019. Rearing time and environment ensured that monarchs remain reproductively active and do not exhibit migratory behavior (Goehring and Oberhauser 2002; Green and Kronforst 2019; Tenger-Trolander and Kronforst 2020). The monarchs from this first batch were used for the female choice experiment (Figure 1a-c).

To generate the second batch, we again mated four unique females to four unique males to create four more distinct lineages, each consisting of full siblings. Up to 200 offspring from each mating pair were raised in the same greenhouse, and again under summer light and temperature conditions (range: 23.5-39.6°C), during September of 2019. As with the first batch, rearing time and environment ensured that monarchs remain reproductively active and do not exhibit migratory behavior (Goehring and Oberhauser 2002; Green and Kronforst 2019; Tenger-Trolander and Kronforst 2020). The monarchs from this second batch were used for the male choice experiment (Figure 1d-f).

All larvae in both batches were raised on the same host plant species, *Asclepias incarnata*. Caterpillars were housed individually on plants that were surrounded by a clear plastic tube (13 cm diameter x 57 cm height) with a netted covering. Upon eclosion from pupae, all adults were weighed and checked for infection by the parasite *Ophryocystis* *elektroscirrha* using established non-invasive methods (de Roode et al. 2007); only uninfected individuals were used in mating trials.

2.2 Experimental design

2.2.1 Overview

The overall goal of our study was to test if monarchs display active inbreeding avoidance when choosing mates. We conducted two experiments, one focused on female choice and the other on male choice. Both experiments involved mating trials where we placed three butterflies in 30 cm (diameter) x 30 cm (height) cylindrical mesh popup insect cages (Carolina Biological Supply Company, Burlington, NC, USA). All cages were kept in walk-in environmental chambers (Environmental Specialties, Inc., Raleigh, NC, USA) set to a 14:10h light/dark cycle at 26°C and 50% relative humidity.

Mating trials were of two main types: mixed and same relatedness (Figure 1). In mixed relatedness trials, the focal individual was simultaneously presented with one sibling and one unrelated member of the opposite sex (Figure 1a,d). Hence, the focal subjects could "choose" a mate based on relatedness. In same relatedness trials, focal individuals were also simultaneously presented with two mating options. However, in these trials, the two mating options were either both siblings of the opposite sex (Figure 1b,e) or both unrelated individuals of the opposite sex (Figure 1c,f). Thus, focal subjects in these trials had only a single choice with respect to mate relatedness. The all-sibling or all-unrelated trials were critical for controlling the effect of mate encounter rate and operational sex ratio on mating preferences. Typically, the sex ratio in mating trials differs between choice tests (2:1 sex ratio, with the subject as the limited sex) and no-choice tests (1:1 sex ratio) (Dougherty

2020). Reducing no-choice trials to a 1:1 sex ratio can be problematic because the decision to reject the only available option has to be weighed against perceived risk of going unmated. In other words, focal subjects may choose to mate with an undesirable option simply because it is better than not mating at all (Dougherty 2020). By exposing focal subjects to only a single potential mate, traditional no-choice trials thus confound two aspects of the social environment that could potentially influence the chooser's behavior (Dougherty 2020; de Boer et al. 2021). We avoided this issue by maintaining a 2:1 sex ratio (with the focal subject as the limited sex) in all trials. Thus, all focal subjects in our study experienced the same mate encounter rates and operational sex ratios.

2.2.2 Experiment details

The first experiment was conducted in June of 2019 and focused on female mate choice (Figure 1a-c). Mating trials contained one female and two male monarchs and consisted of three types: mixed, all-sibling, and all-unrelated (Figure 1a-c). Prior to the start of the experiment, the males in each cage were marked with a 0.25-inch blue or yellow sticker placed on the ventral side of each wing for identification. The combination of stickers provided a unique identifier for each male, and care was taken to randomize color combinations within treatments and relatedness. Females were left unmarked. Mating trials lasted approximately five days, during which monarchs were provided 10% honey water *ad libitum* for food. All cages were spot-checked for matings every evening. This time was chosen because sperm transfer in monarchs occur after dawn in mating pairs that initiated copulation before dawn (Sväd and Wiklund 1988). Butterflies were allowed to mate as many times as they could during the 5-day experiment. Additionally, a random subset of cages was filmed continuously for the entire experiment using high-definition Owl AHD10-841-B cameras. 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 hours per day. These filmed cages allowed us to quantify mating behavior beyond the evening spot-checks. Observers conducted spot-checks and scored the videos without knowing how the males were related to the females.

The second experiment was conducted in October of 2019 and focused on male mate choice (Figure 1d-f). The experimental design was the reciprocal of the female choice experiment described above. Rearing and mating conditions ensured that all monarchs developed and behaved as breeding-generation individuals (Goehring and Oberhauser 2002; Green and Kronforst 2019; Tenger-Trolander and Kronforst 2020).

2.3 Quantification of mating behavior

Male monarchs forgo the chemical or visual courtship that is typical of most butterflies and moths. Instead, it is generally believed that males use a coercive strategy, where they grab females and take them to the ground to force them into copulation (Pliske 1975; Hill et al. 1976). However, despite this male-driven mating behavior, it remains largely unclear which sex is actually “choosier.” Males presumably dictate choice by selecting which females to force into copulation. But females counter male aggression by imposing their own choice with varying degrees of resistance (Frey 1999; Solensky 2004; Solensky and Oberhauser 2005; Agrawal 2017).

For both experiments, we quantified seven measures of mating performance. We broke down monarch mating behavior into two stages: attempt stage and 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 contacts as the monarchs fly around the cage. Females respond to these mating attempts with varying degrees of resistance. 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 two 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 the 5th day after monarchs were placed into cages.

Additionally, we also quantified multiple performance measures 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. Copulations end as soon as the pair separates.

Unlike the attempt stage, we quantified the copulation stage using both spot-checking and video recordings. Specifically, each cage was inspected once each evening between 19:00-20:00h to record which butterflies successfully mated. Monarchs only mate once per day with peak mating activity starting around 16:00 and ending around 19:00h (Oberhauser 1988). All successfully mating pairs will be *in copula* by approximately 19:00h and no additional mating activity happens at night. Pairs that are *in copula* after 19:00h will mate through the evening and typically break up between 02:00-06:00h the following morning (Sväd and Wiklund 1988). Thus, one evening check right before the lights turn off (20:00h) is sufficient to quantify all mating events in the experiment. These nightly checks were used to determine which butterflies were involved in the first mating as well as the total number of times each butterfly copulated over the course of 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 the 6th day after monarchs were placed into cages.

2.4 Statistical analysis

2.4.1 Female choice experiment

We analyzed female mating performance using a series of generalized linear mixed-effects models (GLMM) in R v3.3.3 (R Core Team, 2016) with the 'lme4' package v.1.1e12 (Bates et al. 2014). All models had the same fixed effect structure. Specifically, we modeled mating performance as a function of individual male relatedness (sibling vs. unrelated), trial type (mixed vs. same relatedness) and their interaction. We also included both female mass and her sexual size dimorphism (SSD) with each male as additional model factors to take into account the morphological differences between the choices presented. Moreover, given the physical nature of monarch coercive mating behavior, it seemed likely that body size would play a role in the female's ability to resist male advances. The intercept for all models was set to the behavior quantified in trials where all three butterflies were unrelated (Figure 1c).

We modeled three aspects of attempt performance (Table 1a-c). First, we used a GLMM with a Poisson distribution to predict the total number of attempts females received by each male as the dependent measure while including both cage number and male lineage as random effects. Random effects account for both the multiple attempt totals recorded for each female (i.e., one total from each male) and the possible influence of genetic compatibilities on monarch sexual selection (Mongue et al. 2015). Next, we used a GLMM with binomial distribution and logit link function to predict the attempt acceptance rates females had with each male as the dependent measure while including both cage number and male lineage as random effects (to again account for both the multiple acceptance rates recorded for each female and the possible influence of genetic compatibilities on monarch sexual selection). The attempt acceptance rate is a 2-column variable that column binds (using the command 'cbind') successful attempts and unsuccessful attempts with each male. Finally, we used a GLMM with a gamma distribution and log link function to predict the length of each attempt as the dependent measure while including male ID nested within cage number as random effects (to account for repeated attempts between the same male and female with a cage). The distribution that best fit the data for each of these models was determined using the 'fitdisplus' package v.1.1e12 (Delignette-Muller & Dutang 2015).

We tested initial mate preference by restricting the analysis to the first mating observed in the mixed relatedness cages. In this analysis, we treated the three monarchs in each cage as an experimental unit. The first mating in each of the mixed trials was determined by spot-checking. The proportion of sibling and unrelated males involved in first matings was tested against a random 50-50 mate preference for relatedness using a Chi-squared test with $\alpha = 0.05$.

We then ran three additional models further assessing copulation performance (Table 1d-f) in all trial types. First, we used a GLMM with binomial distribution and logit link function to predict the female's probability of mating with each male as the dependent measure while including cage number and male lineage as random effects (to account for both multiple mating probabilities recorded for each female and possible influence of genetic background on mating behavior). The probability of mating with a given male was recorded as either a "mated" if the female copulated at least once with him, and "unmated" if she never mated with him. Next, we used a GLMM with a Poisson distribution to predict the total number of times females were observed copulating with each male as the dependent measure while including both cage number and male lineage as random effects (to account for both the multiple copulation totals of the female and possible influence of genetic background on mating behavior). Finally, we used a GLMM with a gamma distribution and log link function to model the length of each copulation as the dependent measure while including male ID nested within cage number as a random effect (to account for repeated copulations between the same male and female within a cage). The distribution that best fit the data for each of these models was again determined using the 'fitdisplus' package v.1.1e12 (Delignette-Muller & Dutang 2015).

2.4.2 Male choice experiment

We analyzed male mating performance the same way as the female choice experiment described above (Table 2). The only analytical difference between the experiments was how we modeled copulation lengths. Unlike the female choice experiment, the copulation lengths in the male choice experiment were normally distributed. Thus, we used a linear mixed effects model (LMM) to predict the length of each copulation as the dependent measure while including female ID nested within cage number as a random effect (Table 2f).

3. Results

3.1 Female choice experiment

The female choice experiment included a total of 69 mating trials. These consisted of 44 mixed relatedness trials and 25 same relatedness trials (13 cages contained all siblings and 12 cages contained all unrelated butterflies) (Figure 1a-c). Of these, 57% (25/44) of mixed trials, 38% (5/13) of all sibling trials, and 50% (6/12) of all unrelated trials were filmed continuously for the 5-day experiment.

Mating attempts were quantified from the 36 trials that were filmed. We first analyzed the factors that influenced the total number of attempts the female received from each male. On occasion, some females did not receive a single mating attempt from one or both males in her cage. These zeros were included in the analysis. Thus, we recorded 72 attempt totals from 36 cages (i.e., two totals per cage). The number of attempts with a given male ranged from zero to six. None of the factors tested significantly influenced how many attempts a female received from a particular male (Figure 2a, Table 1a).

Next, we tested how male relatedness influenced female acceptance rates. Attempts are considered accepted when they resulted in copulation. For each trial, observers would determine the female's attempt acceptance rate with each of the two males. If a male never attempted to mate with a female, then we could not calculate an acceptance rate. We obtained 53 success rates from the 36 trials that were filmed. In general, attempts were highly successful (Figure 2b). Across all trials in this experiment, 86.4% (70/81) of attempts ended in copulation. However, acceptance rates did not depend on male relatedness, trial type, or their interaction (Figure 2b, Table 1b). Rates were, however, significantly influenced by body size. Specifically, acceptance rates were positively correlated by both female mass ($P = 0.03$) and her size relative to the male attempting to mate with her ($P = 0.01$) (Table 1b).

Finally, we analyzed factors affecting how long attempts lasted. This analysis included both successful and unsuccessful attempts. Across all 36 filmed trials, we observed 81 total attempts that lasted between 0.4 – 30.3 minutes. None of the factors tested significantly influenced how long attempts lasted (Figure 2c, Table 1c).

We further analyzed how relatedness affects mate preference by restricting our analysis to the first mating observed in the mixed relatedness trials (Figure 1a). Observers spot-checked each of the 44 mixed trials nightly for copulations. We found that 52.3% (23/44) of first matings involved the sibling male and 47.7% (21/44) involved the unrelated male. These proportions did not significantly deviate from random preference (Chi-squared test; $\chi^2 = 0.09$, df = 1, P = 0.76). Moreover, we used spot-checks to also record which males successfully mated at least once during the 5-day experiment. In this analysis, all 138 males in the 69 trials were designated as either "mated" or "unmated". None of the factors tested significantly influenced whether or not a female copulated with a male (Figure 2d, Table 1d).

In addition, we tested if relatedness influenced how often females copulated with each male by recording the number of times we saw each male *in copula* over the course of five days. On occasion, males did not attempt to mate with the female. These zero copulation totals were included in the analysis. Thus, we recorded 138 copulation totals from all 69 cages (i.e., two totals per cage). The number of copulation observations with a given male ranged from zero to three. None of the factors tested significantly influenced how many times females were observed *in copula* with a particular male (Figure 2e, Table 1e). Importantly, among the 36 trials that were filmed, we found that the number of matings recorded from spot-checking in each cage was identical to the number quantified from the corresponding videos. This confirmed that we did not miss any matings by only checking cages a single time per day, and that spot-checking was sufficient to accurately capture which monarchs successfully mated.

Finally, we analyzed factors affecting how long copulations lasted. This analysis could only include the 36 trials that were filmed. Across all trials, we measured the length of 70 copulation bouts that lasted between 8.8 – 63.6 continuous hours. None of the factors tested significantly influenced how long copulations lasted (Figure 2f, Table 1f).

3.2 Male choice experiment

The male choice experiment included a total of 62 mating trials. These consisted of 36 mixed relatedness and 26 same relatedness trials (10 cages contained all siblings and 16 cages contained all unrelated butterflies) (Figure 1d-f). Of these, 64% (23/36) of mixed trials, 50% (5/10) of all sibling trials, and 50% (8/16) of all unrelated trials were filmed continuously for five days. In 10 trials (7 mixed, 1 all-sibling, and 2 all-unrelated) we observed no sexual behaviors among any of the butterflies during the entire experiment (i.e. not a single mating attempt among the three butterflies). While it is unknown why these monarchs showed no inclination to mate, these trials were designated as sexually unreceptive and were removed from all subsequent analyses.

This experiment was analyzed similarly to the female choice experiment described above. We tested how female relatedness influences three aspects of male attempt performance. All attempt measures could only be quantified from the 26 trials that were filmed. We first analyzed the factors that influenced the total number of attempts the males directed toward each female over the course of five days. On occasion, one of the males would not attempt to mate with one of the females. But since there was mating activity from the other male in the cage, these zero attempt totals were included in the analysis. Thus, we recorded 52 attempt totals from 26 cages (i.e., two totals per cage). The number of attempts with a given female ranged from 0 to 12. None of the factors tested significantly influenced significantly influenced the number of times males attempted to mate with a particular female (Figure 3a, Table 2a).

Next, we tested how relatedness influenced the attempt success rates. For each trial, observers would determine the male's attempt success rate with each of the two females. If a male never attempted to mate with a female, then we could not calculate a rate. We obtained 43 success rates from the 26 trials that were filmed. In general, male attempts were unsuccessful. Across all trials, only 18.3% (31/169) of attempts resulted in copulation. There was no significant difference in success rates between siblings and unrelated females in either the mixed or same relatedness trials (Figure 3b, Table 2b). Success rates were, however, significantly influenced by body size where male mass was negatively correlated with success rate ($P = 0.02$).

Finally, we analyzed factors affecting how long attempts lasted. This analysis included both successful and unsuccessful attempts. Across all 26 trials, we observed 169 total attempts that lasted between 0.1 – 67.8 continuous minutes. There was a significant difference in attempt length between all-sibling and all-unrelated trials (Figure 3c, Table 2c). Mean attempt length was longer in all sibling trials than in all unrelated trials ($P < 0.001$).

However, within mixed trials, there was no significant difference in attempt length between sibling and unrelated butterflies. Furthermore, there was a significant interaction between female relatedness and trial type ($P = 0.005$). No aspects of body size significantly influenced how long attempts lasted.

We further analyzed how relatedness affects mate preference by restricting our analysis to the first mating observed in the mixed relatedness trials (Figure 1d). Observers spot-checked each of 20 mixed trials nightly for copulations. We found that 45.0% (9/20) of first matings involved the sibling male and 55.0% (11/20) involved the unrelated male. These proportions did not significantly deviate from random mate preference (Chi-squared test; $\chi^2 = 0.20$, df = 1, P = 0.65), Moreover, we used spot-checks to also record whether or not each female mated at least once during the 5-day experiment. In this analysis, all 104 females in the 52 trials were designated as either “mated” or “unmated”. None of the factors tested significantly influenced likelihood that a male copulated with a particular female (Figure 3d, Table 2d).

In addition, we tested if relatedness influenced how often males copulated with each female by recording the number of times we saw each female *in copula* over the course of five days (Figure 3e, Table 2e). On occasion, males did not copulate with one or both females in their cage. These zero copulation totals were included in the analysis. Thus, we recorded 104 copulation totals from all 52 cages (i.e., two totals per cage). The number of copulation observations with a given female ranged from zero to three. The number of copulations observed was not influenced by the relatedness between males and females. However, trial type had a significant effect on the number of times males copulated with unrelated females ($P = 0.04$). Specifically, males copulated more frequently with unrelated females in the mixed trials than the all unrelated trials. No aspect of body size influenced the likelihood that a male copulated with a particular female. Again, among the 26 trials that were filmed, we found that the number of matings recorded from spot-checking in each cage was identical to the number quantified from the corresponding videos.

Finally, we analyzed factors affecting how long copulations lasted (Figure 3f, Table 2f). The data came from the 26 cages that were filmed. In 12 of these cages, males attempted to mate but were never successful. Thus, the length of copulations was quantified in only 14 trials. Across these trials, we filmed at total of 31 copulation bouts. However, for 10 of these matings, the camera cut out prior to the butterflies separating. This prevented us from determining how long these particular bouts lasted, leaving a dataset that included 21 copulation bouts from 14 cages. Copulations lasted between 0.02 – 32.6 continuous hours and none of the factors tested significantly influenced the length of time males copulated with a particular female.

4. Discussion

Our results show that neither female nor male monarch butterflies actively avoid inbreeding. In the female choice experiment, the first mating in the 44 mixed relatedness trials (Figure 1a) was effectively random, where 52% chose their brother, and 48% chose the unrelated male. Moreover, no aspects of mating performance (i.e., attempts and/or copulations) in these mixed relatedness trials were significantly different between sibling or unrelated pairs (Figure 2; Table 1). This was also true in the same relatedness trials (Figure 1b, c), where we found no significant differences in mating performance between cages with only siblings and cages with only unrelated monarchs. (Figure 2; Table 1).

The lack of inbreeding avoidance was also clear when males were the focal sex (Figure 1d-f). Again, the first mating in the 36 mixed relatedness trials (Figure 1d) indicate random mate choice, where 45% chose their sister, and 55% chose the unrelated female. Additionally, both within and among treatments, nearly all aspects of male mating performance did not significantly differ when mating with sibling or unrelated females (Figure 3, Table 2). The one exception was the influence of relatedness on mean attempt time. Specifically, the length of attempts observed in the all-sibling cages was nearly six times longer than the mixed or all-unrelated cages (Figure 3c; Table 2c). However, this difference is largely attributed to two extreme attempts, where in two all-sibling cages we observed males trying to coerce females into copulation for 46.2 and 64.9 continuous minutes respectively. If these two attempts are removed from the analysis, there is no significant difference in mean attempt length within or among trial types. Importantly, while these two attempts were extreme, it does demonstrate the extent of sexual conflict between the sexes and shows the lengths monarchs will go to try to either force a female into copulation or resist a male's sexual advances.

Importantly, in both experiments we observed typical mating behaviors described from both field and captive monarch studies (Hill 1976; Frey et al. 1998; Frey 1999; Solensky 2004; Solensky and Oberhauser 2004; Brower et al. 2007). Even though our monarchs were confined to small cages, their reduced fight capacity did not hamper their ability or willingness to mate. Indeed, previous studies suggest that mating initiated with aerial captures are quite infrequent. Instead, males are often observed initiating mating attempts by pouncing on a stationary female (Falco 1998; Frey et al. 1998; Solensky 2004). In our cages, aerial pursuits were all but impossible, but males could, and did, initiate attempts by pouncing on females perching on the sides of the cages or feeding. When males did engage in mating, they frequently took females to the ground, which is also typical of wild monarchs (Solensky 2004; Brower et al. 2007). During the ground “wrestling” phase, we observed females deploying the whole battery of resistance behaviors typically seen in wild populations (Frey 1999; Solensky 2004; Brower et al. 2007).

The confined cages also did not influence the effort monarchs put into mating. Coercive attempts across both our experiments lasted an average of 2.37 min (n = 250). This mating effort was nearly identical to the 2.20 min (n = 273) average attempt observed in wild populations (Solensky 2004). Moreover, most of the mating attempts observed across our two experiments ended in failure. We observed males achieving copulation only 40% (101/250) of the time. This is similar to both the 31% (85/273) success rates observed in previous captive studies using larger (1.8 m^3) outdoor cages (Solensky and Oberhauser 2004), as well as the 30-40% success rates reported from wild overwintering populations (Van Hook 1993; Frey 1999; Oberhauser and Frey 1999; Solensky 2004). Thus, the small cages used in our experiments did not appear to significantly influence overall monarch mating behavior, allowing us to analyze the effects of genetic relatedness in a controlled manner that reproduces natural mating behaviors.

Our results indicate that selection for active inbreeding avoidance in monarchs has been historically weak. The willingness to mate with kin is presumably due to monarchs’ reliance on other, more dispersal-based means of avoiding inbreeding. While in general inbreeding depression can reduce the fitness of inbred individuals, in species with dispersal strategies that limit interactions with kin or those found in large, panmictic populations, the risk of inbreeding is too low to drive the evolution of sibling recognition mechanisms

(Szulkin et al. 2013; Duthie et al. 2016; Pike et al. 2021). The high mobility and historically large population sizes of monarchs likely reduce the chances that related individuals interact with each other. As soon as monarchs eclose, they typically disperse from their natal rearing grounds in search of food and mates. In the most extreme cases, some eastern North American monarchs disperse up to 4500 km from their eclosion site to overwintering grounds in central Mexico (Gustafsson et al. 2015; Reppert and de Roode 2018). Indeed, one presumed adaptive function of animal migration is to facilitate admixture of populations and "reshuffle" the gene pool every year to reduce extensive inbreeding within populations (Cresswell et al. 2011). Our data add to the growing number of studies suggesting inbreeding avoidance among animals may not be as widespread as originally presumed (Szulkin et al. 2013; de Boer et al. 2021; Pike et al. 2021).

Although historically monarchs have faced little selective pressure to evolve active inbreeding avoidance mechanisms, the negative consequences of mating with kin remain real (Mongue et al. 2016). Inbreeding could become problematic given that in recent decades, monarch populations throughout North America have undergone severe demographic changes. Previous research suggests that habitat loss and global temperature fluctuations have led to severe population collapse (Forister et al. 2021), at least in western North America. Some estimates of western North American monarch populations have indicated declines exceeding 99% (Pelton et al. 2019). Moreover, increasing global temperatures and planting of non-native milkweed in the southern United States is thought to trigger migratory dropout, where eastern North American monarchs forgo their journey to Mexico and instead establish small, fragmented year-round breeding populations along the Gulf of Mexico and inland Texas (Satterfield et al. 2015, 2018). Similarly, year-round breeding populations are forming in southern California and the Californian Bay Area (Satterfield et al. 2016; James 2021). This rapid population decline, coupled with increased population fragmentation, may increase monarch vulnerability to inbreeding depression. The increased likelihood of mating with relatives may be especially challenging for monarchs given that a single round of full-sibling inbreeding is sufficient to significantly reduce egg viability and adult lifespan (Mongue et al. 2016). Thus, monarchs that transition into pockets of sedentary, year-round breeding populations may no longer be sheltered from inbreeding depression (Semmens et al. 2016).

Indeed, previous studies have shown how inbreeding depression can be particularly problematic in fragmented populations (Schultz et al. 2020). A comprehensive field study of the Glanville fritillary (*Melitaea cinxia*) in Finland found that as populations became small and fragmented, individuals were increasingly forced to mate with kin. Without sufficient emigration, inbreeding depression gradually led to the extinction of 7 of the 42 populations originally sampled (Saccheri et al. 1998). Given the sudden behavioral shifts in movement ecology (Semmens et al. 2016), monarchs could presumably face a similar fate. Interestingly, monarchs have formed viable sedentary populations on islands around the world through independent dispersal events from North America over the last few hundreds of years (Zalucki and Clarke 2004; Zhan et al. 2014). This suggests that these populations have either evolved inbreeding avoidance strategies, that the effects of inbreeding are not severe enough to reduce population health, or that these populations have become more tolerant of inbreeding depression (Kokko and Ots 2006). In some species the effects of inbreeding are mitigated by moderate reductions in population size to purge deleterious alleles. Importantly, previous studies show that these cyclic population declines do not appear to reduce genetic variation enough to cause large drops in fitness (Waser et al. 1986; Facon et al. 2011; Puurtinen 2011). Moreover, selection for inbreeding avoidance is rarely uniform within a species and is instead often population- and context-specific (Pusey and Wolf 1996; Kell and Waller 2002; Pizzari et al. 2004; Herfindal et al. 2014). Testing such differential selection in monarchs would provide an important step in elucidating the potential consequences of the increased inbreeding that will accompany the current shift from migratory to sedentary lifestyles of North American monarchs.

While monarchs did not choose mates based on relatedness, our data do suggest that some components of mating performance are influenced by monarch body size. Body size is a fundamental trait that influences reproductive dynamics in a wide variety of organisms (Hunt et al. 2008; Hunt and Sakaluk 2014). Size can be especially important in coercive mating systems, which involves a physical struggle between males and females. In the female choice experiment, attempt success rates were positively correlated with female mass. While success rates were high in this experiment, this result was not simply because larger females received more attempts. It is possible that larger females are more willing to mate because they are less likely to get injured by a male. Alternatively, larger females can presumably handle more spermatophores than smaller females, and thus may be more willing to accept multiple mating attempts. In extreme cases, females can mate so much that accumulating spermatophores can burst through the abdomen and kill them (Brower et al. 2007). Small females should safeguard against this possibility and limit the number of times they accept mating attempts. Interestingly, we also found that sexual size dimorphism in this experiment was positively correlated with attempt success rate. Specifically, success rates were higher with increasing size dimorphism between the female and male. This result suggest that females may actually be more accepting of smaller males.

Curiously, we saw a similar relationship in the male choice experiment. When cages contained one male and two females, male size was negatively correlated with attempt success rate. In other words, larger males were less likely to achieve copulation during a given attempt. Indeed, the top 25% largest males in this experiment had only a 7% (2/29) attempt success rate. How can the largest males not be successful in a coercive mating system? One possibility is that in some scenarios, females may have preferred smaller males. This again may be due to the female's aversion to injury while copulating. Regardless, our data suggest that body size plays a role in monarch mating dynamics and may females have more control over mating outcomes than previously realized. Future studies should manipulate male and female monarch body size to further identify its influence on mate choice.

Finally, our experiments suggest that monarch mating behavior is affected by the operational sex ratio in mating cages, a phenomenon reported from multiple butterfly species (Puurtinen 2011; Cannon 2020; Holveck et al. 2015; Westerman et al. 2014; Westerman et al. 2019). In our first experiment, when all mating trials consisted of two males and a single female, we observed mating in every single cage. Most butterflies were observed copulating at least once, including 100% (69/69) of females and 68% (94/138) of males. Once *in copula,* pairs remained together for an average (± se) of 22.8 ± 11.6 hours. This high volume of mating was largely due to the high acceptance rates by females. Across all trials, 86% (70/81) of attempts resulted in copulation, which is more than twice as likely as the 30-40% acceptance rates observed in wild populations (Solensky 2004). Of the females that were filmed, 69% (25/36) received two or fewer mating attempts over the course of five days, and only three females were subject to more than four attempts. Moreover, despite high acceptance rates, 64% (44/69) of the females still mated with only one of the two males in the cage, suggesting more complicated choice dynamics that may include some aspects of male-male competition. These data suggest that while mating in this experiment was plentiful, high success rates were not simply due to females being "worn down" by persistent coercion by the two males in a confined space. Rather, male-biased sex ratios appear to make females less choosy, a phenomenon also reported in the model butterfly *Bicyclus anynana* (Holveck et al. 2015).

In contrast to the mating successes observed in the female choice experiment, nearly all measures of mating performance plummeted when the operational sex ratio shifted to two females and one male per cage. In this experiment, only 18% (31/169) of all attempts ended in copulation. This resulted in most of the butterflies going unmated. Across this experiment only 58% (30/52) of males and 36% (37/104) of females were observed copulating. These totals do not include the 10 cages that were removed from the analysis because we observed no mating-related behaviors during the entire experiment. Not only were the butterflies in this experiment less likely to mate, but copulation bouts lasted an average (± se) of 12.8 ± 6.5 hours, which was 44% shorter than in the female choice experiment. This reduction in copulation time likely reflects that, unlike the female choice experiment described above, the single male per cage does not have to deploy mate-guarding tactics to deny a competing male access to the female. The fact that males did not spend nearly as long *in copula* makes it all the more puzzling that they did not achieve more copulations. A 2:1 female biased sex ratio should have provided an ideal scenario to maximize male mating performance. The females, which are presented with only a single option to mate with, would presumably be more willing to mate to avoid the risk of going unmated. Likewise, the singleton males, who do not have to compete with other males for mates, have unlimited access to both females confined to a cage. However, very few males actually achieved copulation with both the females in their cage. Of the 56 males analyzed, 7 mated with both females, 23 mated with only one female, and 22 failed to mate with either female. The inability to achieve copulation was not through lack of trying. The males that were filmed conducted an average (± se) of 6.5 ± 0.9 attempts over the 5-day experiment, nearly three times higher than the males in the female choice experiment. These results compliment previous work showing how butterflies can change their mating behavior in response to social context (Westerman et al. 2014; Westerman et al. 2019). Like many previous monarch studies, we show that females were especially successful at rejecting males (Van Hook 1993; Frey 1999; Oberhauser and Frey 1999; Solensky 2004; Solensky and Oberhauser 2004). Moreover, our fine-scale behavioral analysis provides additional evidence that females may be more in control of the coercive mating attempts than previously realized.

Overall, we conducted the most comprehensive tests of monarch inbreeding avoidance to date. Our data show that North American migratory monarchs, like many butterflies, readily mate with kin. This study can also be added to the growing list of results showcasing animals that do not avoid inbreeding, which further questions its role in the evolutionary trajectories of populations (Robertson et al. 2020; Pike et al. 2021). Our study is consistent with previous work suggesting active inbreeding avoidance should not be considered the default state within populations, but only evolves under particular ecological scenarios (Pike et al. 2021). Since monarchs have historically experienced relaxed selection to actively avoid mating with kin, they may be particularly vulnerable to inbreeding depression during sharp population declines and increasing population fragmentation. This study highlights another possible threat to the persistence of this iconic butterfly.

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Tables

Table 1. Summary of mixed models from the female choice experiment. The intercept for all models was set the mating performance in trials where all three butterflies were unrelated (Figure 1c). We modeled six measures of mating performance as a function of male relatedness (sibling vs. unrelated), trial type (mixed vs. same relatedness) and their interaction. We included both female mass and her sexual size dimorphism (SSD) with each male as additional factors. See Methods for details on random effect structure.

	Behavioral measure	**Fixed effects**	**Estimate**	**Std. Error**	**Test value**	***P value***
a.	**Number of attempts** GLMM n = 72 obs. from 36 cages	Intercept	1.49	1.49	1.00	0.32
		Relatedness	-0.43	0.45	-0.96	0.34
		Trial type	-0.23	0.33	-0.70	0.49
		Female mass	-2.20	2.94	-0.75	0.45
		SSD	-0.02	0.01	-1.87	0.06
		Relatedness x Trial type	0.69	0.52	1.32	0.19
b.	**Attempt success rate** GLMM n = 53 obs. from 36 cages	**Intercept**	-11.58	5.45	-2.12	**0.03**
		Relatedness	21.27	9072.27	0.00	1.00
		Trial type	1.58	1.45	1.09	0.28
		Female mass	26.15	11.67	2.24	**0.03**
		SSD	0.15	0.06	2.78	**0.01**
		Relatedness x Trial type	-22.71	9072.27	-0.00	1.00
c.	**Length of attempts** GLMM n = 81 obs. from 36 cages	Intercept	0.97	1.52	0.64	0.52
		Relatedness	-0.08	0.48	-0.17	0.86
		Trial type	-0.07	0.37	-0.19	0.85
		Female mass	-0.01	3.05	-0.00	1.00
		SSD	-1.15	1.00	-1.15	0.25
		Relatedness x Trial type	-0.09	0.57	-0.15	0.88
d.	**Probability of copulating** GLMM n = 138 obs. from 69 cages	Intercept	1.72	2.59	0.66	0.51
		Relatedness	-0.92	0.69	-1.33	0.18
		Trial type	0.03	0.65	0.05	0.96
		Female mass	-0.55	5.02	-0.11	0.91
		SSD	-0.02	0.02	-1.12	0.26
		Relatedness x Trial type	0.39	0.86	0.45	0.65
e.	**Number of copulations** GLMM n = 138 obs. from 69 cages	Intercept	0.43	0.86	0.50	0.62
		Relatedness	-0.09	0.27	-0.35	0.73
		Trial type	0.08	0.23	0.33	0.74
		Female mass	-0.50	1.64	-0.30	0.76
		SSD	-0.01	0.00	-1.81	0.07
		Relatedness x Trial type	0.02	0.33	0.05	0.96
f.	**Length of copulations** GLMM n = 70 obs. from 36 cages	**Intercept**	3.12	0.76	4.12	**<0.001**
		Relatedness	0.40	0.23	1.72	0.09
		Trial type	0.23	0.18	1.27	0.20
		Female mass	-0.70	1.51	-0.46	0.64
		SSD	0.57	0.49	1.15	0.25
		Relatedness x Trial type	-0.37	0.27	-1.35	0.18

Underlined values indicate $0.05 < P < 0.10$; **Bold values** indicate $P < 0.05$

Table 2. Summary of mixed models from the male choice experiment. The intercept for all models was set the mating performance in trials where all three butterflies were unrelated (Figure 1f). We modeled six measures of mating performance as a function of female relatedness (sibling vs. unrelated), trial type (mixed vs. same relatedness) and their interaction. We included both male mass and his sexual size dimorphism (SSD) with each female as additional factors. See Methods for details on random effect structure.

	Behavioral measure	**Fixed effects**	**Estimate**	**Std. Error**	**Test value**	***P value***
a.		Intercept	1.32	1.05	1.26	0.21
	Number of attempts	Relatedness	0.34	0.48	0.72	0.47
		Trial type	-0.52	0.39	-1.34	0.18
	GLMM	Male mass	-0.22	2.27	-0.10	0.92
	n = 52 obs. from 26 cages	SSD	-0.01	0.00	-1.12	0.26
		Relatedness x Trial type	0.06	0.52	0.11	0.91
b.		Intercept	2.26	1.80	1.26	0.21
	Attempt success rate	Relatedness	0.34	0.83	0.42	0.68
		Trial type	1.23	0.71	1.74	0.08
	GLMM	**Male mass**	-9.92	4.22	-2.35	**0.02**
	n = 43 obs. from 26 cages	SSD	0.01	0.01	0.71	0.48
		Relatedness x Trial type	-0.59	0.98	-0.60	0.55
c.		Intercept	1.32	0.93	1.42	0.16
	Length of attempts	**Relatedness**	1.42	0.36	3.91	**<0.001**
		Trial type	-0.14	0.36	-0.38	0.70
	GLMM	Male mass	-2.68	2.05	-1.31	0.19
	n = 169 obs. from 26 cages	SSD	0.88	0.55	1.58	0.11
		Relatedness x Trial type	-1.38	0.49	-2.80	**0.005**
d.		Intercept	0.58	1.55	0.38	0.71
	Probability of copulating	Relatedness	0.03	0.72	0.04	0.97
		Trial type	1.15	0.62	1.86	0.06
	GLMM	Male mass	-3.74	3.21	-1.16	0.24
	n = 104 obs. from 52 cages	SSD	0.01	0.01	0.68	0.50
		Relatedness x Trial type	-0.68	0.92	-0.74	0.46
e.		Intercept	0.03	1.01	0.02	0.98
	Number of copulations	Relatedness	0.01	0.52	0.02	0.98
		Trial type	0.81	0.40	2.02	**0.04**
	GLMM	Male mass	-2.67	2.13	-1.25	0.21
	n = 104 obs. from 52 cages	SSD	0.01	0.01	0.97	0.33
		Relatedness x Trial type	-0.52	0.64	-0.82	0.41
f.		**Intercept**	28.64	9.20	3.11	**0.006**
	Length of copulations	Relatedness	-5.97	4.58	-1.30	0.21
		Trial type	-1.84	3.64	-0.51	0.62
	LMM	Male mass	-25.47	22.73	-1.12	0.28
	n = 21 obs. from 14 cages	SSD	-3.15	6.83	-0.46	0.65
		Relatedness x Trial type	1.67	5.11	0.33	0.75

Underlined values indicate $0.05 < P < 0.10$; **Bold values** indicate $P < 0.05$

Figure captions

Figure 1. Experimental design. We conducted two experiments to test the role of relatedness on both female (a-c) and male (d-f) mate choice. For each experiment, there were three types of choice trials: mixed, all sibling, and all unrelated. See text for details. For all trial schematics (a-f), males are on top, and females are on bottom.

Figure 2. Results for the female choice experiment. For each panel (a-f), the x-axis refers to the trial type. In mixed relatedness trials (Figure 1a), females are presented simultaneously with one sibling male and one unrelated male. In same relatedness trials, females are presented with either two sibling males (Figure 1b) or two unrelated males (Figure 1c). Light points/bars indicate the mating performance when the female engaged with a sibling, and dark points/bars indicate the mating performance when she engaged with an unrelated male. The fractions on top of the bars of panel (d) indicate the number of males that copulated out of the total that were presented to the females. See Table 1 for mixed model results from each panel.

Figure 3. Results for the male choice experiment. For each panel (a-f), the x-axis refers to the trial type. In mixed relatedness trials (Figure 1d), males are presented simultaneously with one sibling female and one unrelated female. In same relatedness trials, males are presented with either two sibling females (Figure 1e) or two unrelated females (Figure 1f). Light points/bars indicate the mating performance when the male engaged a sibling, and dark points/bars indicate the mating performance when he engaged an unrelated female. The fractions on top of the bars of panel (d) indicate the number of females that copulated out of

the total that were presented to the males. See Table 2 for mixed model results from each panel.

Figures

Figure 1

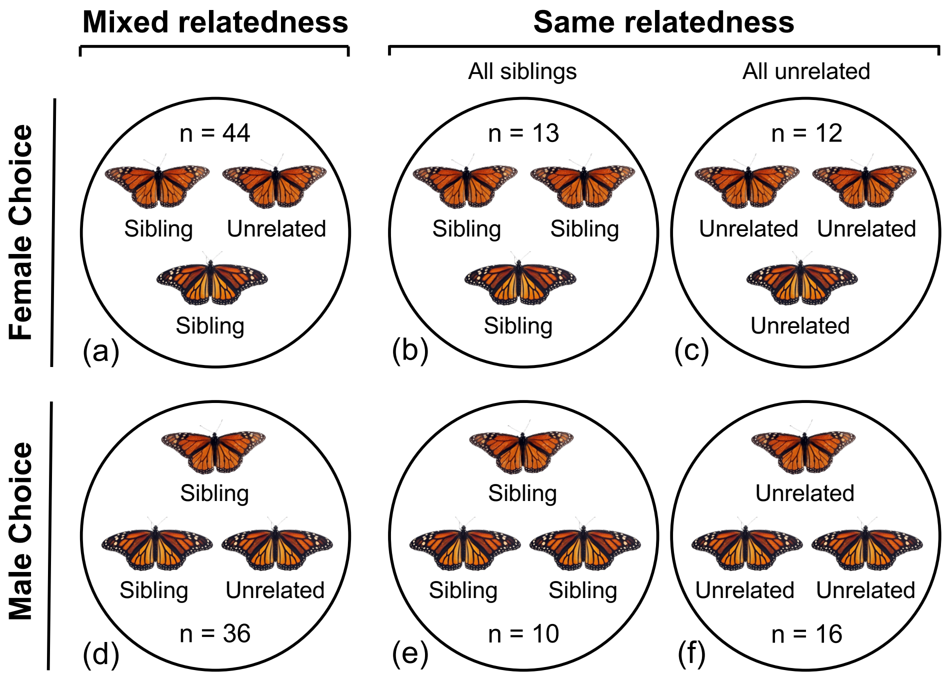


Figure 2

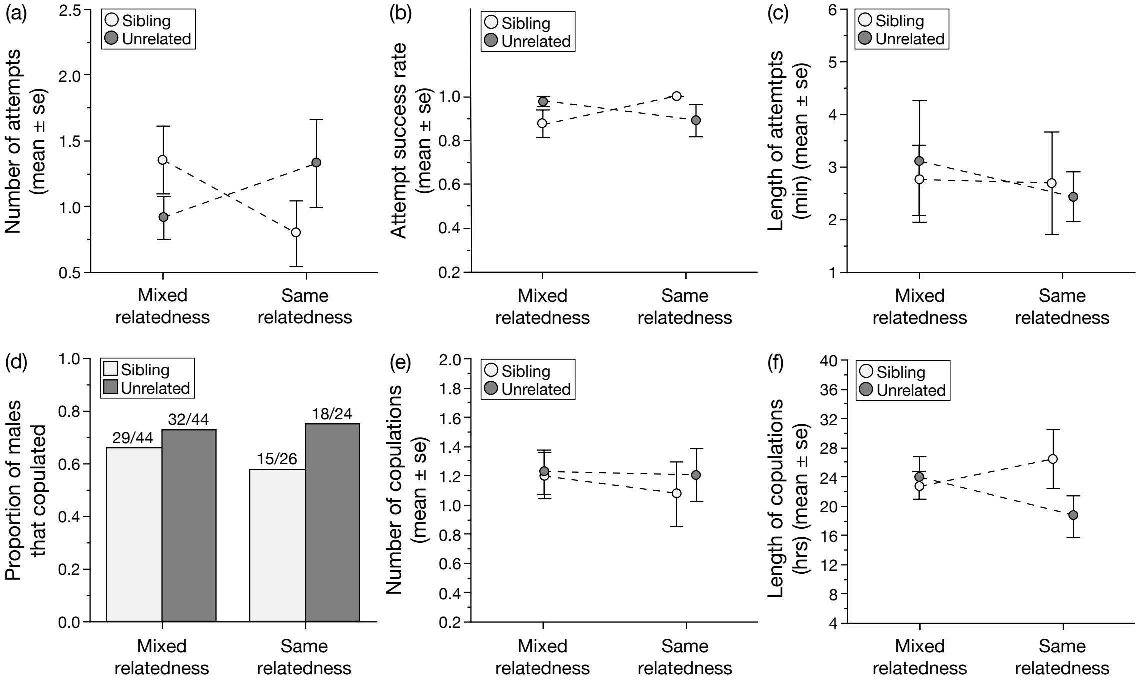
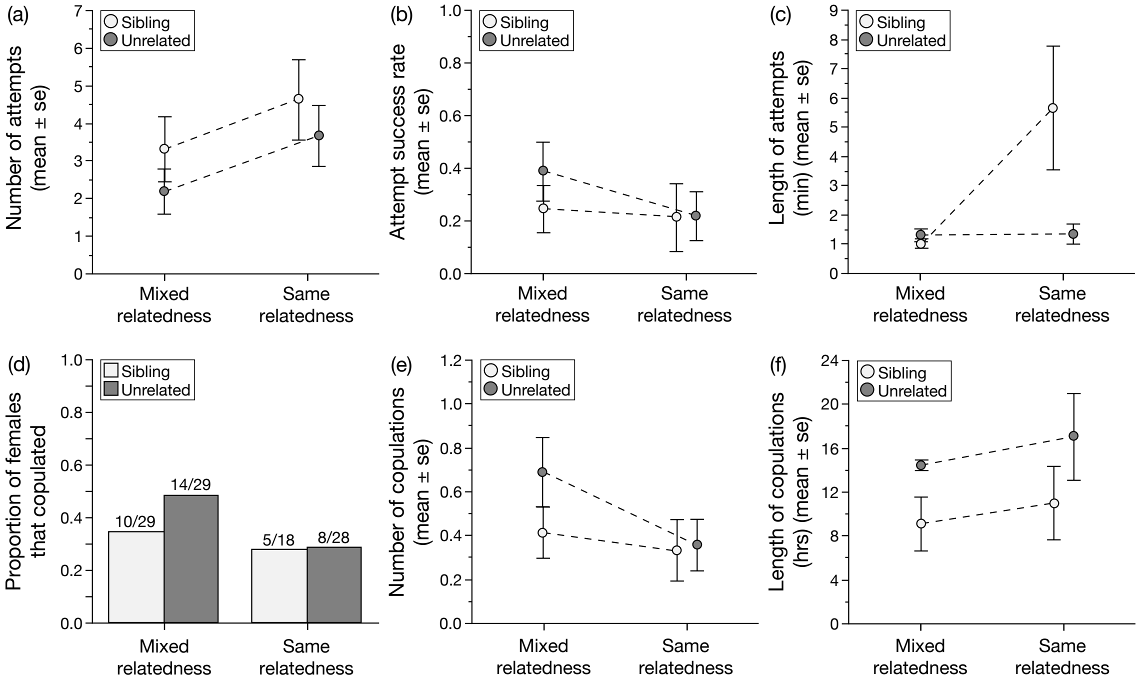

(a)
Number of attempts (mean ± se)
2.5
2.0
1.5
1.0
0.5
○ Sibling
● Unrelated
Mixed relatedness
Same relatedness
(b)
Attempt success rate (mean ± se)
1.0
0.8
0.6
0.4
0.2
○ Sibling
● Unrelated
Mixed relatedness
Same relatedness
(c)
Length of attemtpts (min) (mean ± se)
6
5
4
3
2
1
○ Sibling
● Unrelated
Mixed relatedness
Same relatedness
(d)
Proportion of males that copulated
1.0
0.8
0.6
0.4
0.2
0.0
□ Sibling
■ Unrelated
29/44
32/44
15/26
18/24
Mixed relatedness
Same relatedness
(e)
Number of copulations (mean ± se)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
○ Sibling
● Unrelated
Mixed relatedness
Same relatedness
(f)
Length of copulations (hrs) (mean ± se)
40
36
32
28
24
20
16
12
8
4
○ Sibling
● Unrelated
Mixed relatedness
Same relatedness

Figure 3


(a)
Number of attempts (mean ± se)
0
1
2
3
4
5
6
7
Sibling
Unrelated
Mixed relatedness
Same relatedness
(b)
Attempt success rate (mean ± se)
0.0
0.2
0.4
0.6
0.8
1.0
Sibling
Unrelated
Mixed relatedness
Same relatedness
(c)
Length of attempts (min) (mean ± se)
0
1
2
3
4
5
6
7
8
9
Sibling
Unrelated
Mixed relatedness
Same relatedness
(d)
Proportion of females that copulated
0.0
0.2
0.4
0.6
0.8
1.0
Sibling
Unrelated
10/29
14/29
5/18
8/28
Mixed relatedness
Same relatedness
(e)
Number of copulations (mean ± se)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Sibling
Unrelated
Mixed relatedness
Same relatedness
(f)
Length of copulations (hrs) (mean ± se)
0
4
8
12
16
20
24
Sibling
Unrelated
Mixed relatedness
Same relatedness