FACTORS DETERMINING HETEROSPECIFIC MATING SUCCESS

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IN THE SIBLING FRUIT FLY SPECIES

DROSOPHILA PSEUDOOBSCURA AND *D. PERSIMILIS*

By

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ABSTRACT

Patterns of heterospecific matings are important to understand in order to learn about speciation. I proposed a classical signal detection model as a representation of heterospecific mating patterns in both sexes. From the model I proposed that males would be able to achieve successive heterospecific mating success, as those males were more similar to conspecifics. I also proposed that restrictive females that rejected heterospecific matings would also be more likely to reject conspecific matings. I used the sibling species *Drosophila pseudoobscura* and *D. persimilis* to examine heterospecific mating patterns in males and females. I found that males previously accepted as heterospecific mates were more likely to achieve heterospecific matings than males that had previously been rejected as heterospecific mates. This was most likely due to decreased courtship by rejected males. Males rejected heterospecifically but mated conspecifically also were less likely to achieve heterospecific matings than males that had initially been accepted as heterospecific mates. This indicated that the same males were able to achieve successive heterospecific matings, despite rejected males also having had initial mating success. This was again likely influenced by decreased courtship in rejected males. Furthermore, I found that females that rejected heterospecific males were also more likely to reject conspecific males than naive females. There was also a non-significant trend of females that previously accepted heterospecific males were more likely to again accept heterospecific males than females that had previously rejected heterospecific males.

ACKNOWLEDGEMENTS

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INTRODUCTION

Examples of closely related, yet distinct, species exist in abundance. A prime example exists within *Drosophila*, with its thousands of species that are sexually isolated, even in sympatry (Dobzhansky, 1972). Sibling species are indistinguishable morphologically, but still recognized as discrete species due to sexual isolation. In contrast, individuals within other species can be highly polymorphic and persist in producing viable progeny. Biochemical and genetic analyses are commonly used to better illustrate the relationship between two individuals; other animals frequently make similar judgements with finely tuned biological machinery using multiple and overlapping cues, and occasionally heterospecifics are treated as conspecifics. The frequency with which heterospecific mating occurs is genetically variable, in both sexes (Carracedo & Casares, 1985; Jamart et al., 1993). Allopatric populations, which are separated by a geographical barrier, that later come again into sympatry, where a geographical barrier no longer exists, must have divergence in behavioural and ecological patterns in order for speciation or replacement to occur, if reproductive isolation has not yet been established.

The frequency of heterospecific mating could be a factor of male phenotype. Males that behave and appear similarly to males of a separate, closely related species could have higher heterospecific mating success than males that are more dissimilar to the closely related species (Fig. 1). The two curves represent the distribution of male phenotypes of two hypothetical populations, A on the left and B on the right. The overlap of the curves indicates that certain phenotypes are present in both populations. A female from population B rejects a male with a phenotype below a threshold phenotype, and is represented on the graph by all males to the left of the vertical line indicating decision criteria. This would imply that some males would repeatedly be able to achieve heterospecific matings, because their phenotype is sufficiently far enough along the scale to consistently pass threshold decision criteria. Alternatively, simply having mating experience may make a male more attractive to a female (Wade and Pruett-Jones, 1990). A female may detect olfactory cues from the male that has recently mated. Males that have experience courting may have their pheromonal profiles or behaviours altered after the acceptance or rejection as mates by females. The alteration in pheromonal profile would more likely be a change in composition as opposed to the absolute amount of hydrocarbons, as there has been found to be no link between amount of cuticular hydrocarbons and frequency of male heterospecific mating success between *Drosophila simulans* and *D. melanogaster* (Carracedo et al., 2003). Cuticular hydrocarbons from females signal mated status and deter male courtship (Ferveur, 2005). However, cues of previous mating experience in males can also be detrimental for future mating possibilities, as this decreases male mating success, and females show a preference for virgin males (Markow et al., 1978).

Heterospecific mating success can also be a consequence of female permissiveness. Female fruit flies exhibit a variation of preferences for male phenotypes, such as courtship songs, and often preferences are consistent within females (Isoherranen et al., 1999). Some females within the population may be more permissive than other females (Fig. 1). A distinction is made between permissiveness, which is the female

response to a heterospecific signal, and receptivity, which is the female response to a conspecific signal (Lynch et al., 2006). Restrictive females that reject heterospecific mating attempts may also be more likely to reject conspecific mating attempts, because of high threshold decision criteria.

The closely related species *D. pseudoobscura* and *D. persimilis* diverged approximately 1 million years previously (Wang and Hey, 1996), and are visually indistinguishable. Both allopatric and sympatric populations occur in the natural environment. The two species differ in their cuticular hydrocarbon profiles and courtship song patterns, and females use both cues to differentiate among males (Wallace & Dobzhansky, 1946). Males court females of various species indiscriminately initially, and learn to reduce their courtship intensity of heterospecific species (Dukas, submitted). Learning is mediated both by behaviour and cuticular pheromones (Siwicki et al., 2005). The species are reproductively isolated due to polymorphic chromosomal inversions coding for sterility; female hybrid progeny of the two species are fertile, while male progeny are sterile (Brown et al., 2004). Hybrid progeny are rarely found in the field, but mitochondrial DNA analyses of sympatric populations show commonalities while allopatric populations do not, an indication that gene flow does occur (Powell, 1983). However, heterospecific matings between female *D. pseudoobscura* and male *D. persimilis* occur more often in the laboratory than in the field. This is partially due to experimental protocol in the laboratory, which separates sexes within hours after eclosion, and subsequently tests flies that have had little to no experience with the opposite sex (Dukas, submitted). These conditions are quite different from those in the natural environment, where both sexes are free to interact with each other during maturation.

I used *D. pseudoobscura* and *D. persimilis* to examine heterospecific mating patterns of both males and females. First I investigated the subsequent heterospecific mating successes of males that were previously either accepted or rejected as heterospecific mates, in order to elucidate whether males were able to achieve consecutive heterospecific matings. I predicted that males previously accepted as heterospecific mates would have greater subsequent heterospecific mating success than males previously rejected as heterospecific mates. I also compared the heterospecific mating successes of males that previously mated heterospecifically to that of males rejected heterospecifically and mated conspecifically, in order to eliminate the possibility that previous mating experience by males played a role in heterospecific mating success. Again I predicted that males with heterospecific mating success would subsequently have greater heterospecific mating success than males that were rejected heterospecifically and then mated conspecifically. Female heterospecific mating patterns were first examined by comparing the heterospecific mating frequencies of females that had previously rejected heterospecific mates to naïve females. I predicted that females that rejected heterospecific mates would also reject conspecifics as mates with a greater frequency than naïve females. I also mated females either heterospecifically and conspecifically, and tested them five days later with heterospecific and conspecific males. I predicted that females initially mated with heterospecific males would exhibit a higher frequency of heterospecific rematings than females that initially rejected heterospecific males and

mated with conspecific males. I also predicted that the remating frequencies with conspecific males would be similar regardless of females' first mate.

GENERAL METHODS

I used allopatric populations of *D. persimilis* and *D. pseudoobscura* provided by the *Drosophila* Tucson Stock Center. *Drosophila pseudoobscura* were initially collected in Tucson, Arizona in 2004, and *D. persimilis* were initially collected on Santa Cruz Island, California in 2004. The flies were maintained in large population cages housed in distinct environmental chambers at temperatures of 21.5 -25°C and 70% humidity, on a 12-hour light dark cycle, with lights on at 1000 hours. Flies were fed standard fly medium, consisting of yeast, sucrose, dextrose, cornmeal, agar, methyl paraben, and ethanol (see Dukas in press for further details).

I collected virgin flies within eight hours of eclosion. Flies were anaesthetized with CO_2, separated by sex, placed in groups of 20 in 40 cm^3 vials each containing 5 cm^3 of standard fly medium and kept in the environmental chambers. One day before the start of an experiment, I moved males into individual vials containing standard food medium because such isolation dramatically increases male courtship and mating success (Noor 1996, Dukas unpublished data). I conducted experiments from approximately 0800 –1130 hours, which corresponded to dawn and early morning hours, the time period in which flies demonstrate maximum courtship and mating behaviour (Mayr, 1946; Dukas unpublished data). In all four experiments, the heterospecific pairings involved female *D. pseudoobscura* and male *D. persimilis*.

EXPERIMENT 1: THE HETEROSPECIFIC MATING SUCCESS OF MALES THAT WERE PREVIOUSLY EITHER ACCEPTED OR REJECTED BY HETEROSPECIFIC FEMALES

Methods

I predicted that males that were initially rejected as heterospecific mates would subsequently also have lower mating success than accepted males. One replicate was conducted per day, and replicates were performed on consecutive days, for a total of four replicates. Each replicate was planned to consist of 80 trials, for a total of 320 trials. However, due to the relatively rare occurrence of heterospecific mating, and subsequent difficulty in obtaining mated males, a total of 280 males were tested, 140 in each treatment condition.

Phase 1: Females were six days old on the day of the experiment, and males were four days old. A single heterospecific female was added to a vial already containing a single male. Pairs remained in vials for 30 minutes, after which the females were removed and the males left alone for approximately 20 minutes. About 190 vials per day were set up at intervals and observed for mating over a three-hour period in total. Due to the large number of rejected males, only a random selection was tested in phase 2.

Phase 2: A single male from phase 1 was placed in a fresh empty vial along with two naïve virgin heterospecific females. All vials were observed for 30 minutes, and

mating was recorded using a custom-designed computer program. In addition, randomly selected vials were observed for the first 15 minutes of the test phase, and courtship behaviour was recorded onto a computer. Courtship behaviour included following of the female, wing vibrations, and attempts to mount the female by the male. Courtship and mating behaviour observations allowed for the calculation of courtship and mating durations and latencies. Courtship proportions were calculated as the total amount of time spent courting by the male divided by the total amount of observation time before mating, as behavioural observations were ceased after mating completed. In trials that mating did not occur, the total time spent courting was divided by the length of the observation period, which was 15 minutes. The observer was blind to the identity of the males, as all vials were labelled using only numbers. The trials were in randomized order and counterbalanced. For all experiments, binary logistic regression was conducted on mating data, and ANOVAs conducted on log transformed mating latencies and arcsine square root transformed courtship proportions.

Results
Males that were previously accepted as heterospecific mates had significantly higher heterospecific mating frequencies in the test phase than males that were previously rejected by heterospecific females (logistic regression: Wald: χ_1^2=6.844, p= 0.009; Fig. 2a). The previously accepted males also spent significantly more time courting females in the test phase compared to the previously rejected males ($F_{1, 119}$= 21.807, p<0.001; Fig. 2b).

EXPERIMENT 2: MALES PREVIOUSLY EITHER ACCEPTED BY HETEROSPECIFIC FEMALES OR REJECTED BY HETEROSPECIFIC FEMALES AND MATED WITH CONSPECIFIC FEMALES

Methods
This experiment was similar to experiment 1, with the exception that in phase 1, males that were rejected were then mated with conspecific females. I compared heterospecific mating success of heterospecifically mated males to heterospecifically rejected, conspecifically mated males, in an attempt to eliminate reduced courtship by rejected males. I predicted that males that had previously mated heterospecifically would have a higher mating frequency than males that had been rejected heterospecifically and mated conspecifically. It was also predicted that males of both treatments would spend similar proportions of time courting, because in both treatments males had achieved mating success. The experiment was planned to consist of 660 trials, however, a total of only 520 trials were conducted, 260 per treatment, short again due to the infrequency of heterospecific mating.

Results
Males that were previously accepted by heterospecific females had significantly higher mating frequencies in the test phase than males that were previously rejected by heterospecific females and mated with conspecific females (logistic regression: Wald: χ_1^2

=6.379, p = 0.012; Fig. 3a). The males previously accepted by heterospecific females also spent more time courting heterospecific females compared to the previously rejected males that mated conspecifically ($F_{1, 171}$=5.268, p=0.023; Fig. 3b).

EXPERIMENT 3: REJECTING FEMALES VERSUS NAÏVE FEMALES

Methods

I predicted that female *D. pseudoobscura* that initially rejected male *D. persimilis* would have longer mating latencies and lower mating frequencies in the test phase than naïve female *D. pseudoobscura*. Females were again given the opportunity to mate heterospecifically, and females that rejected heterospecific males as mates were subsequently tested with conspecific males. Naïve females were also placed alone in vials, and then tested with conspecific males. Mating behaviour was recorded, and the protocol blind and counterbalanced. The experiment was planned to consist of four replicates, with 80 trials per replicate and 40 trials per treatment within a replicate, for a total of 640 trials; in actuality, 452 trials were conducted, due to high heterospecific mating rates in the first phase.

Results

Females that previously rejected heterospecific males had significantly lower mating frequencies with conspecific males than naïve females (logistic regression: Wald: χ_1^2= 4.451, p = 0.035; Fig. 4a). The conspecific males, however, spent similar amounts of time courting females of the two treatments ($F_{1, 54}$ = 0.087, p = 0.769; Fig. 4b).

EXPERIMENT 4: HETEROSPECIFICALLY MATED FEMALES VERSUS CONSPECIFICALLY MATED FEMALES

Methods

I predicted that females initially mated with heterospecific males, because of low threshold criteria, would exhibit a higher frequency of heterospecific rematings than females that initially rejected heterospecific males and mated with conspecific males. I also predicted that the remating frequencies with conspecific males would be similar regardless of females' first mate.

In this experiment, I gave four-day old females the opportunity to mate heterospecifically. Females that rejected heterospecific males were given the chance to mate with conspecific males. I transferred all females that mated into small cages and provided them with fresh food daily. When the females were nine-day old, I tested their remating propensity with either heterospecific or conspecific males. I had a total of four treatment conditions: (1) Females mated with heterospecific males and tested for remating with heterospecific males, (2) females mated with heterospecific males and tested for remating with conspecific males, (3) females mated with conspecific males and

tested for remating with heterospecific males, and (4) females mated with conspecific males and tested for remating with conspecific males.

Behavioural observations were conducted and analyzed in a similar manner to the previous experiments. I ran 314 out of a possible 320 trials.

Results

The interaction between the female's first mating and subsequent test mating was non-significant (logistic regression: Wald: $\chi_1^2 = 2.880$, p= 0.090; Fig. 5a). There was also no significant interaction between the proportion of time spent courting by males in phase 1 and phase 2 ($F_{1, 124}= 0.908$, p= 0.343; Fig. 5b).

DISCUSSION

The results reported in experiment 1 showed that males that were previously accepted as heterospecific mates achieved significantly more matings than males that were previously rejected as heterospecific mates. These results showed that the same males were able to achieve multiple successive heterospecific matings, supporting the possibility that some heterospecific males meet some female threshold criteria necessary for mating. Furthermore, the difference in mating success of males either previously accepted or rejected as heterospecific mates was accompanied by a difference in their subsequent courtship effort. Males that had previously been rejected subsequently courted significantly less than males that had previously been accepted as heterospecific mates. The males rejected in the first phase of the experiment could have learned to avoid heterospecific courtship, which resulted in a decrease in courtship behaviour in the second phase of the experiment, and therefore also a decrease in mating success. This is consistent with results found in *D. melanogaster* and *D. simulans*, where males also learned to reduce heterospecific courtship with experience (Dukas, 2004). Because the first phase of the experiment was only 30 minutes in length, it is possible that this was not a sufficient amount of time for learning to take place.

Another potential explanation for the variance in heterospecific mating success of males in experiment 1 was the possibility that females detected that the males had previously mated (Wade and Pruett-Jones, 1990). Experiment 2 sought to eliminate the possibility that females detected that males had previously been either accepted or rejected as mates, through olfactory cues and/or behavioural differences of the male, by mating rejected males with conspecific females in the first phase. Once again, males initially accepted as heterospecific mates had a significantly higher mating success than males initially rejected as heterospecific mates, even though rejected males had successfully mated with conspecific females. However, again males that had initially been rejected as heterospecific mates had significantly shorter courtship durations in the test phase than males that had initially been accepted. It was interesting that the positive reinforcement provided by the conspecific females in the first phase did not negate the effect on male behaviour of rejection by heterospecific females. The behaviour of *D. pseudoobscura* females may have acted as a fairly aversive stimulus, as females often hit

and kick males with their legs in a typical display of rejection (Brown, 1964). There was indication that the same males were able to achieve successive heterospecific matings and therefore more likely meet threshold criteria, however, the results may have been skewed by rejected males learning and subsequently decreasing courtship directed towards heterospecific females.

Experiment 3 showed that females that rejected heterospecific males were also more likely to reject conspecific males than naive females. There was no significant difference in courtship durations by the males between acceptor and rejector females. The results of this experiment showed that some females are consistently more permissive, while others are more restrictive in their mating criteria. This is similar to a fixed threshold model of mate choice strategy, wherein females sample males and mate with the first male to meet the threshold criteria (Jennions and Petrie, 1997), except applied to heterospecific mate choice as well. Experiment 4 showed a non-significant trend for the previous mating history of females influencing mating success in the test phase. Females that previously accepted heterospecific mates were more likely to later on accept heterospecific mates than females that originally rejected heterospecific mates. This occurred despite rejector females experiencing higher rates of courtship than acceptor females. The variation in permissiveness in females may be related to the variation in speed with which they reached sexual maturity. In crosses between *D. melanogaster* and *D. simulans*, the faster a female reached sexual maturity, the more likely the female was to hybridize (Carracedo and Casares, 1987; Carracedo et al. 1991).

Females vary in their abilities to discriminate among males (Wood & Ringo, 1980). Discrimination abilities are inherited in a dominant fashion, and this variance likely furthers speciation after reproductive isolation has occurred, with poor discriminators selected against (Ortiz-Barrientos et al., 2004). The model presented in Figure 1 plays a role in situations when the identity of a given male is ambiguous, and in this range females vary in the level of permissiveness with regards to the phenotypes of potential mates. The level of permissiveness was not simply a function of the discrimination ability of the female, as this would lead to the prediction that the superior discrimination ability of restrictive females would lead to greater receptivity with regards to conspecific males than naïve females. On the contrary, restrictive females were also less receptive when paired with conspecific mates (Fig. 4a).

It may be expected that the overall level of permissiveness would be lower in sympatric populations than in allopatric populations, due to reinforcement. Reinforcement theory proposes a mechanism for speciation, whereby two previously allopatric closely related species come into sympatry, and natural selection acts against hybrids to further diverge the species. The theory was first proposed as an alternative to the idea that physiological isolation between species is a byproduct of genetic divergence (Dobzhansky, 1940). Recent evidence concerning sexual isolation between *D. pseudoobscura* and *D. persimilis*, contrary to opposing reports (Noor, 1995; Lorch and Servedio, 2005; Noor and Ortíz-Barrientos, 2006), has suggested that sexual isolation between the two species is similar in sympatric and allopatric populations (Anderson and Kim, 2005), and therefore it is also possible that due to gene flow, overall levels of permissiveness do not differ between sympatric and allopatric populations.

Reinforcement is not a universal feature of sympatric populations (Coyne et al., 2002); it is possible that high levels of permissiveness were selected against and helped maintain sexual isolation in sympatric populations immediately after speciation, and that gene flow later eliminated differences in permissiveness between allopatric and sympatric populations. On the other hand, because the two species are so recently diverged, it is possible that reinforcement, that is natural selection against permissive females, has not yet had an effect. Therefore an experiment demonstrating differing levels of permissiveness in allopatric and sympatric populations would help in the understanding of speciation, while a negative result would not necessarily negate the effect of female permissiveness on speciation.

I have presented evidence that a female's heterospecific permissiveness is related to its conspecific receptivity. This may imply that permissiveness is related to other factors that affect female receptivity, such as the availability of males, and may vary not only between individuals, but also as a function of other such factors. The interaction between permissiveness and receptivity is another indication that the level of permissiveness may vary between allopatric and sympatric populations (Pfennig, 2000).

I have presented evidence that the same males are able to achieve successive heterospecific matings. It is unclear whether this characteristic was due to the variation in male phenotypes, or whether learning by rejected males was the key factor in the difference in heterospecific mating success between accepted and rejected males. Furthermore, I found that females vary in heterospecific permissiveness, and that permissiveness was linked to conspecific receptivity, providing support for threshold mating criteria determining heterospecific mating success. Additional investigations could elucidate the pattern of permissiveness when comparing allopatric and sympatric populations of fruit flies.
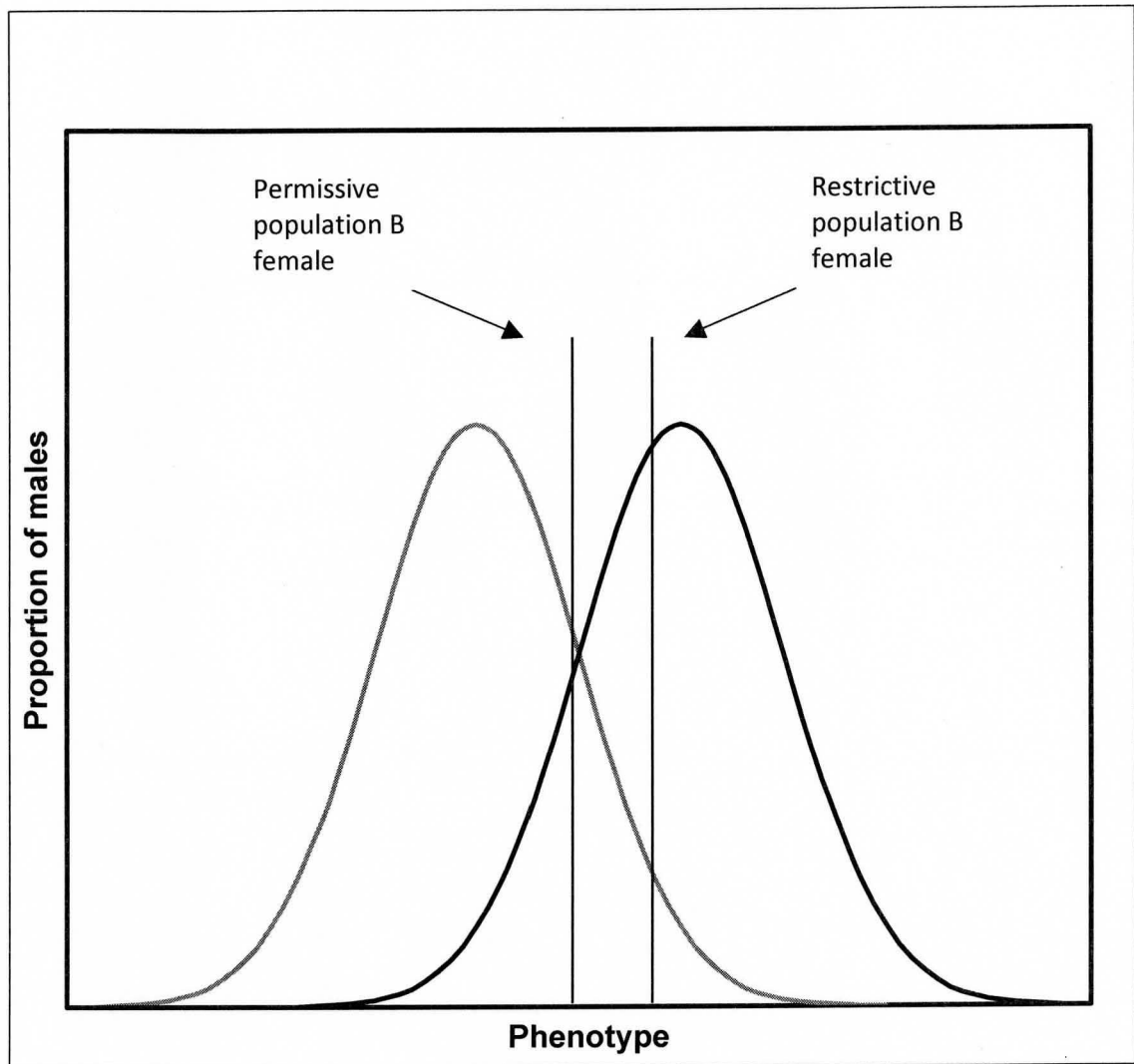
Fig.1: A representation of possible male phenotypes and their frequencies, from two hypothetical populations, A in gray and B in black. Solid vertical lines represent threshold mating criteria of females with varying levels of permissiveness.
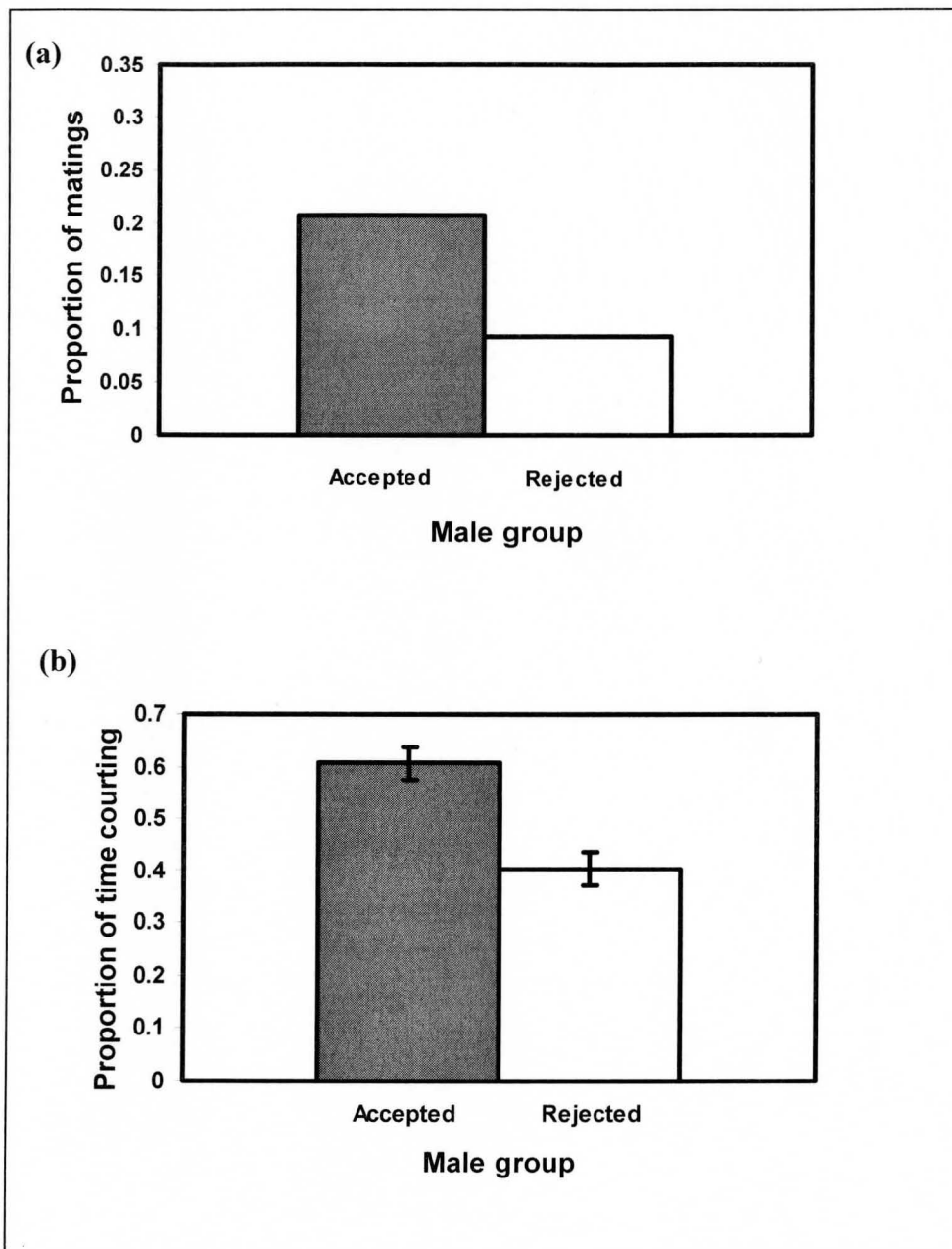
Fig. 2: (a) The heterospecific mating success of males that were previously either accepted (grey bar) or rejected (white bar) by heterospecific females (N= 280), and **(b)** the corresponding mean (±1 SE) proportion of time a subset of these males spent courting females (N= 120).
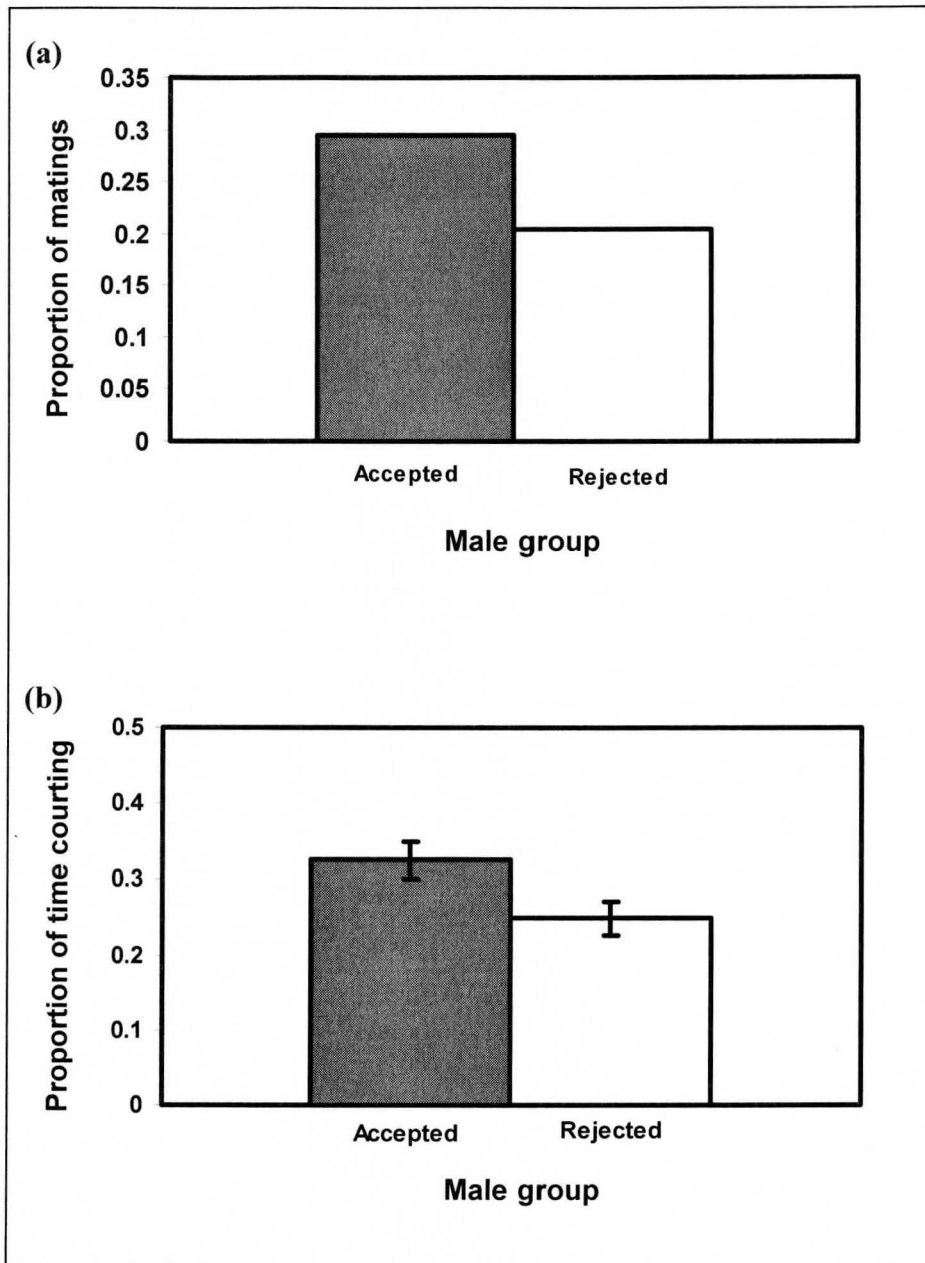
Fig. 3: (a) The proportion of accepted (grey bar) and rejected then mated conspecifically (white bar) males that mated heterospecifically in the test phase (N = 520). **(b)** The mean proportion of time spent courting females (±1 SE) by accepted (grey bar) and rejected (white bar) males (N= 172).
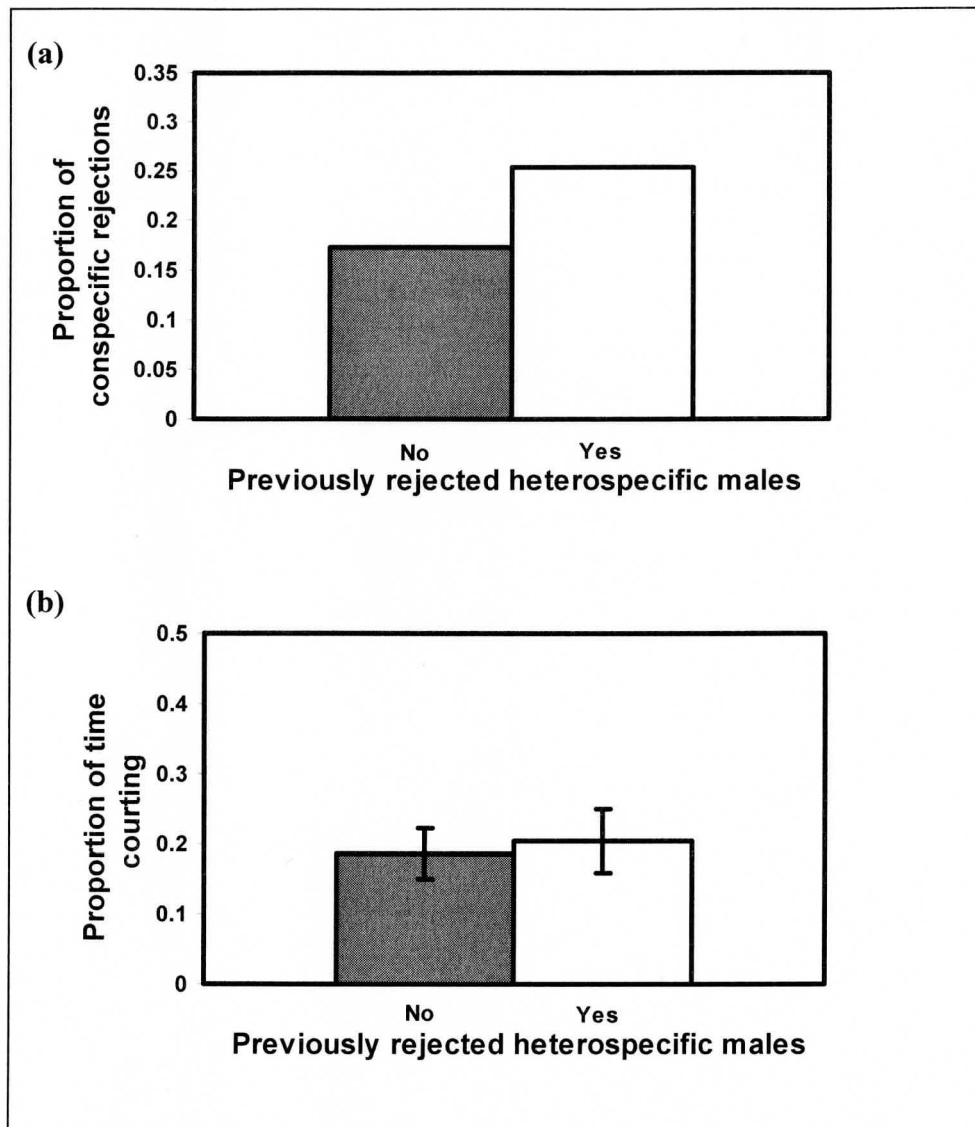
Fig. 4: (a) The proportion of conspecific matings by control females (grey bar) and females that previously rejected heterospecific males (white bar) (N= 452), and **(b)** the mean proportion of time (±1 SE) the conspecific males spent courting each female category (N=55).
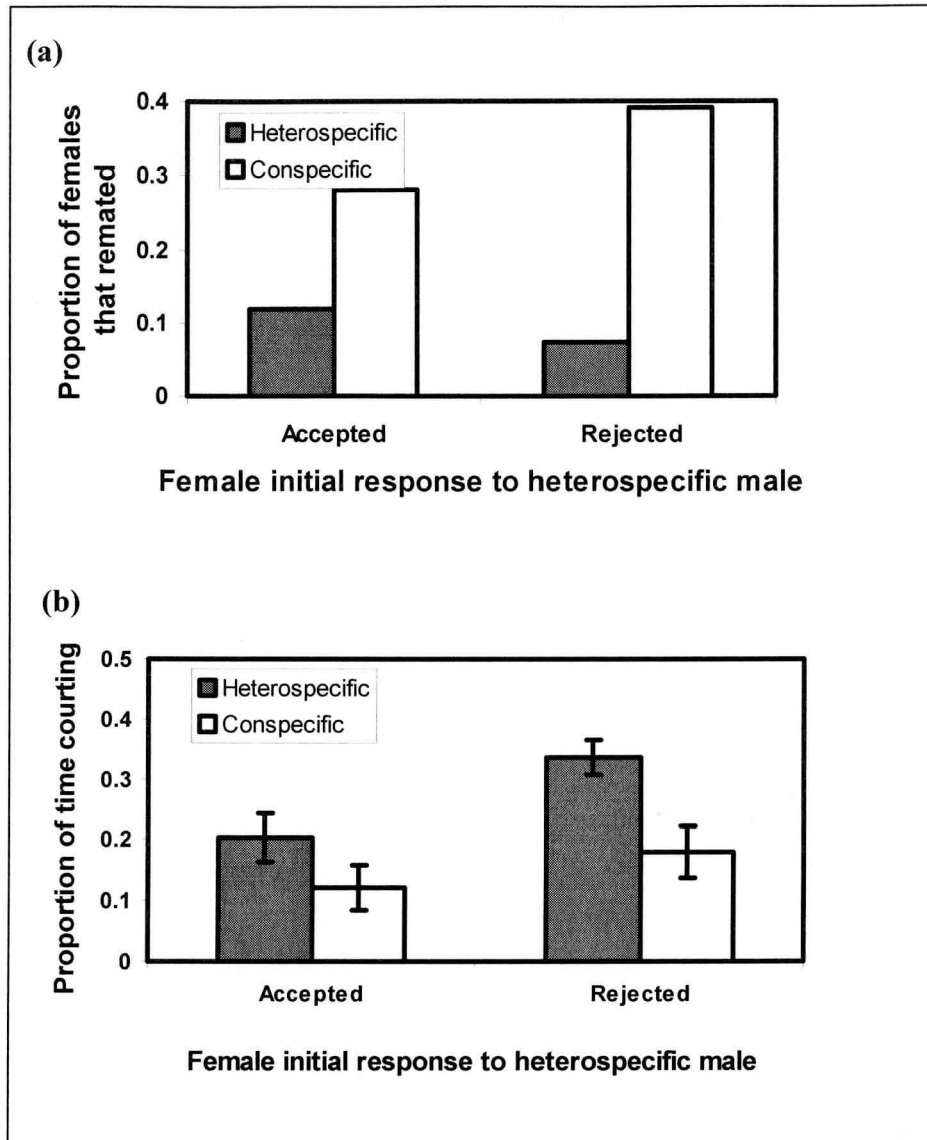
Fig. 5: **(a)** The proportion of heterospecifically (grey bar) and conspecifically mated (white bar) females in the test phase (N= 314). **(b)** The mean proportion of time (±1 SE) males spent courting heterospecifically mated (grey bar) and conspecifically mated (white bar) females (N= 125).

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Appendix A. Heterospecifically mated males versus virgin males

Experiment 5

Rationale

This was the first experiment conducted in the series. Its aim was to investigate whether males with heterospecific mating success were more likely to again achieve heterospecific mating success than naïve males. Males chosen as heterospecific mates may be better quality males overall, or they may more resemble (in physical appearance, behaviour, and/or pheromonal profile), the conspecific species than other heterospecific males. On the other hand, females may detect that a male had previously mated heterospecifically, and engage in mating with that male in a form of mate choice copying. However, results were obscured because of female preference for virgin males. Female *D. melanogaster* have the ability to discriminate between experienced and naïve males, and are less likely to mate with experienced males because of their immediate reduced fertility (Markow et al., 1978). Subsequent experiments that I conducted mated rejected males with conspecific females in an attempt to control for differences that might have been caused by rejection, and in the process also eliminated virgin males from being tested.

Methods

This experiment was similar in set up to experiment 1, with the exception that rejected males were not used in the test phase. Naïve males were used in place of the rejected males. Males were randomly selected to either be paired with a heterospecific female or remain naïve. I predicted that males recently mated with heterospecific females would have higher heterospecific mating frequencies than naïve, virgin males, because successful males would be more likely to pass female threshold decision criteria. Furthermore, successful males could have residual olfactory cues that signal them as attractive mates to females. It was also predicted that proportion of time spent courting by males in each of the treatments would be similar. The experiment consisted of four replicates; one replicate was conducted per day, and replicates were performed on consecutive days. Each replicate consisted of 60 trials. Males were randomly assigned to either the treatment condition or the control condition. A total of 240 males were tested, 120 males per condition.

Results

Males with experience mating with heterospecific females had similar heterospecific mating frequencies in the second phase compared to virgin males (logistic regression: Wald: $\chi_1^2 = 0.078$, p= 0.780; Fig. 6a). There was also no significant difference in courtship by the males in the second phase ($F_{1,60}= 0.356$, p=0.553; Fig. 6b).
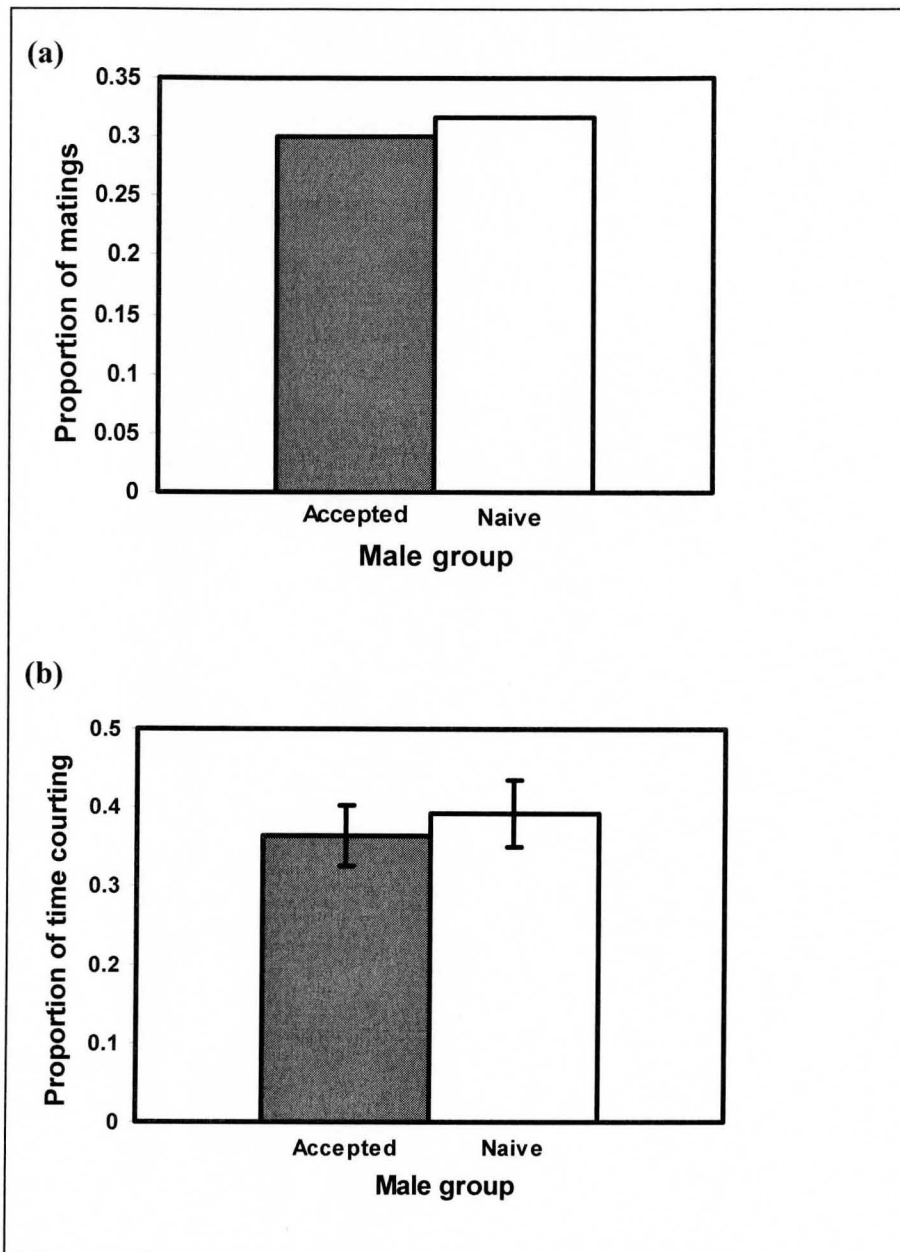
(a)

(b)

Fig. 6: (a) The heterospecific mating success of males that were previously either accepted by heterospecific females (grey bar) or naive (white bar) (N= 240), and **(b)** the corresponding mean (±1 SE) proportion of time a random subset of these males spent courting females (N= 60).

Discussion

This experiment did not support the hypothesis that some males have higher heterospecific mating success than others, by virtue of quality, phenotype, or mate choice copying. The lack of difference between the two treatment groups was most likely due to the preference of females for virgin males over experienced males. Males have temporary reduced fertility after multiple matings due to depletion of the accessory glands, and require approximately 3 hours to recover (Markow et al., 1978).

Appendix B. Heterospecifically mated males versus conspecifically mated males

Experiment 6

Rationale

The results of experiment 2 showed that previously heterospecifically mated males had greater mating success than males previously rejected heterospecifically. However, these results may have occurred because of some difference between males that were mated heterospecifically and males that were mated conspecifically, as opposed to a difference in males that were initially either accepted or rejected as heterospecific mates. Heterospecific females could have possibly been cued that a male had previously mated with a heterospecific or conspecific female, and some form of mate choice copying may have taken place. In order to investigate this further, males in this experiment were randomly selected to be paired with either a heterospecific or conspecific female in the first phase. Males that were rejected, either heterospecifically or conspecifically, were not tested.

Methods

This experiment was set up in a similar manner to the previous experiments. In one condition, randomly selected males were placed in vials with a heterospecific female. The second condition consisted of randomly selected males placed in vials with a conspecific female. If mating did not occur within 30 minutes, the vial was discarded. The experiment consisted of eight replicates in total. Two replicates were performed per day, over four consecutive days. Each replicate was planned to consist of 40 trials, for a total of 320 trials. However, due once again to the relatively rare occurrence of heterospecific mating, a total of only 292 trials were conducted, 146 per treatment.

Results

There was no significant difference in heterospecific mating success between males initially mated heterospecifically or conspecifically (logistic regression: Wald: $\chi_1^2 = 0.592$, p= 0.442; Fig.7a). There was also no significant difference in the proportion of time spent courting between males in the two treatment groups in the test phase ($F_{1,163} = 1.556$, p= 0.214; Fig.7b).
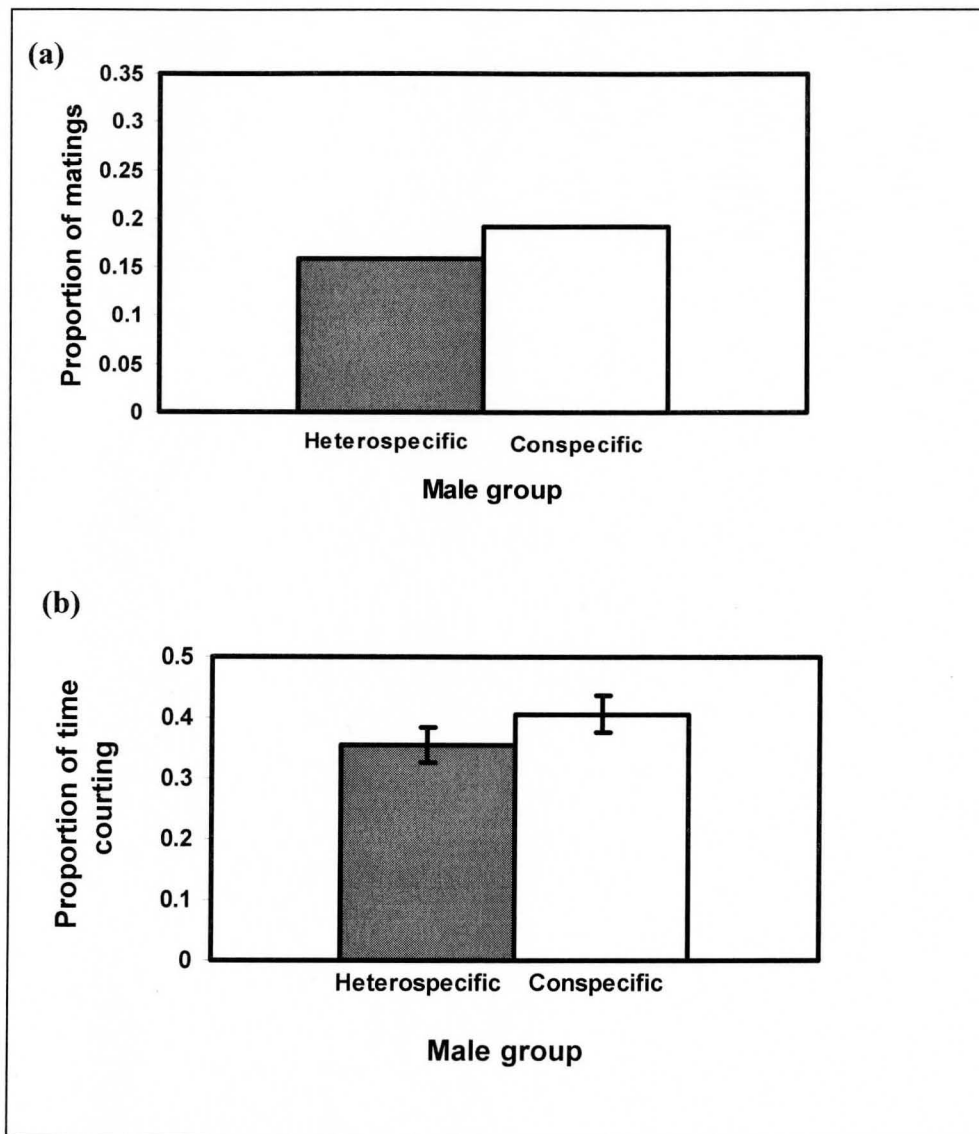
Fig. 7: (a) The heterospecific mating success of males that were previously either mated with a heterospecific (grey bar) or conspecific (white bar) female (N= 292), and **(b)** the corresponding mean (±1 SE) proportion of time a random subset of these males spent courting females (N= 164).

Discussion

The results from this experiment did not indicate a significant difference in heterospecific mating success between males previously mated with either heterospecific or conspecific females. These results suggested that male attractiveness did not change solely as a function of the species with which they had previously mated. In fact, although the difference was non-significant, males that had mated conspecifically had greater heterospecific mating success than males previously mated heterospecifically.