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Sexual conflict in the alfalfa leafcutting bee, *Megachile rotundata*, and the effect of the haplodiploid genetic system

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
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by

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For Mom

“It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change.”
-- Charles Darwin

“It’s time to move on. It’s time to get going. What lies ahead, I have no way of knowing.”
-- Tom Petty

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ABSTRACT OF THE DISSERTATION

Sexual conflict in the alfalfa leafcutting bee, *Megachile rotundata*,
and the effect of the haplodiploid genetic system

by

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Doctor of Philosophy in Biology
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Sexual conflict, when the evolutionary interests of males and females diverge, is a major force driving the evolution of males and females. Males evolve harmful traits that benefit males at the expense of female fitness. Females evolve counter-adaptations to reduce the harmful trait's effects, leading to a sexual arms race. However, this evolution can be influenced by the species' genetic system, the organization and transmission of genes. In this dissertation, I focus on the dynamics of sexual conflict and its origin in haplodiploid systems. The genetic system affects the likelihood that new traits invade and spread and should affect the sexual arms race in a species. Using a theoretical model, I simulated the appearance of a new harmful male trait and female resistance to that trait in different genetic systems and found that haplodiploid systems were more likely to be invaded by these male and female traits, suggesting that haplodiploid species may more often exhibit sexual arms races. Female resistance was also more effective in haplodiploid systems. The remainder of this dissertation focuses on the alfalfa leafcutting bee (*Megachile rotundata*). Males exhibit a form of sexual conflict called sexual harassment, where males coerce females to mate through repeated attempts. I measured the fecundity of females housed under various sex ratios, which affected harassment rates. Females harassed more produced fewer offspring, demonstrating the cost harassment imposes on female fecundity. I examined how harassment indirectly affects offspring by increasing females' production of early-emerging offspring that developed as adults that same season. These offspring have fewer foraging opportunities than overwintering offspring that develop and emerge the following season. I infer that harassment creates poor foraging conditions for females, so females produce more early-emerging offspring to make the "best of a bad situation." Finally, I examined the males' perspective, testing for male mate preferences through laboratory choice tests. Larger males attempted to mate more and had an unexpected preference for previously mated females over virgin females. I demonstrate the impact of a species' genetics on its evolution and behavior as well as the value of haplodiploid species as model systems for sexual conflict research.

CHAPTER 1

GENERAL INTRODUCTION

Sexual conflict has received much attention over the past few decades. Sexual conflict occurs when the genetic interests of males and females diverge (Arnqvist & Rowe 2005; Parker 2006). This differs from the more traditional view of sexual selection in which there is a more harmonious pairing of males and females. In the traditional view, males evolve traits that indicate benefits or qualities he will transmit to a female or her offspring, while females evolve preferences for those traits (Andersson 1994). Under sexual conflict, however, males evolve traits to exploit females and increase their own reproductive success at the expense of female fitness. Females are then expected to evolve counter-adaptations to reduce the costs imposed by males on female fitness, which then reduces male fitness. A sexual arms race, called sexually antagonistic coevolution, can result, with male traits and female resistance becoming quite elaborate and extreme.

For example, in the fruit fly *Drosophila melanogaster*, seminal substances of the male induces changes in female reproductive behavior and physiology to increase the reproductive success of the male (Chapman et al. 1995). However, the substances are somewhat toxic to females, and females that mate with multiple males suffer reduced longevity and other harmful effects. Females have evolved some resistance to neutralize these substances under certain circumstances (Rice 1996; Holland & Rice 1999), which puts selective pressure on males to evolve more effective toxins.

Many aspects of sexual conflict and sexually antagonistic coevolution have been studied both empirically and theoretically. This dissertation focuses on how the genetic system of a species can affect the origin and outcome of sexual conflict and on the dynamics of conflict in a haplodiploid model system, the alfalfa leafcutting bee (*Megachile rotundata*).

One form of sexual conflict is sexual coercion (Clutton-Brock & Parker 1995), where males use physical force to coerce females into mating. Females can pay costs as a result of coercive male behaviors through increased physical injuries, increased predation risks, decreased foraging efficiency, and energetic and time costs. Sexual harassment is one type of sexual coercion where males make repeated attempts to mate (Clutton-Brock & Parker 1995), and females can resist by fleeing from approaching males (Thornhill 1980) or avoiding areas where males are found (Stone 1995).

Table 1.1 - Definitions of the genetic systems and sex determination systems that are discussed.
Genetic systems
haploid - having one of each type of chromosome **diploid** - having a pair of each type of chromosome **haplodiploid** - females have a pair of each chromosome (i.e., diploid) and males have one of each chromosome (i.e., haploid) **Diploid mechanisms of sex determination**
XX/XY - sex chromosomes are X and Y; males are the heterogametic sex (i.e., XY)
ZZ/ZW - sex chromosomes are Z and W; females are the heterogametic sex (i.e., ZW)

Theoretical work has led to the suggestion that females may benefit from harmful male behavior indirectly by producing sons that inherit their mate's traits (Kokko et al. 2003; Parker 2006). For example, the most resistant females can only be subdued by the strongest, most aggressive males, and her sons would inherit the genes for strength and aggression.

Model systems are needed where direct measures of fitness (e.g., fecundity or longevity) can be quantified to assess the exact costs imposed on females by males. Individuals and their offspring need to be easily identified and monitored. If any possible indirect benefits of conflict to females are to be measured, the fitness of the offspring and grandoffspring must also be assessed, so that the indirect benefits females receive can be compared to the costs she pays directly.

The haplodiploid genetic system

To date, most research on sexual conflict has been on diploid species (Arnqvist & Rowe 2005). However, research has shown that the genetic system (Table 1.1), organization of genetic material and how it is transmitted (Bull 1983), can have a big impact on the evolutionary dynamics of a species. For example, haplodiploid systems with single-locus complimentary sex determination are more prone to extinction than diploid systems due to the production of sterile diploid males (Zayed & Packer 2005). Beneficial X-linked traits evolve faster than autosomal traits (Mank et al. 2007), and because the transmission of sex-linked genes in XX/XY sex determination systems is identical to the transmission of autosomal genes in haplodiploid genetic systems, this suggests the same would be true of traits in haplodiploids. Reeve & Pfennig (2003) used mathematical simulations to demonstrate how the genetic system can affect the likelihood of elaborate male secondary sexual characters invading a population and spreading.

The genetic system can also impact the way that females might receive indirect benefits of resistance. In haplodiploids, females develop from fertilized eggs and males from unfertilized eggs. Thus, it is not be possible for a female's sons to inherit strength and aggression from her mate because males have no fathers. Such traits can only be inherited from the males' grandfathers. Any indirect benefits females receive would have to be gained through fitter daughters or possibly grandoffspring, where grandsons could exhibit traits from her mate.

Therefore, haplodiploid females might have an advantage over males in a sexual arms race that diploid females do not. Within each patriline, any sex-limited male traits can only be expressed every other generation. If these male traits (i.e., coercion) are in conflict with female traits (e.g., resistance), the female trait should be able to spread through a population faster and be more of an impediment to the male trait.

In Chapter 2, I created two-locus, individual-based models and simulated the evolution of a harmful male trait and female resistance to that trait in haploid, haplodiploid, and diploid populations. These simulations suggested that the dynamics of sexual arms races may be very different in haplodiploid species. For example, harmful male traits can invade new populations more easily in haplodiploids than diploids. Interestingly however, haplodiploidy may also limit how far the conflict can escalate because female resistance traits will more readily evolve in response to increasingly harmful male traits. Haplodiploid study systems should provide new insights into the evolution of sexual conflict, and I illustrated their value by uncovering how sexual conflict affects female fecundity and offspring development in addition to how male mating behavior is not necessarily a frantic rush to mate with any and every female within sight.

Study species

The alfalfa leafcutting bee (*Megachile rotundata*) is a haplodiploid solitary bee (Figure 1.1). Their mating behavior had been previously unstudied, though anecdotal evidence suggested that males chase and harass females to mate and never court or entice females into mating (Gerber & Klostermeyer 1972). Females appeared to flee from and resist all mating attempts, even when they are virgins and need to mate at least once before they can begin producing daughters. If the male's behavior is true sexual

Figure 1.1 – A female alfalfa leafcutting bee.

harassment, there should impose a cost on female fitness. These male chases seemed like they should cost females quite a bit of time, having to flee from males while trying to forage and build nests.

The female's nest-building behavior provided an excellent opportunity to quantitatively measure the impact of harassment directly on a component of female fitness – fecundity. females build linear nests in pre-existing tunnels and cut leaves that are formed into individual cells (Gerber & Klostermeyer 1972; Richards 1984). In general, only one female builds cells in each tunnel at a time, so the owner of each tunnel (and thus the mother of all the eggs laid in that tunnel) can be identified. They are mass provisioning, so they gather nectar and pollen, place it in the cell, lay an egg, and seal off that cell to being work on the next. The cell and provisions therein represent the entire parental investment of the female. The amount provisioned for an egg can greatly affect its fitness (Gerber & Klostermeyer 1972; Klostermeyer et al. 1973), so any disruptions to a female's ability to forage and provision efficiently are important.

Cost of harassment

In Chapter 3, I measured the cost of sexual harassment to females' fecundity by placing males and females in large, outdoor cages at different male: female sex ratios (Figure 1.2 & 3.1, Table 3.1). I predicted that the sex ratios would affect the rate of harassment of females and females that were harassed more often would produce fewer offspring due to the disruptions of their foraging trips. Alfalfa leafcutting bees make an excellent system because the offspring production of each female can be tracked on a

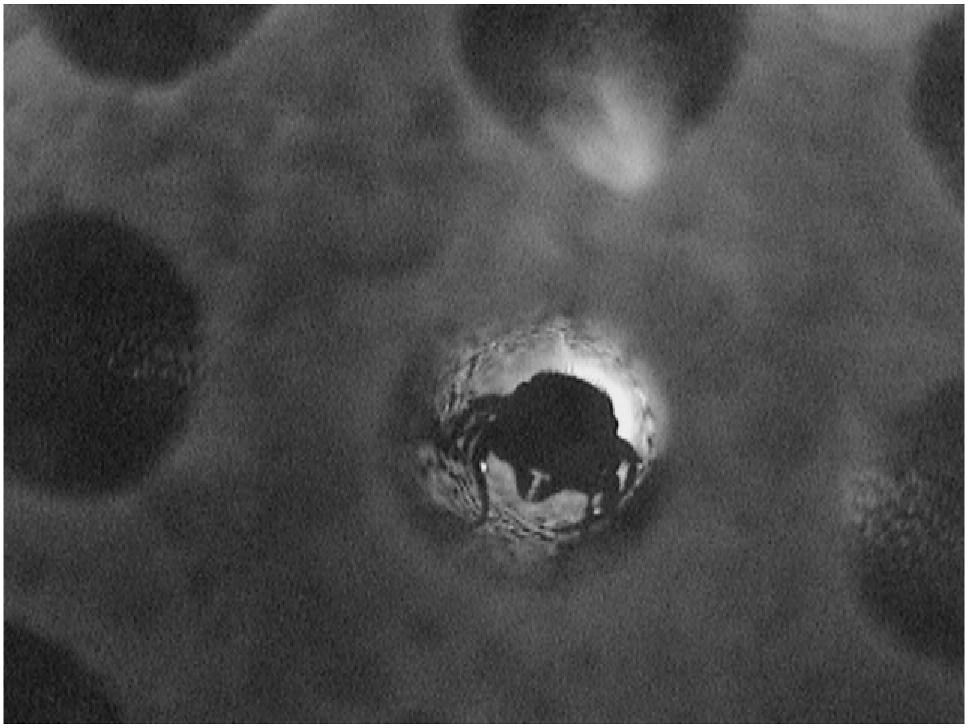

Figure 1.2 – A female alfalfa leafcutting bee sitting in a nest tunnel in an artificial, polystyrene nest block.

daily basis. Females that were harassed more frequently did, in fact, take longer foraging trips, which resulted in a slower rate of offspring production. Female fecundity was reduced by harassment by males.

Indirect effect of harassment on offspring type

In Chapter 4, I explored how harassment can also affect females indirectly by influencing their offspring's development. Most alfalfa leafcutting bee offspring develop from eggs into pre-pupae and then undergo diapause, overwintering as pre-pupae until the following season. However, many offspring instead continue to develop past the prepupal stage and emerge as adults that same season (Parker 1978; Tepedino & Parker

1988). These early emerging offspring typically do not do as well as their overwintering siblings because they emerge late in the season when floral resources are declining.

Research has focused on the proximate causes of early emerging offspring. The incidence of diapause appears to be maternally induced in response to environmental factors such as food availability, ambient temperature, and photoperiod (Parker & Tepedino 1982). Because harassment by males can indirectly affect food availability by limited females' access to floral resources, I tested whether early emerging offspring were produced to make the "best of a bad situation" or whether they were produced as extra, marginal offspring (Mock & Forbes 1995) when conditions were favorable. Because harassment can affect how favorable or unfavorable foraging conditions are for a female, I hypothesized that harassment would affect the proportion of offspring that did not overwinter and emerged early.

High rates of harassment created poor foraging conditions for females, as did high ambient temperatures and low floral resources. It was harder for females to forage efficiently, so they took longer to forage for leaves and nest provisions. As a result, the longer it took females to provision and seal off a cell, the more they risked losing the cell and provisions to predators, scavengers, desiccation, or other usurping females. Females made the best of this bad situation by laying an egg and sealing off these less provisioned cells, leading them to develop into early emerging offspring. Females in good situations (e.g., low harassment rates, high temperature, high floral resources) produced fewer early emerging offspring. Thus, females create early emerging offspring in response to poor foraging conditions.

Male mate preferences

The behavior of males in species with sexual coercion is often assumed to be scramble competition with indiscriminate attempts to mate with any and all females (or anything resembling a female, including other males). However, males often express mating preferences even when there is heavy competition for access to females.

In Chapter 5, I found surprising evidence that certain males prefer to mate with previously mated females over virgin females. I first paired virgin males with virgin females of various sizes and monitored their behavior for an hour. The body size of the male and female had an effect on their physical interactions and mating attempts. Next, I placed virgin males into the bottom of a Y-shaped choice arena where they were given a choice of two females, one a virgin and one mated. I measured the time males spent in close proximity to each female and found that larger males preferred mated females. This is unusual in that males should gain more by preferring virgins (e.g., lack of sperm competition, greater future reproductive potential). In addition, males tended to prefer larger females, and the time he spent near the virgin female depended on virgin and mated females' body sizes. These preferences should affect the interactions between males and females in the wild. Males are not simply patrolling the nest and foraging sites searching for any possible female to pounce on. Rather, males may preferentially shift their mating attempts to certain types of females. Females will not necessarily all receive the same level of attention from males, so certain females may have an advantage.

The purpose of this dissertation is to examine how sexual conflict can be different in a haplodiploid system and explore the dynamics of sexual conflict in an individual haplodiploid model system. I demonstrate how a species' genetic system can impact the appearance and evolution of conflict. I also highlight the value of using haplodiploid species as model systems. The effects of sexual conflict can be measured quantitatively, even indirect effects on offspring. In addition, the behavior of males cannot be ignored. While males certainly seem more zealous than females, they are not necessarily as indiscriminate as they might appear. There are many haplodiploid species exhibiting behavior that resembles sexual conflict waiting to be studied. My research should shed light on the benefits of using these species to answer questions about the evolution of sexual conflict.

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CHAPTER 2

THE ORIGIN AND EVOLUTION OF SEXUAL CONFLICT WITH A HAPLODIPLOID GENETIC SYSTEM

ABSTRACT

The organization of genetic material and how it is transmitted (i.e., a species' genetic system) may drive the direction of evolution. In particular, it can affect the likelihood of a new mutant allele successfully invading and spreading through a population. Our goal was to determine the impact this could have on sexual conflict and sexual arms races (a.k.a., sexually antagonistic coevolution). We created a two-locus, individual-based model to simulate the appearance of two new antagonistic traits: (1) a new aggressive, harmful male trait, the A allele at first locus, and (2) a new female resistance trait, the R allele at second locus. We simulated the appearance of a new A-bearing male or R-bearing female into a population and measured the change in its frequency at the end of 100 generations. Simulations revealed that these two traits were more likely to invade haplodiploid than diploid populations. In haplodiploids, the female resistance trait was more effective in preventing the invasion of male aggression, and the male aggression trait was less effective in preventing the invasion of female resistance. These results suggest that haplodiploid species may be more likely to be involved in sexual arms races and that haplodiploid females may be at more of an advantage (or less of a disadvantage) than their diploid counterparts.

This sets the stage for more complex simulations of a continuing sexual arms race with more elaborate traits appearing and evolving as well as empirical tests of our results with comparative studies of insect taxa.

INTRODUCTION

The organization of genetic material, how it is transmitted (Bull 1983), and mechanism of sex determination (Bull 1983; Cook 2002; Kraak & Pen 2002) can all significantly impact the evolution of a species. Haplodiploid systems, where males develop from unfertilized eggs and females from fertilized eggs, can evolve quite differently from diploid systems. For example, a positive benefit from haplodiploidy is reduced inbreeding depression over diploid ones (Henter 2003). However, theoretical work predicts that haplodiploid systems with single-locus complimentary sex determination are more prone to extinction due to the production of sterile diploid males (Zayed & Packer 2005). Haplodiploid genetic systems can also facilitate sexual conflict over sex allocation and brood sex ratios (Shuker et al. 2009).

In diploid systems, beneficial mutations (for either sex) on sex chromosomes are more efficiently fixed and evolve faster than traits on autosomal chromosomes (Mank et al. 2007). Because traits on sex chromosomes are transmitted identically to all autosomal traits in haplodiploid systems, the entire genome may evolve as if sex-linked. New mutant genes may, therefore, increase in frequency or reach fixation faster in haplodiploid systems.

The likelihood of a new trait successfully invading can also depend on the genetic system through protected invasion (Reeve 1993; Reeve & Shellman-Reeve 1997). In simulations of haplodiploid systems, rare alleles for female parental care were less likely to be lost than male parental care alleles (i.e., traits in diploid females were better protected from loss than those in the haploid males). Overall, female parental care alleles appeared less likely to be lost in haplodiploid systems than in diploid ones. The protected invasion hypothesis therefore predicts that haplodiploid systems are generally more invadable, with a bias towards gene fixation of female-expressed traits.

The protected invasion hypothesis was also applied to sexual selection to explain the bias in the prevalence of elaborate male secondary sexual traits between different taxa (Reeve & Pfennig 2003). Simulated male (i.e., XX/XY) and female heterogametic (i.e., ZZ/ZW) systems showed that rare alleles coding for male traits are better protected in ZZ/ZW systems (birds) than in XX/XY systems (mammals), and this protection is stronger in diploid than in haplodiploid systems. A comparative analysis, primarily focused on vertebrate taxa, found that males in ZZ/ZW systems evolved more secondary sexual traits than in XX/XY system (Reeve & Pfennig 2003). However, Mank et al. (2006) found no correlation between the genetic system of sex determination and male secondary sexual traits in actinopterygiian fishes, a clade that includes both ZZ/ZW and XX/XY species.

Sexual conflict occurs when males evolve adaptations to increase their mating success at the expense of female fitness (Arnqvist & Rowe 2005; Parker 2006). Male traits that harm or manipulate females can invade and spread in a population under certain conditions (Parker 1979). These male adaptations can be behavioral (e.g., sexual harassment: Chapter 3), morphological (e.g., grasping hooks: Arnqvist 1997), and chemical (e.g., "toxic" sperm: Holland & Rice 1999). Females evolve counter-adaptations to reduce these male-induced costs to their fitness, which can also be behavioral (e.g., male avoidance: Stone 1995), morphological (e.g., anti-grasping spines: Arnqvist et al. 1997), and chemical (e.g., "toxic" sperm resistance: Holland & Rice 1999). Unlike traditional sexual selection, sexual conflict is expected to result in a sexual arms race known as sexually antagonistic coevolution. Theoretical exploration of this arms race found that the probability of the harming allele spreading and avoiding extinction differed across XX/XY and ZZ/ZW systems (Andrés & Morrow 2003). This predicted difference strongly suggests that haplodiploidy would affect the evolutionary dynamics of sexual conflict.

We use a two-locus, individual-based model approach to determine how the genetic system can affect sexual conflict over mating by testing its impact on the spread of new antagonistic alleles at two loci. At the first locus, an aggressive male mating behavior allele (A) increases male fitness (e.g., increased mating success) but at the expense of female fitness (fecundity). At a second locus, a female resistance allele (R) increases successful female resistance to male aggression. Avoiding aggressive, A-bearing males maintains higher female fecundity by preferentially mating with non-aggressive males, but at a cost that a proportion of R-bearing females will not mate at all due to their avoidance behavior. This represents the cost of female resistance frequency observed in nature (Smuts & Smuts 1993; Olsson 1995; Darden & Croft 2008). Each of these traits was sex-limited, so only males expressed the aggression trait and females the resistance trait. Our simulations incorporated randomness to mimic a realistic environment. Females' mates were selected randomly based on the frequency of each male genotype, and offspring sex and genotype was randomly selected based on the probability of producing each sex and genotype.

Because genes in haplodiploids are more protected from loss and can evolve faster than diploid genes (Mank et al. 2007), we predict that the new male and female traits will invade and spread faster in haplodiploid than in diploid simulations.

A very interesting consequence of haplodiploidy is that a female's sons do not inherit genes from her mate. Thus, any advantageous (for males) trait in a female's mate can only be expressed in her grandsons. On the other hand, sex-limited female traits are potentially expressed every generation. Also because of haplodiploidy, all genes spend two-thirds of their time across generations in females rather than males. On the face of it, this suggests females have the advantage in the arms race between new male and female traits. Female traits should spread through a population faster and be more of an impediment to the male trait in haplodiploids than in diploids.

We tracked the fitness of given traits by measuring grandoffspring. The mean grandoffspring produced by males in populations with and without resistant females was compared as well as the change in grandoffspring produced by females in populations with and without aggressive males. We predicted the opposite sex's traits to have less of an impact on fitness in haplodiploid than diploid populations.

Finally, because sex ratio can vary widely in haplodiploid Hymenoptera (Bourke & Franks 1995; Crozier & Pamilo 1996) and in some diploid taxa (Werren et al. 1988), we compared the invasion probability of alleles within sex-biased populations.

METHODS

A single individual carrying a new mutant allele was introduced to a population of 2000 individuals with an approximate 1:1 sex ratio. We tracked the frequency of the A and R alleles over 100 nonoverlapping generations in haploid, haplodiploid, and diploid genetic systems. Either a new A-bearing male (A if haploid and Aa if diploid) was introduced to a population with some pre-existing frequency of the R allele, or a new R-bearing female was introduced to a population with some pre-existing frequency of the A allele. At the start of each trial, individuals were randomly assigned to be males or females based on the sex ratio to create some variation between trials. Separate simulations were conducted with the new mutant alleles (A and R) as completely dominant (CD; e.g., phenotype of heterozygote Aa individuals same as homozygous AA individuals) and semi-dominant (SD; e.g., phenotype of heterozygote Aa individuals is intermediate of homozygous AA and aa individuals). Heterozygous individuals in semi-dominant simulations expressed the intermediate of whichever allele is being discussed. Further mention of A- or R-bearing individuals refer to a phenotypic expression of the allele equivalent to that of a homozygous individual.

The mating advantage of A-bearing males, *m*, gave them a greater chance of mating with any given female (Table 2.1). To calculate the probability that an A-bearing male mated, we multiplied the frequency of that male's genotype by *m* and rescaled to the new maximum frequency using this equation (in a haploid system):

$$\frac{(\text{freq. of A-males}) * m}{(\text{freq. of A-males} * m) + (\text{freq. of a-males})} \tag{2.1}$$

For example, if *m* equaled 1.5 and the frequency of A-bearing males was 0.5, the probability of an A-bearing male would increase from 0.5 to 0.6. The cost to female fecundity, *c*, determined the new brood size of females that mated with an A-bearing male. For example, in our simulations females each produced 10 offspring. However, if *c* equaled 0.8, a female that mated with an A-bearing male would produce 80% of the offspring that she would otherwise produce, or 8 offspring.

The phenotypic effect of the female resistance trait R included the degree of avoidance, *v* (Table 2.1). This reduced the odds that an R-bearing female would mate with an A-bearing male. R-bearing females were more likely to mate with a-bearing males than r-bearing females were. To calculate the probability that an A-bearing male would mate with an R-bearing female, we used the following equation (in a haploid system):

$$\frac{\text{Equation 2.1 for A-males} * \text{v}}{(\text{Equation 2.1 for A-males} * \text{v}) + (\text{Equation 2.1 for a-males})} \tag{2.2}$$

For example, if *m* equaled 1.5, *v* equaled 0.6, and the proportion of A-bearing males was 0.5, then the probability of an A-bearing male mating with an r-bearing female was 0.6. However, his probability of mating with an R-bearing female dropped to 0.47.

All r-bearing females mated once. Some R-bearing females, however, would not mate at all. For example if the female cost of resistance (*f*) equaled 0.8, only 80% of the R-bearing females would mate.

The genotypes of the starting individuals were chosen randomly based on the starting frequencies of the A and R alleles. Male genotype frequencies were measured by calculating the proportion of males with each genotype. Weighted proportions were calculated based on the mating advantage (*m*) using equation (2.1). A second set of weighted proportions were calculated using equation (2.2) and were used when R-bearing females mated. Ten offspring were produced and their sexes each determined randomly based on a global 1:1 sex ratio. The genotype of each offspring was also randomly based on the probability of each genotype being produced from the combination of the parental genotypes. For example, if an AARr female mates with an AARR male, on average one-half of the offspring would be AARR and one-half would be AARr. The parents were then discarded. Offspring were then chosen at random to make up the 2000 individuals of the next generation, and the process was repeated for 100 total generations. All non-integer values for number of males, females, or offspring were rounded down the nearest whole number. Each trial was repeating using the same variables for 30 trials, and the means of these trials were used in our analyses.

In addition, we ran the same simulations under various starting frequencies of A and R for only two generations to calculate the average number of grandoffspring produced by each individual of each sex and genotype.

To assess the effect of sex ratio, we ran additional simulations using a 3:1 (male: female) male-biased sex ratio and a 1:3 female-biased sex ratio. For simulations with a new mutant A-bearing male, we used the following variables: $m = 3.0$, $c = 0.6$, $f = 1.0$, $v = 0.8$, initial frequency of R = 0.5. For simulations with a new mutant R-bearing female, the following variables were used: $m = 1.5$, $c = 0.6$, $f = 1.0$, $v = 0.6$, initial frequency of A = 0.5.

We tested the effects of the genetic systems and simulation variables using Analysis of Variance (ANOVA) in SPSS (Version 15, SPSS Inc., Chicago, IL, U.S.A.). We then conducted post hoc pairwise comparisons of the estimated marginal means of our five genetic systems. A Bonferroni correction was used to control the Type I error rate. Only results from the SD haplodiploid and diploid simulations are discussed below. Observed patterns between CD systems were similar to those observed between SD systems unless specified in the Appendix.

RESULTS

Results are summarized in Table 2.2.

New A-bearing mutant male

The probability of the A-allele successfully invading was significantly affected by the genetic system, the male mating advantage (m), and the cost of mating with an aggressive male (c), with m having the strongest effect (ANOVA: $F_{9,32} = 37.867$, $R^2 = 0.890$, $p < 0.0001$; Table 2.3). Post hoc pairwise comparisons of marginal genetic system means revealed that the mean probability of A-allele invasion was 0.297 in haplodiploids and 0.210 in diploids (ANOVA: $F_{1,32} = 9.617$, p = 0.0040). In the trials where the A-allele invaded, the final frequency of the allele after 100 generations was significantly affected by the two male trait variables (*m* and *c*), but not the genetic system (ANOVA: $F_{9,22} = 7.186$, $R^2 = 0.642$, p = 0.0001; Table 2.4). The male mating advantage, *m*, had the largest effect (Table 2.4).

The probability that the new A-allele successfully invaded populations where R-bearing females were present was significantly affected by the genetic system, all four simulation variables, and the initial frequency of the R-allele (ANOVA: $F_{14,741} = 650.502$, $R^2 = 0.923$, $p < 0.0001$; Table 2.5 & Figure 2.1). The genetic system had the largest effect followed by the male mating advantage, *m* (Table 2.5). Post hoc pairwise comparisons of genetic system means showed that the mean probability of invasion by the A-allele was 0.705 in haplodiploid simulations and 0.130 in diploid simulations (ANOVA: $F_{1,741} = 6597.832$, $p < 0.0001$).

Among simulations where the A-allele successfully invaded, the genetic system and only *m* affected the final frequency of the A-allele after 100 generations (ANOVA: $F_{14,542} = 2.437$, $R^2 = 0.028$, p = 0.0025; Table 2.6). The largest effects were due to the genetic system and the male mating advantage, *m* (Table 2.6). The mean frequency of the A-allele was 0.999 in haplodiploid simulations and 0.978 in diploids (ANOVA: $F_{1,542} = 14.465$, $p = 0.0002$).

We compared the coefficients from multiple regressions that we conducted on each genetic system's data using the four simulation variables and the initial frequency of the A-allele as main effects. To increase the chances that the A-allele went extinct by 1%, the initial frequency of the R-allele needed to increase by only 0.027 in haplodiploids, but in diploids the initial R-allele frequency needed to increase by 0.185. Thus, haplodiploid populations with R-bearing females were less likely to be invaded by A-bearing males than diploid populations with an identical proportion of R-bearing females.

New R-bearing mutant female

The probability that the new R-allele invaded populations with A-bearing males was higher in haplodiploid than diploid simulations and was also affected by the male mating advantage (m), the female cost of resistance (f), and the initial frequency of the A-allele (ANOVA: $F_{14,741} = 160.066$, $R^2 = 0.747$, $p < 0.0001$; Table 2.7 & Figure 2.2). The female cost of resistance, f, and the genetic system had the largest effects (Table 2.7). Pairwise comparisons of genetic system means showed that the probability that R-allele invaded was 0.294 in haplodiploids and 0.092 in diploids (ANOVA: $F_{1,741} = 264.282$, $p < 0.0001$).

The final frequency of the R-allele in simulations where the R-allele did not go extinct was affected by the genetic system, the male mating advantage variable (m), and the initial frequency of the A-allele (ANOVA: $F_{12,234} = 89.208$, $R^2 = 0.811$, $p < 0.0001$; Table 2.8). The largest effects were due to the genetic system and the male mating advantage, m (Table 2.8). The final frequency of the R-allele was 0.962 in haplodiploid simulations and 0.190 in diploid simulations (ANOVA: $F_{1,234} = 964.194$, $p < 0.0001$).

Multiple regressions conducted on each genetic system's data revealed that the A-allele was less effective in preventing the spread of the R-allele in haplodiploid simulations than in diploid simulations. To decrease the final frequency of the R-allele by 1% after 100 generations, the initial frequency of the A-allele had to increase by 0.09 in haplodiploids, but only 0.01 in diploids.

Differences in A- and R-alleles

In both haplodiploid and diploid simulations, the male allele was more likely to invade (Paired t-test: haplodiploid: $t = 19.178$, $p < 0.0001$; diploid: $t = 2.452$, $p = 0.0147$) and had a higher final frequency (Paired t-test: haplodiploid: $t = 3.697$, $p < 0.0001$; diploid: $t = 32.801$, $p < 0.0001$) than the female allele (Table 2.9).

Three simulation variables (m, c, and f) and the genetic system had a significant effect on the difference in the probability of invasion between the two alleles (ANOVA: $F_{13,742} = 85.946$, $R^2 = 0.594$, $p < 0.0001$; Table 2.10). The largest effects were due to the genetic system and the male mating advantage, m (Table 2.10). Pairwise comparisons revealed that the difference in the probability of invasion by each allele was greater in haplodiploids than diploids by 0.375 (ANOVA: $F_{1,742} = 404.436$, $p < 0.0001$). The difference in the final frequencies of A and R was significantly affected by the two mating probability variables (m and f) and the genetic system (ANOVA: $F_{12,187} = 104.915$, $R^2 = 0.862$, $p < 0.0001$; Table 2.11), and the difference in the final frequencies of the two alleles was greater in diploids by 0.831 (ANOVA: $F_{1,187} = 1044.076$, $p < 0.0001$). The largest effects were due to the genetic system and the male mating advantage, m (Table 2.11).

Grandoffspring of males with and without R-bearing females

We also calculated the difference in grandoffspring produced by males when the frequency of the R-allele was 0 and when it was 0.5 under identical conditions (i.e., same values for the simulation variables *m*, *c*, *f*, and *v*). The genetic system and two simulation variables (*c* and *f*) had a significant effect on these differences (ANOVA: $F_{13,364}$ = 144.329, $R^2 = 0.832$, $p < 0.0001$; Table 2.12). The largest effects were due to the female cost of resistance (*f*) and the genetic system (Table 2.12). Pairwise comparisons showed that males in diploid simulations lost an average of 12.02 more grandoffspring due to the presence of R-bearing females than males in haplodiploid simulations (ANOVA: $F_{1,1730}$ = 1917.296, $p < 0.0001$).

The mean difference in grandoffspring produced between homozygous A-males and homozygous a-males when the frequency of R was 0.5 was significantly affected by three simulation variables (*m*, *c*, and *f*) and the genetic system (ANOVA: $F_{13,364} = 98.803$, $R^2 = 0.771$, $p < 0.0001$; Table 2.13) and was greater in diploids than haplodiploids by 13.943 offspring (ANOVA: $F_{1,1730} = 405.268$, $p < 0.0001$). The largest effects were due to the male mating advantage, *m*, and the cost of mating with an A-male, *c* (Table 2.12).

Grandoffspring of females with and without A-bearing males

We calculated the difference in grandoffspring produced by females when the frequency of the A-allele was 0 and when the frequency was 0.5 under identical conditions. We found that the genetic system and all four simulation variables had a significant effect on these differences (ANOVA: $F_{13,364} = 388.125$, $R^2 = 0.930$, $p < 0.001$;

Table 2.14). The largest effects were due to the cost of mating with an A-male, *c*, and the female cost of resistance (*f*) (Table 2.14). Pairwise comparison of marginal genetic system means showed that females in diploid simulations lost an average of 5.02 more grandoffspring due to the presence of A-bearing males than females in haplodiploid simulation (ANOVA: $F_{1,1730} = 396.853$, $p < 0.0001$).

The difference in grandoffspring produced between homozygous R-females and homozygous r-females when the frequency of A was 0.5 was significantly affected by all four simulation variables and the genetic system (ANOVA: $F_{13,364} = 332.782$, $R^2 = 0.920$, $p < 0.0001$; Table 2.15) and was greater in haplodiploids than diploids by 3.326 offspring (ANOVA: $F_{1,1730} = 242.834$, $p < 0.0001$). The largest effects were due to the cost of mating with an A-male (*c*) and the female cost of resistance (*f*) (Table 2.15).

Sex ratio

In general, as the sex ratio moved from female- to male-biased, the probability of invasion by either allele (A or R) decreased in both genetic systems (Figure 2.3 & 2.4). In trials where A invaded, it went close to fixation, and there was no difference in the final frequency of A in the two genetic systems. However, the frequency of the R-allele was affected by the sex ratio and genetic system (ANOVA: $F_{3,111} = 23.426$, $R^2 = 0.371$, $p < 0.0001$; Figure 2.5). In haplodiploids, the final frequency of R was highest in female-biased and 1:1 sex ratios. In diploids, the highest frequency of R occurred in female-biased sex ratio, while the lowest frequency of R occurred in the 1:1 sex ratio.

DISCUSSION

Invasion by the male aggression and female resistance alleles appear more likely in haplodiploid than diploid populations. The male aggression trait, however, was less able to hinder the spread of female resistance with haplodiploids than with diploids. This suggests that haplodiploid species might be more likely to undergo a sexual arms race (i.e., sexually antagonistic coevolution) because female resistance can invade populations with aggressive male mating behaviors in place.

Female resistance reduced the probability of invasion by male aggression more with haplodiploidy than diploidy. Thus, if females in a haplodiploid species have evolved resistance to previous harmful male traits, that population is more resistant to invasion by new harmful male traits than in diploids. Overall, females in haplodiploid species may be more likely to 'win' a sexual arms races and have an evolutionary advantage over males (Arnqvist & Rowe 2002) than in diploid species.

In both genetic systems, the male trait was more likely to invade and spread faster to a higher frequency than the female trait. However, there was less of a difference in the probability of invasion in diploid simulations. Also, the difference in the final frequencies of the two alleles was actually greater in diploids than haplodiploids. This could be due to haplodiploid females being able to better control the spread of the male allele, keeping the frequencies closer together.

The probability of invasion and spread of the A-allele was most affected by the male mating advantage (*m*) and the genetic system. The invasion and spread of the R-allele was most affected by the male mating advantage (*m*), the female cost of resistance (*f*), and the genetic system. This again confirms the major influence that the genetic system has on the invasion and spread of the new alleles. It also is clear that the male mating advantage would affect the spread of the A-allele, but not necessarily that it could inhibit the spread of the R-allele. It is possible that, depending on the level of avoidance by R-bearing females, A-bearing males would still find themselves carrying the R-allele and their mating advantage would then help its spread.

The number of grandoffspring of males and females was increased more by the presence of the opposite sex's new allele (i.e., A or R) in haplodiploid systems. However, the male aggression allele appeared less effective at increasing male fitness in haplodiploid simulations, which supports the idea that haplodiploid females were better able to control the spread of the male allele. Likewise, the female resistance allele increased female fitness more in haplodiploid simulations. The cost of mating with A-bearing males (*c*) and, to a lesser extent, the female cost of resistance (*f*) had large effects on the effectiveness of the A- and R-alleles in increases the number of grandoffspring produced when the opposite sex's allele was present in the population. These variables both had the potential to decrease the number of offspring (and thus grandoffspring) that were produced, so it is not surprising they had the largest effects.

Sex ratio did have an impact on the invasion, and female-biased sex ratios seem much more likely to be invaded by both harmful and resistance alleles. Thus, species with female-biased sex ratios, with either genetic system, should be more likely to exhibit a sexual arms race. We found interesting results in the final frequencies of the female resistance allele (Figure 2.5). In diploid populations, the frequency of R was the lowest in cases where there was a 1:1 sex ratio. This would predict that males have their greatest advantage (or smallest disadvantage) in a sexual arms race when there is no sex-ratio bias. Females do better with biased sex ratios. In contrast to diploids, males in haplodiploid systems have their greatest advantage (or smallest disadvantage) with male-biased sex ratios.

In all sex ratios, haplodiploidy was more likely to be invaded with higher final frequencies of the female resistance allele, which suggests that the spread of these alleles is more likely in haplodiploids.

We modeled the female resistance behavior as an avoidance of A-bearing males. However, in many cases, females can resist males by counter-acting their tactics. For example, in *Drosophila melanogaster*, females can evolve resistance to the harmful effects of males' 'toxic sperm' (Rice 1996; Holland & Rice 1999). Females can mate with those males with reduced male-imposed costs. Resistant females in our model could reduce the mating advantage of A-males through avoidance, but could do nothing to reduce the fecundity cost when they did mate with A-males. It is unclear how successful a female trait that reduced the male's fecundity cost would be compared to our avoidance trait.

Our results should also apply to species with paternal genome loss (PGL, a.k.a., paternal genome elimination). Under most forms of PGL, sons and daughters both result from fertilized eggs, but the paternal genes are not transmitted or are inactivated in sons (Bull 1983; Herrick & Segar 1999). Thus the transmission of genes in PGL is functionally identical to that in haplodiploidy.

Our model treated the cost of resistance by females (*f*) as a constant. There are situations where avoidance of aggressive males could result in a constant cost. For example, if females resist by avoiding a safer foraging site with low predation where aggressive males are present, they instead forage at less safe sites with high predation. Females avoid the safer site regardless of how many aggressive males are there. As a result, the resistant females pay a cost in terms of higher predation risk that is independent of the frequency of aggressive males (e.g., Darden & Croft 2008). On the other hand, there would certainly be situations where cost would be a function of the frequency of aggressive males. For example, if the rate of mating attempts each female experiences is a function of the frequency of aggressive males and mating attempts increase the risk of predation, the frequency of aggressive males would thus positively affect the predation risk (e.g., Rowe 1994).

The female cost of resistance variable could also represent either the death of the female or a mating disadvantage and lack of mating. For a diploid organism with non-overlapping generations, these are essentially equivalent events. However, in haplodiploids, virgin females can and often do reproduce, producing only sons. Female traits that increase avoidance of aggressive males could still be passed on by unmated females through sons. This is an additional advantage to haplodiploid females over diploid females that was not incorporated into our model. Future simulations will include these additional complexities.

A comparative analysis of sexual conflict and sexually antagonistic coevolution and their relationship to genetic system in taxa with a diversity set of genetic systems would be informative. Diploidy, haplodiploidy and PGL are common throughout many insect taxa (Bull 1983; Normark 2003) making them the ideal study group.

There are several insect taxa with mixed genetic systems with cyclic or facultative switches between haplodiploid/diploid systems and those without males (i.e., thelytoky) (Normark 2003). Species where males are relatively new would be excellent systems to test predictions. In addition, in species with single-generation cycling between haplodiploidy and thelytoky, males should be at an even greater disadvantage in a sexual arms race, which could be tested both empirically and theoretically.

Many insects have short generation times, making longterm evolutionary studies possible. Predictions on the influence of sex ratio on sexual arms races could be tested in taxa with a wide variety of sex ratios such as the haplodiploid Hymenoptera (Bourke & Franks 1995; Crozier & Pamilo 1996) and the diploid genus *Drosophila*, which has genetic sex ratio distorters (Werren et al. 1988; Curtsinger 1991). Using a system with short generation times such as *Drosophila*, one could even manipulate the sex ratios and measure changes in the sexual arms race.

APPENDIX

Analyses were repeated using all three genetic systems (haploid, haplodiploid, and diploid) and both the SD and CD versions of haplodiploid and diploid. Only relationships between haplodiploid and diploid systems that deviated from previously reported patterns are discussed. When the dominance of a haplodiploid or diploid system is not specified, both versions are being discussed together because they are not significantly different.

New A-bearing mutant male

In populations with or without R-bearing females, the probability of invasions in diploid (CD) simulations was not significantly different from haplodiploids and haploids. This could be explained by the fact that the phenotypic expression of traits in the CD diploid system resembles haploidy because individuals only need an allele on one of their two chromosomes to express the trait.

New R-bearing mutant female

The probability of invasion by the new R-allele was higher in haploid than all other genetic systems (ANOVA: $F_{4,1880} = 128.45$, $p < 0.0001$). Any female with the R-allele immediately expressed it in haploids and would pass the R-allele to 100% of their offspring. Diploid (CD) females would also express the resistance trait with only one allele, but there would only be a 50% chance that a heterozygote would pass on the R-allele to each offspring. The final frequency of the R-allele was highest in haplodiploid and lowest in diploid systems (CD and SD) (ANOVA: $F_{4,672} = 514.17$, $p < 0.0001$).

Grandoffspring of males with and without R-bearing females

The difference in grandoffspring by males when the frequency of R was 0 and when it was 0.5 was lowest in haploid systems (ANOVA: $F_{4,4366} = 1745.35$, $p < 0.0001$).

The presence of resistant females hurt haploid males the least possibly because male aggression traits were more easily expressed. Haploids were possibly hurt less than CD haplodiploid and diploids because in haploid simulations there were lower proportions of females expressing resistance. For example, in a haploid population with R equal to 0.5, the proportion of resistant females is 0.5. In a diploid (CD) population, it was 0.75, so those males are more likely to encounter resistant females immediately expressed.

The difference between grandoffspring produced by homozygous A-males and homozygous a-males was still greater in diploid (SD) than haplodiploid systems by 13.94 grandoffspring, but it was greater in haplodiploid than diploid (CD) systems by 27.75 grandoffspring (ANOVA: $F_{4,4366} = 428.79$, $p < 0.0001$). The difference in diploids (SD) was greater than diploids (CD) by 41.70 grandoffspring.

Grandoffspring of females with and without A-bearing males

The difference between grandoffspring produced by homozygous R-females and homozygous r-females in haploid simulations did not significantly differ from that in diploid (CD) simulations. Again, because heterozygous females express their resistance to the same degree that a homozygous female does, diploid (CD) simulations act similarly to haploid ones.

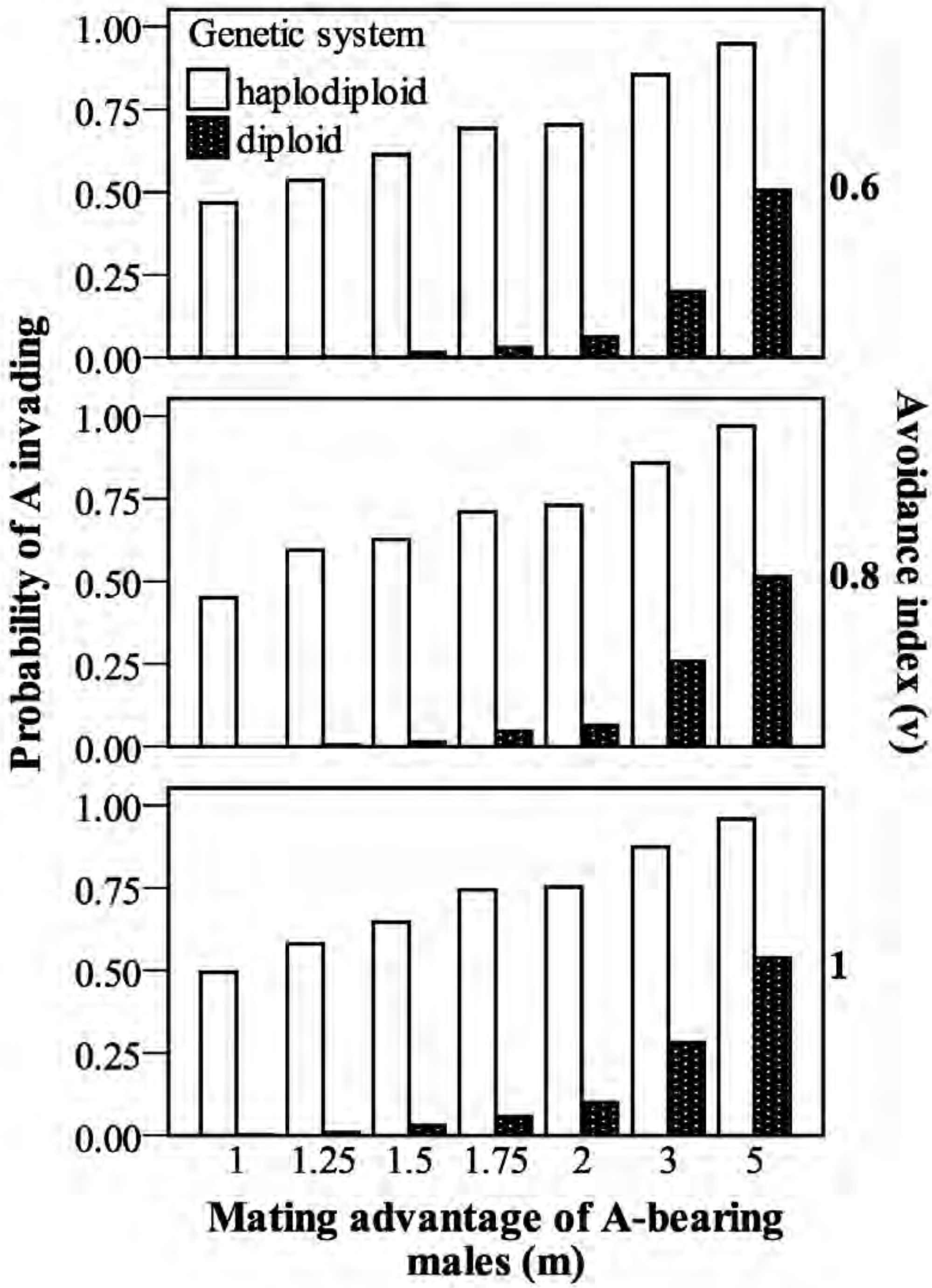

Figure 2.1 – The A-allele is more likely to invade haplodiploid systems than diploid systems. The avoidance index has a small effect on the invasion probability (Table 2.5).

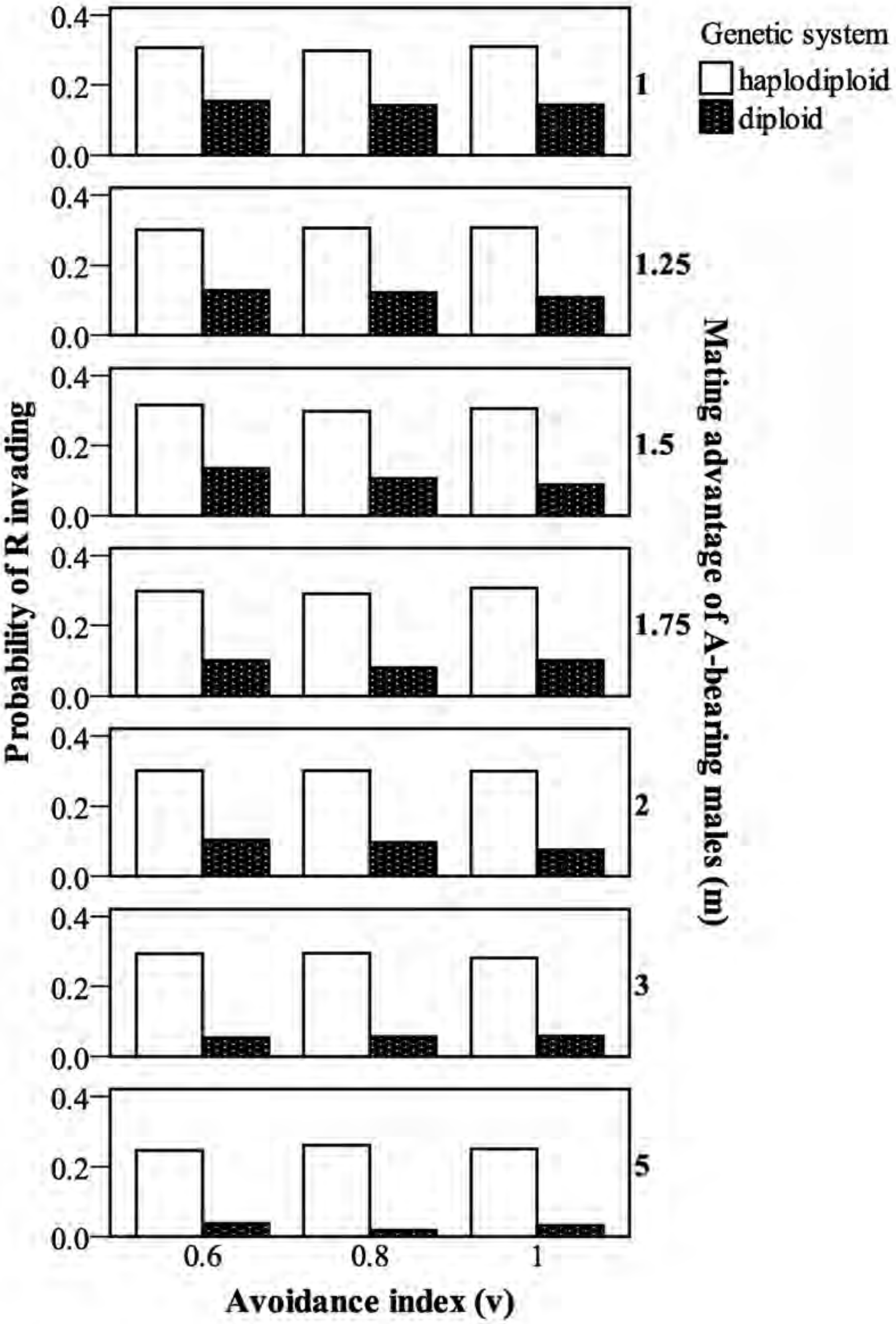

Figure 2.2 – As the mating advantage to A-bearing males increases, the probability of invasion by the R-allele decreases at all levels of avoidance and genetic systems.

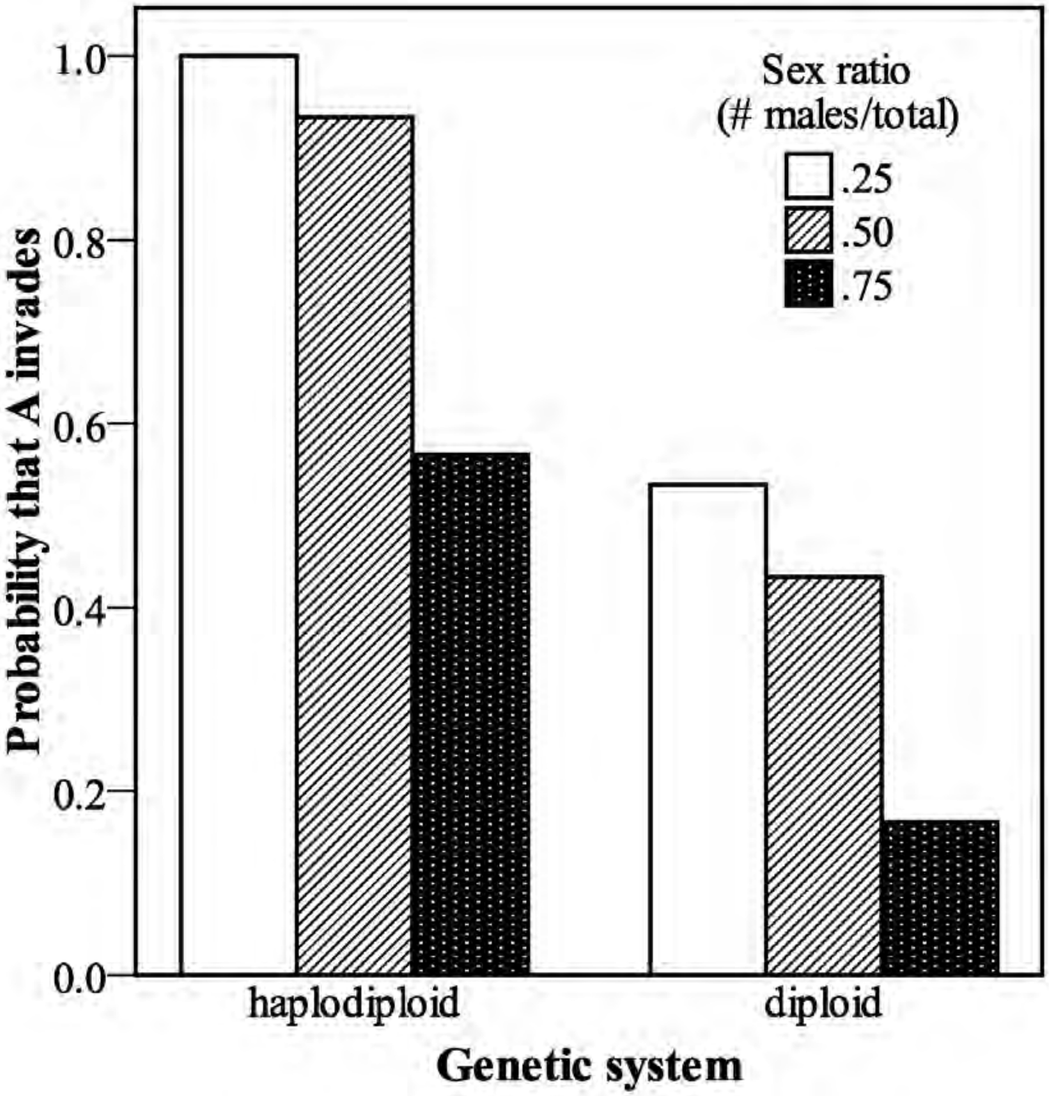

Figure 2.3 – The probability of invasion by the A-allele increases as populations become more female-biased. Also, A is more likely to invade haplodiploid than diploid populations.

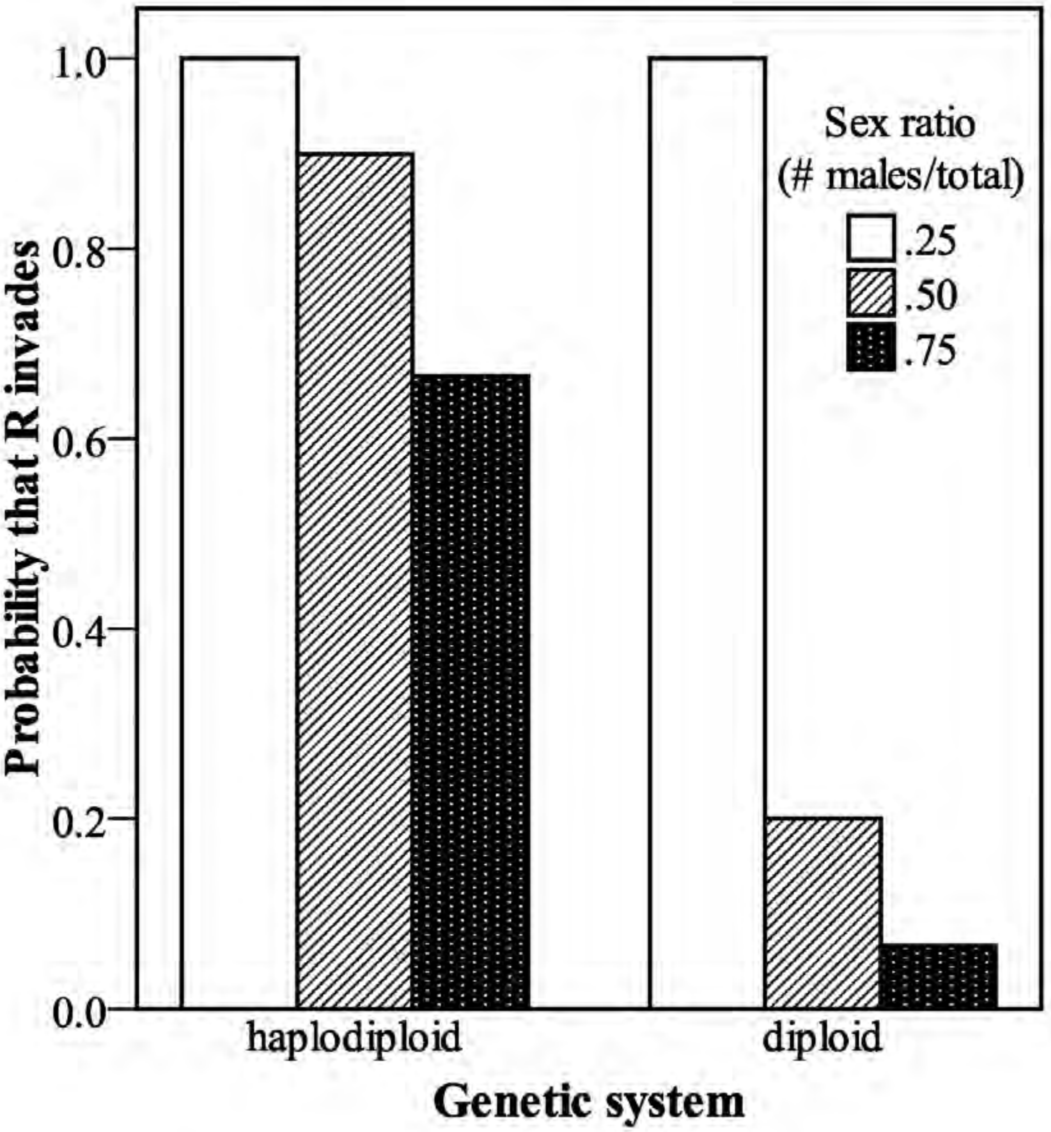

Figure 2.4 – The probability of invasion by the R-allele increases as populations become more female-biased. Also, R is more likely to invade haplodiploid than diploid populations.

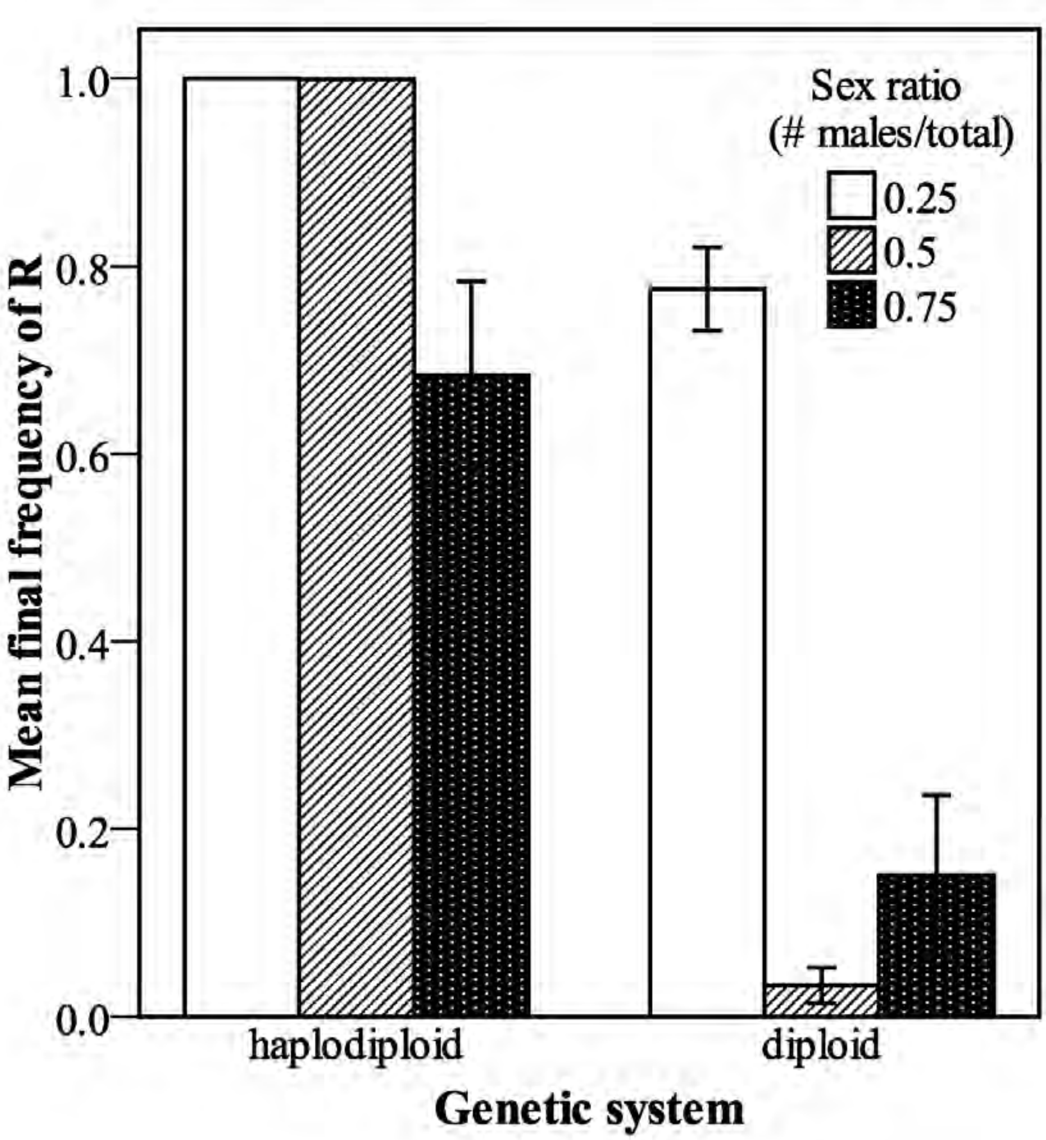

Figure 2.5 – The mean final frequency of the R-allele after 100 generations is affected by sex ratio and genetic system. In haplodiploids, male-biased sex ratios produce the lowest frequency of R. In diploids, the lowest R frequency is found in the 1:1 sex ratios. Error bars represent ± SE.

Table 2.1 - Descriptions of the four simulation variables used. The values of these variables affected the phenotypic expression of male aggressive behavior (A) and female resistance (R).

Variable name	Symbol	Range	Values used	Description
Male trait variables				
mating advantage	*m*	≥ 1	1, 1.25, 1.5, 1.75, 1, 2, 3, 5	The mating advantage of an A-bearing male. Increased the probability of a male of that genotype mating.
cost to females	*c*	0 - 1	0.6, 0.8, 1	The cost paid by a female that mates with an A-bearing male. The number of offspring she produced was calculated as 10 * *c*.
Female trait variables				
cost of resistance	*f*	0 - 1	0.6, 0.8, 1	The cost of resistance for an R-bearing female. Decreased the probability of mating.
avoidance index	*v*	0 - 1	0.6, 0.8, 1	Degree that R-bearing females avoid mating with A-bearing males. The frequency of A-bearing males was multiplied by *v* and rescaled.

Table 2.2 - A summary of the comparisons that were made and the direction of the differences between the haplodiploid (HD) and diploid (D) genetic systems.

Comparison	Outcome
Male aggression (A)	
Probability of invasion	higher in HD
Final frequency	higher in HD*
Grandoffspring lost by females due to presence of A-allele	fewer grandoffspring lost in HD
Difference in grandoffspring between A- and a-males	higher in HD
Female resistance (R)	
Probability of invasion	higher in HD
Final frequency	higher in HD
Grandoffspring lost by males due to presence of R-allele	fewer grandoffspring lost in HD
Difference in grandoffspring between R- and r-females	higher in HD
Differences in A- and R-alleles	
Most likely to invade	A-allele in both genetic systems
Difference in probability of invasion	greater difference between A's and R's probability of invasion in HD
Sex ratio as it moves from female- to male-biased	
Probability of invasion of either allele	decreases in both genetic systems
Final frequency of A-allele	NS in both genetic systems
Final frequency of R-allele	decreases in HD; highest at 1:1 in D

* A is close to fixation in both genetic systems

Table 2.3 - ANOVA showing the effect of the genetic system and male trait variables on the probability of invasion by the A-allele in populations without any R-bearing females ($R^2 = 0.890$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.914	2.836	9	0.315	37.867	< 0.0001		
Intercept	0.910	2.692	1	2.692	323.500	< 0.0001		0.89
m	0.907	2.603	6	0.434	52.138	< 0.0001	1	-0.77
							1.25	-0.69
							1.5	-0.67
							1.75	-0.64
							2	-0.53
							3	-0.33
							5	0.00
c	0.365	0.153	2	0.076	9.178	0.0007	0.6	-0.15
							0.8	-0.08
							1	0.00
genetic system	0.231	0.080	1	0.080	9.617	0.0040	HD	0.09
							D	0
Error		0.266	32	0.008				
Total		5.794	42					
Corrected Total		3.102	41					

Table 2.4 - ANOVA showing the effect of the genetic system and male trait variables on the final frequency of the A-allele after 100 generations in trials where it did not go extinct ($R^2 = 0.642$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.746	3.123	9	0.347	7.186	0.0001		
Intercept	0.864	6.747	1	6.747	139.756	< 0.0001		1.18
m	0.730	2.872	6	0.479	9.913	< 0.0001	1	-1.17
							1.25	-0.86
							1.5	-0.31
							1.75	-0.32
							2	-0.17
							3	0.00
							5	0.00
c	0.421	0.774	2	0.387	8.014	0.0024	0.6	-0.42
							0.8	-0.15
							1	0.00
genetic system	0.005	0.006	1	0.006	0.121	0.7310	HD	-0.03
							D	0.00
Error		1.062	22	0.048				
Total		23.047	32					
Corrected Total		4.185	31					

Table 2.5 - ANOVA showing the effect on the percent of trials where A-allele did not go extinct ($R^2 = 0.923$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.925	86.081	14	6.149	650.502	< 0.0001		
Intercept	0.950	131.806	1	131.806	13944.588	< 0.0001		1.12
m	0.738	19.704	6	3.284	347.431	< 0.0001	1	-0.50
							1.25	-0.45
							1.5	-0.41
							1.75	-0.36
							2	-0.34
							3	-0.18
							5	0.00
c	0.046	0.340	2	0.170	17.990	< 0.0001	0.6	-0.05
							0.8	-0.01
							1	0.00
f	0.283	2.770	2	1.385	146.504	< 0.0001	0.6	-0.13
							0.8	-0.12
							1	0.00
v	0.018	0.129	2	0.065	6.837	0.0011	0.6	-0.03
							0.8	-0.02
							1	0.00
initial freq. of R	0.100	0.775	1	0.775	81.956	< 0.0001	0.2	0.06
							0.5	0.00
genetic system	0.899	62.364	1	62.364	6597.832	< 0.0001	HD	0.57
							D	0.00
Error		7.004	741	0.009				
Total		224.891	756					
Corrected Total		93.085	755					

Table 2.6 - ANOVA showing the effect on the mean final frequency of A-allele for trials where A did not go extinct ($R^2 = 0.035$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.059	0.110	14	0.008	2.437	0.0025		
Intercept	0.996	390.094	1	390.094	120942.489	< 0.0001		1.00
m	0.026	0.047	6	0.008	2.425	0.0253	1	-0.02
							1.25	-0.03
							1.5	-0.01
							1.75	-0.01
							2	-0.01
							3	0.00
							5	0.00
c	0.002	0.004	2	0.002	0.588	0.5557	0.6	0.00
							0.8	0.01
							1	0.00
f	0.008	0.014	2	0.007	2.229	0.1086	0.6	0.01
							0.8	0.01
							1	0.00
v	0.004	0.007	2	0.004	1.127	0.3247	0.6	0.00
							0.8	0.01
							1	0.00
initial freq. of R	0.000	0.000	1	0.000	0.024	0.8773	0.2	0.00
							0.5	0.00
genetic system	0.026	0.047	1	0.047	14.465	0.0002	HD	0.02
							D	0.00
Error		1.748	542	0.003				
Total		552.741	557					
Corrected Total		1.858	556					

Table 2.7 - ANOVA showing the effect on the percent of trials where R-allele did not go extinct ($R^2 = 0.747$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Val ue	B
Corrected Model	0.752	65.353	14	4.668	160.066	< 0.0001		
Intercept	0.566	28.170	1	28.170	965.936	< 0.0001		0.59
m	0.024	0.521	6	0.087	2.979	0.0070	1	0.09
							1.25	0.07
							1.5	0.07
							1.75	0.06
							2	0.06
							3	0.03
							5	0.00
c	0.000	0.005	2	0.002	0.082	0.9216	0.6	0.01
							0.8	0.00
							1	0.00
f	0.723	56.340	2	28.170	965.936	< 0.0001	0.6	-0.58
							0.8	-0.58
							1	0.00
v	0.000	0.009	2	0.005	0.161	0.8511	0.6	0.01
							0.8	0.00
							1	0.00
initial freq. of A	0.034	0.770	1	0.770	26.416	< 0.0001	0.2	0.06
							0.5	0.00
genetic system	0.263	7.707	1	7.707	264.282	< 0.0001	HD	0.20
							D	0.00
Error		21.610	741	0.029				
Total		115.133	756					
Corrected Total		86.963	755					

Table 2.8 - ANOVA showing the effect on the mean final frequency of R-allele for trials where R did not go extinct ($R^2 = 0.811$). R did not invade in trials for every value of *f*. HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.821	40.760	12	3.397	89.208	< 0.0001		
Intercept	0.902	81.709	1	81.709	2145.955	< 0.0001		0.66
m	0.230	2.656	6	0.443	11.627	< 0.0001	1	0.33
							1.25	0.27
							1.5	0.21
							1.75	0.17
							2	0.14
							3	0.09
							5	0.00
c	0.022	0.202	2	0.101	2.657	0.0723	0.6	0.07
							0.8	0.04
							1	0.00
f	0.000	0.000	0	-	-	-	0.6	-
							0.8	-
							1	-
v	0.001	0.006	2	0.003	0.080	0.9232	0.6	0.01
							0.8	0.00
							1	0.00
initial freq. of A	0.174	1.878	1	1.878	49.325	< 0.0001	0.2	0.17
							0.5	0.00
genetic system	0.805	36.712	1	36.712	964.194	< 0.0001	HD	0.77
							D	0.00
Error		8.910	234	0.038				
Total		134.751	247					
Corrected Total		49.670	246					

Table 2.9 - Paired T-tests conducted on each genetic system individually. The paired differences represent the value for the A-allele (male) minus that for the R-allele (female). Thus, a higher mean difference represents a greater value for the A-allele.

	Paired differences				
	Mean	Std. Deviation	t	df	Sig.
Probability of invasion					
haplodiploid	0.411	0.416	19.178	377	< 0.0001
diploid	0.036	0.283	2.452	377	0.0147
Final allele frequency					
haplodiploid	0.045	0.136	3.697	125	< 0.0001
diploid	0.895	0.235	32.801	73	< 0.0001

Table 2.10 - ANOVA showing the effect on the difference in the probability of invasion (frequency of A minus R) ($R^2 = 0.594$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.601	73.408	13	5.647	85.946	< 0.0001		
Intercept	0.436	37.660	1	37.660	573.201	< 0.0001		0.62
m	0.357	27.027	6	4.504	68.559	< 0.0001	1	-0.60
							1.25	-0.52
							1.5	-0.48
							1.75	-0.41
							2	-0.39
							3	-0.22
							5	0.00
c	0.010	0.470	2	0.235	3.578	0.0284	0.6	-0.06
							0.8	-0.02
							1	0.00
f	0.281	19.084	2	9.542	145.235	< 0.0001	0.6	0.28
							0.8	0.37
							1	0.00
v	0.005	0.255	2	0.127	1.938	0.1447	0.6	-0.04
							0.8	-0.03
							1	0.00
genetic system	0.353	26.572	1	26.572	404.436	< 0.0001	HD	0.37
							D	0.00
Error		48.750	742	0.066				
Total		159.818	756					
Corrected Total		122.158	755					

Table 2.11 - ANOVA showing the effect on the difference in final frequencies (frequency of A minus R) ($R^2 = 0.862$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.871	34.821	12	2.902	104.915	< 0.0001		
Intercept	0.765	16.850	1	16.850	609.209	< 0.0001		0.15
m	0.146	0.882	6	0.147	5.316	< 0.0001	1	-0.16
							1.25	-0.22
							1.5	-0.14
							1.75	-0.18
							2	-0.14
							3	-0.09
							5	0.00
c	0.003	0.018	2	0.009	0.328	0.7210	0.6	0.02
							0.8	0.02
							1	0.00
f	0.032	0.172	1	0.172	6.218	0.0135	0.6	0.11
							0.8	-
							1	0.00
v	0.016	0.082	2	0.041	1.479	0.2306	0.6	-0.04
							0.8	0.02
							1	0.00
genetic system	0.848	28.877	1	28.877	1044.076	< 0.0001	HD	-0.84
							D	0.00
Error		5.172	187	0.028				
Total		65.791	200					
Corrected Total		39.993	199					

Table 2.12 - ANOVA showing the effect on the mean grandoffspring males produce without R-bearing females minus mean grandoffspring produced when the frequency of R = 0.5 ($R^2 = 0.832$). HD = haploidiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.838	26660.570	13	2050.813	144.329	< 0.0001		
Intercept	0.849	29052.006	1	29052.006	2044.574	< 0.0001		-1.89
m	0.004	22.996	6	3.833	0.270	0.9509	1	0.81
							1.25	0.62
							1.5	0.51
							1.75	0.41
							2	0.37
							3	0.20
							5	0.00
c	0.221	1467.097	2	733.549	51.624	< 0.0001	0.6	-4.82
							0.8	-2.63
							1	0.00
f	0.797	20299.565	2	10149.782	714.304	< 0.0001	0.6	17.93
							0.8	9.74
							1	0.00
v	0.001	2.933	2	1.466	0.103	0.9020	0.6	-0.21
							0.8	-0.07
							1	0.00
genetic system	0.485	4867.980	1	4867.980	342.591	< 0.0001	HD	-7.18
							D	0.00
Error		5172.194	364	14.209				
Total		60884.770	378					
Corrected Total		31832.764	377					

Table 2.13 - The effect of genetic system and simulation variables on the mean difference in grandoffspring produced by homozygous A males and homozygous a males when the frequency of R was 0.5 ($R^2 = 0.771$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.779	302106.636	13	23238.972	98.803	< 0.0001		63.98
Intercept	0.617	138095.397	1	138095.397	587.130	< 0.0001	1	-66.55
m	0.663	168740.912	6	28123.485	119.571	< 0.0001	1.25	-55.33
							1.5	-46.19
							1.75	-39.00
							2	-32.55
							3	-16.02
							5	0.00
c	0.447	69182.052	2	34591.026	147.068	< 0.0001	0.6	-33.07
							0.8	-18.32
							1	0.00
f	0.040	3606.396	2	1803.198	7.667	0.0005	0.6	-7.56
							0.8	-4.02
							1	0.00
v	0.000	2.286	2	1.143	0.005	0.9952	0.6	-0.12
							0.8	0.07
							1	0.00
genetic system	0.414	60574.990	1	60574.990	257.542	< 0.0001	HD	-25.32
							D	0.00
Error		85614.300	364	235.204				
Total		525816.333	378					
Corrected Total		387720.936	377					

Table 2.14 - ANOVA showing the effect on the mean grandoffspring females produce without A-bearing males minus mean grandoffspring produced when the frequency of A = 0.5 ($R^2 = 0.930$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.933	49938.312	13	3841.409	388.125	< 0.0001		
Intercept	0.983	211197.415	1	211197.415	21338.788	< 0.0001		15.59
m	0.107	433.222	6	72.204	7.295	< 0.0001	1	-3.37
							1.25	-2.47
							1.5	-1.87
							1.75	-1.46
							2	-1.13
							3	-0.46
							5	0.00
c	0.923	43208.229	2	21604.114	2182.819	< 0.0001	0.6	26.16
							0.8	14.07
							1	0.00
f	0.614	5722.457	2	2861.229	289.090	< 0.0001	0.6	-9.53
							0.8	-4.97
							1	0.00
v	0.003	12.100	2	6.050	0.611	0.5432	0.6	-0.44
							0.8	-0.21
							1	0.00
genetic system	0.135	562.304	1	562.304	56.814	< 0.0001	HD	2.44
							D	0.00
Error		3602.635	364	9.897				
Total		264738.362	378					
Corrected Total		53540.947	377					

Table 2.15 - The effect of genetic system and simulation variables on the mean difference in grandoffspring produced by homozygous R females and homozygous r females when the frequency of A was 0.5 ($R^2 = 0.920$). HD = haplodiploid, D = diploid.

Source	Partial Eta Squared	Type III Sum of Squares	df	Mean Square	F	Sig.	Value	B
Corrected Model	0.922	38612.528	13	2970.194	332.782	< 0.0001		
Intercept	0.959	75544.383	1	75544.383	8464.039	< 0.0001		-5.04
m	0.043	145.960	6	24.327	2.726	0.0133	1	-1.95
							1.25	-1.49
							1.5	-1.07
							1.75	-0.85
							2	-0.58
							3	-0.35
							5	0.00
c	0.578	4441.718	2	2220.859	248.826	< 0.0001	0.6	8.39
							0.8	4.49
							1	0.00
f	0.911	33279.650	2	16639.825	1864.336	< 0.0001	0.6	-22.91
							0.8	-13.06
							1	0.00
v	0.038	127.300	2	63.650	7.131	0.0009	0.6	1.40
							0.8	0.92
							1	0.00
genetic system	0.160	617.901	1	617.901	69.230	< 0.0001	HD	2.56
							D	0.00
Error		3248.822	364	8.925				
Total		796662.982	1750					
Corrected Total		335712.050	1749					

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CHAPTER 3

SEXUAL HARASSMENT BY MALES REDUCES FEMALE FECUNDITY

ABSTRACT

Under sexual conflict, males evolve traits to increase their mating and reproductive success that impose costs on females. Females evolve counter-adaptations to resist males and reduce those costs. Sexual harassment is a form of sexual conflict in which males make repeated, costly attempts to mate. Costs to female foraging or predation risk have been measured in several species, but quantitative measurements of direct fitness costs are rare. In the alfalfa leafcutting bee (*Megachile rotundata* Fabricius; Hymenoptera: Megachilidae), males harass females, and females resist all mating attempts. We placed bees in large, outdoor cages with different male-biased sex ratios. Harassment rate, nest progression, offspring production, temperature and food availability were measured daily for seven days. Harassment rates were highest at intermediate sex ratios. Harassment reduced the number of foraging trips and increased the duration of foraging trips made by females. Females produced offspring at a slower rate when subjected to higher rates of harassment. This demonstrates a direct link from sex ratio to harassment to female fitness under natural conditions. We also discuss an alternative explanation that female resistance is a mechanism for mate choice for high quality males, which would require that indirect benefits accrue either through daughters or in grandsons because all sons in haplodiploid species arise from unfertilized eggs.

INTRODUCTION

Sexual conflict can drive the evolution of males and females in ways completely different from traditional mate choice. Under traditional mate choice, males evolve traits to lure and entice females, and female preferences evolve because choosy females receive direct and/or indirect benefits from males (Andersson 1994). In contrast, under sexual conflict, males evolve adaptations that increase their own fitness while imposing costs on females. Females then evolve counter-adaptations to resist mating attempts, which, in turn, reduces the fitness of these manipulative males. Though these male-induced costs have been measured in several species, there is currently a debate over whether examples of sexual conflict represent true conflict. This is due to the possibility that female resistance may be a mechanism for mate choice to only allow the highest quality males to mate (Eberhard 2002; Chapman et al. 2003; Kokko et al. 2003; Eberhard 2005; Parker 2006; Peretti & Cordoba-Aguilar 2007). If females receive indirect benefits through offspring, the observed female resistance behaviors may actually function to screen out lower quality males. Females that are highly resistant to coercive males would end up mating with only the most coercive males. If coercion ability in males is heritable, those highly resistant females would produce highly coercive sons. Females could thus “gain by losing” through this “sons effect” (a.k.a., “sexy son”) benefit (Weatherhead & Robertson 1979; Wedell & Tregenza 1999; Huk & Winkel 2008). Females could also receive good genes benefits by mating with the most vigorous, aggressive males. Such indirect benefits to females are thought to be weak compared to the direct costs because they are expressed only through sons (Parker 2006). Females may also receive direct benefits due to their resistance by avoiding low quality males that do not provide high direct benefits such as nuptial gifts (Thornhill 1980). It is essential that costs *and* benefits to females are measured in the same species to determine whether direct costs are outweighed by indirect benefits (Eberhard 2005; Hosken & Tregenza 2005). If the indirect benefits do outweigh the costs, this would suggest that female choice is operating. If not, then sexual conflict is operating (Parker 2006). Both female choice and sexual conflict could be operating simultaneously, but the net cost or benefit would indicate which is primarily responsible for the evolution of male and female traits.

One form of sexual conflict is sexual coercion, where males attempt to copulate through physical force and harassment (Clutton-Brock & Parker 1995). Through harassment, males make repeated, costly mating attempts, which induce females to mate rather than continue resisting. The cost of male harassment to females has been measured in several species in terms of physical injuries to the female (Rowe et al. 1994; Blanckenhorn et al. 2002; Mühlhäuser & Blanckenhorn 2002), increased predation (Rowe et al. 1994; Mühlhäuser & Blanckenhorn 2002) and foraging costs (Rowe et al. 1994; Stone 1995; Schlupp et al. 2001). Rowe et al. (1994) found that changes in the population sex ratio in water striders resulted in higher rates of harassment and higher potential costs to females. The few studies that directly measured fitness in terms of longevity and fecundity were performed in the laboratory under artificial conditions and measured costs by pairing the subjects (e.g., a male and a female vs. two females) (McLain & Pratt 1999; Meader & Gilburn 2008; Sakurai & Kasuya 2008; Gay et al. 2009).

Our study species was the alfalfa leafcutting bee (*Megachile rotundata* Fabricius; Hymenoptera: Megachilidae), a solitary bee. Males pursue females at their nests and foraging sites. Females put up active resistance to all mating attempts and usually mate only once (Gerber & Klostermeyer 1972; Blanchetot 1992), though they are capable of mating multiply. Thus, if males impose a fitness cost on females, this can be easily observed and quantified because females build linear nests making daily measurements of reproduction possible. The frequency of harassment from male bees can be manipulated under natural conditions in outdoor cages by varying the sex ratio within the species' normal range. If male harassment impairs a female's foraging ability, then females housed with relatively more males should be harassed more frequently and need to take more or longer foraging trips to build and provision each cell. As a result, the more frequently harassed females should produce offspring at a slower rate. Reduced fecundity would represent a quantifiable measure of the direct fitness cost of sexual conflict, measured in interacting groups of bees under natural conditions.

METHODS

Study species

Alfalfa leafcutting bees are sexually dimorphic being easily distinguished by color and size (Gerber & Akre 1969; Akre et al. 1982; Richards 1984), with females an average of 1.2 times larger than males (Klostermeyer & Gerber 1969; Klostermeyer et al. 1973). After emergence as adults, most females live approximately 30 days, and males live 15-23 days, though many individuals of either sex live longer (Richards 1984). Adult sex ratios range from 1:1 to 5:1 (males: females) depending on environmental and nesting conditions of the parents (Gerber & Klostermeyer 1972; Richards 1993; Pitts-Singer & James 2005) and drops towards the end of the season when males die off before females (Richards 1984). Females nest gregariously (under wild and captive conditions) and build linear nests in pre-existing tunnels. Females forage for leaves, nectar and pollen near their nests. The tunnels are lined with leaf-cuttings used to form individual brood cells, which are provisioned with nectar and pollen. A single egg is laid in each cell, which is then sealed off with leaf discs before the initiation of the next cell (Gerber & Klostermeyer 1972; Richards 1984).

The mating system appears to be a form of scramble competition, and the male mating strategy resembles sexual coercion through harassment with apparent attempts at forced copulations (Gerber & Klostermeyer 1972). Males patrol and chase females near nesting and foraging sites, and they pounce on females found resting, foraging at flowers, entering nest tunnels, or flying nearby. This harassment appears to interfere with females' nesting activities (Gerber & Klostermeyer 1972). Once a male captures a female, he moves to dorsally mount the female and copulate (Wittmann & Blochtein 1995).

The females' behavior suggests intense resistance to all mating attempts by males. When a female is seized, a struggle ensues as females try to dislodge males using rapid abdominal thrusts (Wittmann & Blochtein 1995) and leg kicks (Rossi, B. H. personal observation). These struggles can last from a few seconds to several minutes and end after copulation or with the male dislodged. Most females will mate with only one male within the first few days post-eclosion before nest-building begins (Gerber &

Klostermeyer 1972; Richards 1984) providing them with a lifetime supply of sperm (Richards 1994), though some females may mate multiply (Blanchetot 1992). Observations suggest that females may become more resistant to mating attempts after they mate (Gerber & Klostermeyer 1972).

Many features of struggles during sexual encounters remain unexplained and may include a combination of male coercive and luring behaviors. When mounting a female, alfalfa leafcutting bee males press their front legs over the female's eyes and antennae. Odor glands on the front legs may be used to send signals to the female through her antennae, perhaps to stimulate her rather than physically overcome her resistance(Wittmann & Blochtein 1995). Males will also beat their wings intermittently throughout the event (Rossi, B. H. personal observation).

General procedure

In the summers of 2006 and 2007, eight 2 x 6 x 6m (H x W x L) outdoor screened cages were placed in a field of alfalfa (*Medicago sativa*) in Logan, UT, U.S.A. and each was equipped with a small domicile that housed a Polystyrene nest board with prefabricated tunnels (Figure 3.1). Nest tunnels were 10 cm deep and 6mm in diameter. We cut nest boards to size so that four nest tunnels were provided for each female and two nest tunnels per male to prevent overcrowding and provide space for both sexes to rest in tunnels at night (Stephen 1981). Paper straws were inserted in the available nest tunnels to allow progress of each nest to be monitored, as described below. Alfalfa leafcutting bees forage close to their nest (Richards 1984), so this cage setup resembled their natural conditions.

Bees were obtained from a commercial bee supplier (JWM Leafcutters, Inc., Nampa, ID, U.S.A.) as pre-pupae in leaf-covered cocoons. Alfalfa leafcutting bees overwinter as pre-pupae and are stored in this state over the winter season (Gerber & Klostermeyer 1972). Pre-pupae were incubated (in staggered batches of approximately 60 bees) individually in clear gelatin capsules (size 00, Capsuline Inc., Pompano Beach, FL, U.S.A.) at 30 degrees C for 2-3 weeks until they emerged as adults (Pankiw et al. 1979; Richards 1984). Only bees (males and females) of equal age were used in each cage for each trial.

We uniquely color-marked each female upon emergence and took four body size measures – fresh weight at emergence, head width, intertegular width (Cane 1987) and wing length. Virgin males and females were released into cages and allowed to freely interact, mate, examine nest tunnels, forage and build nests. Variations in sex ratio and bee density represented different treatment conditions (Table 3.1) and included possible sex ratios of 0.5:1, 3:1 and 4:1 (male: female) and bee densities of 8, 10, 12 and 16 total bees (males and females). This is similar to what has been done in studies of sexual harassment in water striders (Rowe et al. 1994). The bee density was varied to control for the possible effect of overcrowding in the cages.

we monitored the bees' activities at the nest box for 2-3 days until at least 75% of the females had initiated nests. Females do not initiate nests until after they have mated. Frequent chases and occasional mountings of females by males were observed, but it is unknown if these resulted in successful copulations. We then monitored the nesting females for 7 days. In total, we monitored 34 females in eight different cages. To measure male harassment, we counted the number of male-initiated chases of any females within 50 cm of the nest in 10 minute observations twice a day in each cage. Observations were made from 1000 to 1500 hours (MDT), the bees' active period (Klostermeyer & Gerber 1969). The exact time of observation was varied from day to day to represent every part of the active period in the data sets and ensure that each cage was observed during the same times of day. "Harassment rate" was defined as the mean number of male-initiated chases per day divided by the number of females (known to be present that day).

We videotaped (using Sony Digital-8 camcorders) each nest box for one hour each day during one of three time periods: 1000-1130, 1130-1230 and 1230-1500 hours. We used the number of times that each female entered and exited her nest to calculate the number of trips taken per hour, the average duration of foraging trips, and the total time spent foraging during the hour.

We also measured other aspects of the female nesting behavior. The type of foraging trip (e.g., for leaves or pollen/nectar) was recorded by noting whether the female performed a specific "turn-around" maneuver after arriving. When a female has collected pollen, it is held in the hairs of her scopa, or pollen-carry apparatus, on the underside of the abdomen. The female first enters her nest head-first, so to regurgitate nectar into the cell. Then the female backs out of the tunnel, turns around and moves into the tunnel abdomen-first, so that she can scrape pollen from the scopa and pack it into a mass provision. Thus, if this turn-around maneuver is observed, the female must have just been on a nectar/pollen-collecting trip. Pollen and nectar are usually collected on the same trips (Klostermeyer & Gerber 1969; Klostermeyer et al. 1973). Also, we counted the number of “mistakes” females made as they returned to their tunnel. Because females usually work on only one nest at a time (Klostermeyer & Gerber 1969), if a female entered a tunnel that was not her nest prior to finding her own nest tunnel, this was counted as a mistake.

Ambient temperature and food availability (floral resources) were monitored because they are well-known to positively affect bee activity levels (reviewed in Willmer & Stone 2004) and reproduction (Kim 1996; Richards 1996; Kim 1999; Peterson & Roitberg 2006). Ambient temperature was monitored using a Hobo data-logger (Onset Computer Corp., Pocasset, MA, U.S.A.) that was placed inside each domicile in each cage. For analyses, we used the mean temperature for each day during the bees’ active period from 1000 to 1500 hours.

We estimated the floral resources by counting open, unvisited flowers every other day in four 0.25 m^2 quadrats placed in four evenly-spaced locations within each cage. When an alfalfa flower is visited by a bee, pressure on the keel petal causes the flower to “trip,” meaning the sexual column is released. The bee can then collect both pollen and nectar from the flower (Larkin & Graumann 1954). Thus, we used untripped flowers, identified by the exposed sexual columns, as representative samples for the floral resources available to bees. We conducted the first flower survey before bees were introduced to ensure floral resources were adequate for nesting success to occur and determine the maximum floral resources available to bees.

Males and females were counted every night in nest tunnels. Though the numbers of males and females remained relatively stable, the sex ratios and bee densities did vary from the initial starting values. A mean sex ratio and bee density was calculated for each cage and each female (across the days she lived), and those values were used in our analyses. Also at night, we removed each nest tunnel's paper straw and measured the distance from the back end of the nest to the end of any nest construction to determine the progress made each day by each bee. From these data was calculated the mean nest progression (mm) for each female.

To determine total offspring production, we removed and x-radiographed each nest at the conclusion of trials. X-radiography has no significant, negative effects on developing offspring (Stephen & Undurraga 1976; Maki et al. 1990). In the x-ray images, developing offspring are clearly visible within the individual cells (Figure 3.2). The nest-building distances from each day were compared to the x-ray images to measure offspring production, which was defined as the number of offspring each female produced each day. An offspring (of either sex) was counted if it developed to at least the prepupal stage. It was not possible, from the x-radiographs, to identify offspring that died before developing to this stage because females will also produce cells without an egg (Pitts-Singer 2004). All adult bees were removed and frozen, so we could take further morphological measurements that were not used in these analyses.

Data analysis

To determine which factors affected variables measured at the cage (treatment) (e.g., harassment rate and food availability), we conducted linear and nonlinear (when appropriate) regression analyses level. $N = 8$ unless otherwise indicated.

To determine the factors affecting variables measured at the individual female level (e.g., nest progression, offspring production, and female foraging variables), we used a linear regression model, the Generalized Linear Model (GLM) (Laird 2004) with robust standard errors adjusting for possible cage effects (SPSS 15, SPSS Inc., Chicago, IL, U.S.A.). $N = 34$ unless otherwise indicated.

The values for sex ratio and bee density differed from their initial starting values (Table 3.1) due to disappearances of males or females and were calculated as means over the seven days. Five females in three of the cages were not present for the entire seven days, because they either died or escaped, so separate harassment rates, temperatures and floral resources were calculated for each of them only using measures from days they were present.

RESULTS

Factors affecting harassment rate

The male: female sex ratio did not affect mean harassment rate (Linear regression: $F_{1,6} = 3.913$, $R^2 = 0.294$, $p = 0.0953$; power = 0.38; Figure 3.3), though the trend was in the expected direction. The distribution suggested that a quadratic fit was more appropriate. Using this model, sex ratio did affect harassment rate, with the highest harassment rates at intermediate sex ratios (Quadratic regression: $F_{1,5} = 7.336$, $R^2 = 0.746$, $p = 0.0326$).

Mean harassment rate increased with mean temperature (Linear regression: $F_{1,6} = 18.209$, $N = 8$, $R^2 = 0.835$, $p = 0.0053$), but not with food availability (Linear regression: $F_{1,5} = 1.201$, $N = 7$, $p = 0.3231$). Total bee density (includes both males and females) had no significant effect on harassment (Linear regression: $F_{1,6} = 1.572$, $R^2 = 0.076$, $p = 0.2566$; power = 0.19) or offspring production rate (GLM: B+SE = -0.037+0.050, $R^2 = 0.035$, $p = 0.4687$).

Effect of harassment and sex ratio on fecundity

As mean harassment rate increased, offspring production decreased (GLM: B+SE = -0.351+0.042, $R^2 = 0.442$, $p < 0.0001$; Figure 3.4) and nest progression decreased (GLM: B+SE = -3.192+0.341, $R^2 = 0.483$, $p < 0.0001$). Offspring production increased with nest progression (GLM: B+SE = -3.192+0.341, $R^2 = 0.725$, $p < 0.0001$).

As sex ratio increased, offspring production decreased (GLM: B+SE = -0.116+0.060, $R^2 = 0.165$, $p = 0.0523$; Figure 3.5). However, the relationship may not be best described as linear. Therefore, we added a second order term of sex ratio as a main affect, thus enabling the testing of a quadratic model using a linear GLM. This significantly improved the fit of the model with the lowest offspring production at intermediate sex ratios (GLM: $R^2 = 0.481$, $N = 34$; Figure 3.5, Table 3.2).

Effect of harassment on female foraging behavior

Mean harassment rate had a negative effect on the mean number of nest visits (GLM: B+SE = -1.343+0.378, $R^2 = 0.214$, $p = 0.0004$) and foraging trips (GLM: B+SE = -1.879+0.580, $R^2 = 0.218$, $p = 0.0012$) per female per day. Offspring production was positively affected by the number of nest visits (GLM: B+SE = 0.134+0.026, $R^2 = 0.299$, $p < 0.0001$) and foraging trips (GLM: B+SE = 0.060+0.019, $R^2 = 0.271$, $p = 0.0014$).

Mean harassment rate had a positive effect on the mean durations of nest visits (GLM: B+SE = 48.650+17.910, $R^2 = 0.236$, $p = 0.0066$) and foraging trips (GLM: B+SE = 117.663+46.550, $R^2 = 0.269$, $p = 0.0115$). Offspring production was lower in nest visits and foraging trips of longer durations (nest visits: GLM: B+SE = -0.002+0.001, $R^2 = 0.206$, $p = 0.0341$; foraging trips: GLM: B+SE = -0.002+0.0002, $R^2 = 0.459$, $p < 0.0001$).

There was no significant effect of mean harassment rate on total times spent in the nest (GLM: B+SE = -89.032+68.994, $p = 0.1969$) or on foraging trips (GLM: B+SE = 85.102+66.425, $p = 0.2001$) per day. Offspring production was not significantly affected by total times spent in the nest (GLM: B+SE < 0.001+0.0002, $p = 0.1083$) or on foraging trips (GLM: B+SE < 0.001+0.0002, $p = 0.1083$).

Mean harassment rate positively affected the proportion of pollen trips (GLM: B+SE = 0.046+0.017, $R^2 = 0.035$, $p = 0.0077$), but did not affect offspring production (GLM: B+SE = -0.033+0.212, $R^2 = 0.026$, $p = 0.8758$). Harassment did not affect the number of mistakes a female made when returning to the nest (GLM: B+SE < 0.001+0.027, $p = 0.9964$).

Effect of temperature, floral resources and female body size

As mean temperature increased, offspring production decreased (GLM: B+SE = -0.053+0.014, $R^2 = 0.297$, $p = 0.0002$). Nest progression also decreased as mean temperature increased (GLM: B+SE = -0.525+0.079, $R^2 = 0.389$, $p < 0.0001$).

Data analyses from the seven cages in which floral resources were recorded revealed that offspring production increased with the mean number of untripped flowers (GLM: B+SE = 0.019+0.010, $N = 30$, $R^2 = 0.160$, $p = 0.0404$). Bee density did not affect the number of untripped flowers (Linear regression: $F_{1,5} = 3.512$, $N = 7$, $R^2 = 0.30$, $p > 0.1198$), nor was there any significant effect of sex ratio on the mean number of untripped flowers per female (Linear regression: $F_{1,5} = 0.019$, $N = 7$, $R^2 < 0.01$, $p > 0.8944$). All cages used in our analyses contained at least 1650 untripped flowers per female each day, the minimum number of flowers needed by a female to produce a single provision (Cane 2005), and contained from 4113 to 39287 untripped flowers each day. Thus, females did not appear to have been limited by floral resources.

A principal component analysis of the female body size measurements was conducted (SPSS 15) and produced three independent measures of body size (Table 3.3). There was no significant effect of any of the direct adult female body size measures or any of the principal component measures of body size on offspring production (GLM: $p > 0.05$).

DISCUSSION

Our results demonstrate that male harassment imposes a fecundity cost on female alfalfa leafcutting bees. Importantly, this cost is directly related to sex ratio, where intermediate sex ratio treatments result in the highest harassment rates. Sex ratio is known to affect costs of harassment (Rowe et al. 1994), and harassment is known to negatively affect female longevity (Meader & Gilburn 2008) and fecundity (McLain & Pratt 1999; Sakurai & Kasuya 2008). However, this is the first demonstration of a direct link between sex ratio and fecundity under field conditions within realistic sex ratio bounds. By resisting mating attempts and fleeing from males, females made fewer foraging trips and took longer to make the number of foraging trips necessary to build each cell. Thus, the females harassed more frequently built their nests and laid eggs at a slower rate resulting in lower reproductive success.

The foraging costs that resulted from females fleeing males are similar to those observed in seed-eating true bugs (McLain & Pratt 1999) and the solitary bee *Anthophora plumipes* (Stone 1995). Because females are not known to mate while nest-building, this resistance likely serves to reduce the cost of male mating attempts. If females did not flee and allowed approaching males to mount them, they would have to endure even longer time costs as they worked to dislodge males. Fleeing reduces these potential time costs by preventing the mounting of males. The cost of male mating attempts probably cannot be completely eliminated by females because males patrol in areas essential to females such as their nests and foraging sites.

Male harassment may impact female foraging through time costs of fleeing males and the gathered resources that are lost. We observed that females returning to nests with a leaf piece would often drop it when pounced upon or chased by a male. Once she escaped, she then had to retrieve a new leaf piece, so a part of that foraging trip had to be repeated. There was no correlation between harassment and "mistakes", or females entering the wrong nest cavity upon return from a foraging trip, so a female's ability to correctly identify her own nest cavity did not seem affected by pressure to flee harassing males.

Harassment rates peaked in the 3:1 sex ratio cages. The slightly reduced harassment rate in the 4:1 cages might have been due to male-male competition. At high densities, male bees are known to directly compete more with other males to maintain access to areas containing females such as nest or foraging sites (Thornhill & Alcock 1983; Larsson 1991; Stone et al. 1995; Willmer & Stone 2004). While we only measured male chases of females, males did chase other males. We did not mark males, so it is unclear if these chases were attempts to defend territories, attempts to exclude other males from the male's current vicinity, or mistaken mating attempts. In any case, when the male density is very high, male-male chases may increase, which would reduce the frequency of male chases of females. A similar pattern was found in mosquitofish (Smith 2007), where male-male agonistic displays were more frequent and copulation attempts less frequent at higher male densities.

Environmental factors also influenced reproductive success. Food availability correlated with offspring production, which was expected based on previous work on alfalfa leafcutting bees (Peterson & Roitberg 2006) and its sister species *M. apicalis* (Kim 1996; Kim 1999). Also, food limitation did not influence our results because all cages had at least the minimum required flowers for each female each day to build cells and produce offspring. In all the experiments we observed females foraging throughout the cages. Indeed, males patrolled and chased females everywhere in the cages. There were no areas that were free of males, and thus females could not shift foraging areas to avoid males

Mean temperature reduced nest and offspring production, though temperature was previously shown to increase nest and offspring production in alfalfa leafcutting bees (Richards 1996). Additional studies of solitary bees that found positive effects of temperature on bee activity (Stone et al. 1995; Abrol 1998) were conducted at lower temperatures. The bees in our experiments may have suffered from overheating (Willmer & Stone 2004) with some cages reaching maximum temperatures as high as 44º C.

Adult body size did not correlate with offspring production in our study. This is similar to findings in another cavity-nesting, solitary megachilid, *Osmia lignaria* Say (Tepedino & Torchio 1982). However, in alfalfa leafcutting bees (Klostermeyer et al. 1973) and *M. apicalis* (Kim 1997), body size did have an effect. Also, when bees were selected for each cage, attempts were made to keep the average fresh weight of each cage's females close to that of the other cages. We often needed to use whatever bees had emerged within the last few days to ensure bees in each cage were of identical ages. To detect the effects of body size on offspring production in the context of high and low harassment rates, we would need to test many females in more cages with intentionally-selected larger- or smaller-sized females.

We did not examine the longevity of females, another component of fitness. While it is possible that the females harassed at a higher rate may have ended up living longer and making up the difference in fecundity, we would predict that if the energetic costs of escaping harassing males had any effect on longevity, it would be to reduce it (due to physical injuries and increased predation risk), not increase it. The relationship between factors promoting female longevity and lifetime fecundity are fertile grounds for future investigation.

Indirect benefits in a haplodiploid system

If there are indirect benefits of female resistance, they would come from those first few days post-eclosion when females do mate with a single male despite appearing resistant to all mating attempts. Now that there is confirmation that costs exist, this experiment can be repeated and multiple generations monitored to measure possible indirect benefits of female resistance.

Hymenoptera are haplodiploid, meaning sons develop from unfertilized eggs and daughters from fertilized eggs. As a result, a female's sons do not inherit any genes from her mate. Any "sons effect" must be expressed through grandsons (i.e. a "grandsons effect"). Nevertheless, the male effect is still genetically similar to diploid organisms because the relatedness of a haplodiploid father to his grandson is the same as a diploid father to his son ($r = 0.5$). Alternatively, indirect benefits could appear as a "daughters effect” in which daughters inherit increased strength or vigor that aggressive, coercive males might possess. Thus, females could accrue benefits from mating with effectively harassing males either through daughters or grandoffspring. This study has shown that females pay an immediate cost from male harassment. This opens the opportunity for future work to explore whether they can recoup those costs through the reproductive success of their daughters and especially through the reproductive success of grandsons (e.g., Chapter 2). If the costs are outweighed by such indirect benefits, then the function of female resistance would be for mate choice in addition to reducing male-induced costs of sexual conflict.

Figure 3.1 - The outside (top) and inside (bottom) of the outdoor cages, including the artificial nest block.

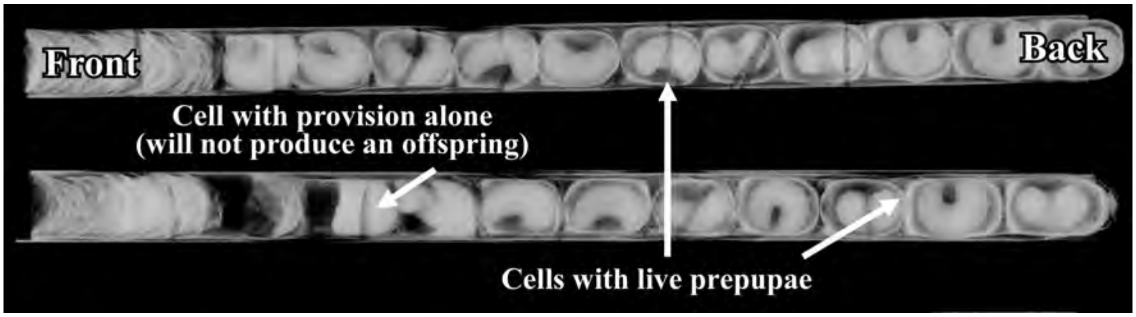

Figure 3.2 - X-Radiographs of nest straws were used to count the number of offspring produced based on the length of nest that was built each day. Cells with live prepupe can be distinguished from cells that just contain provision and will not produce a live offspring (either because the female never laid an egg in that cell or the offspring died).

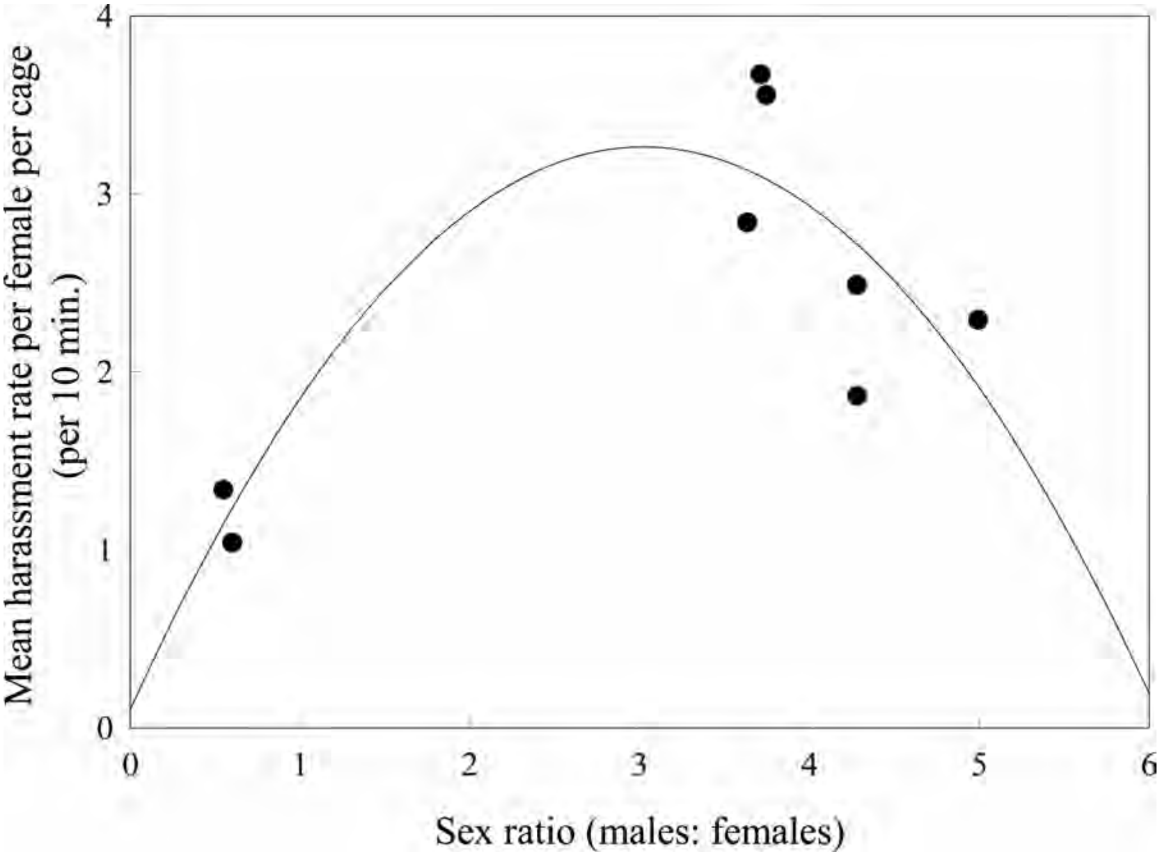

Figure 3.3 - Effect of sex ratio (males: females) on the mean harassment rate (Quadratic regression: $F_{1,5} = 7.336$, $R^2 = 0.746$, $p = 0.0326$). Harassment rate was highest at intermediate sex ratios.

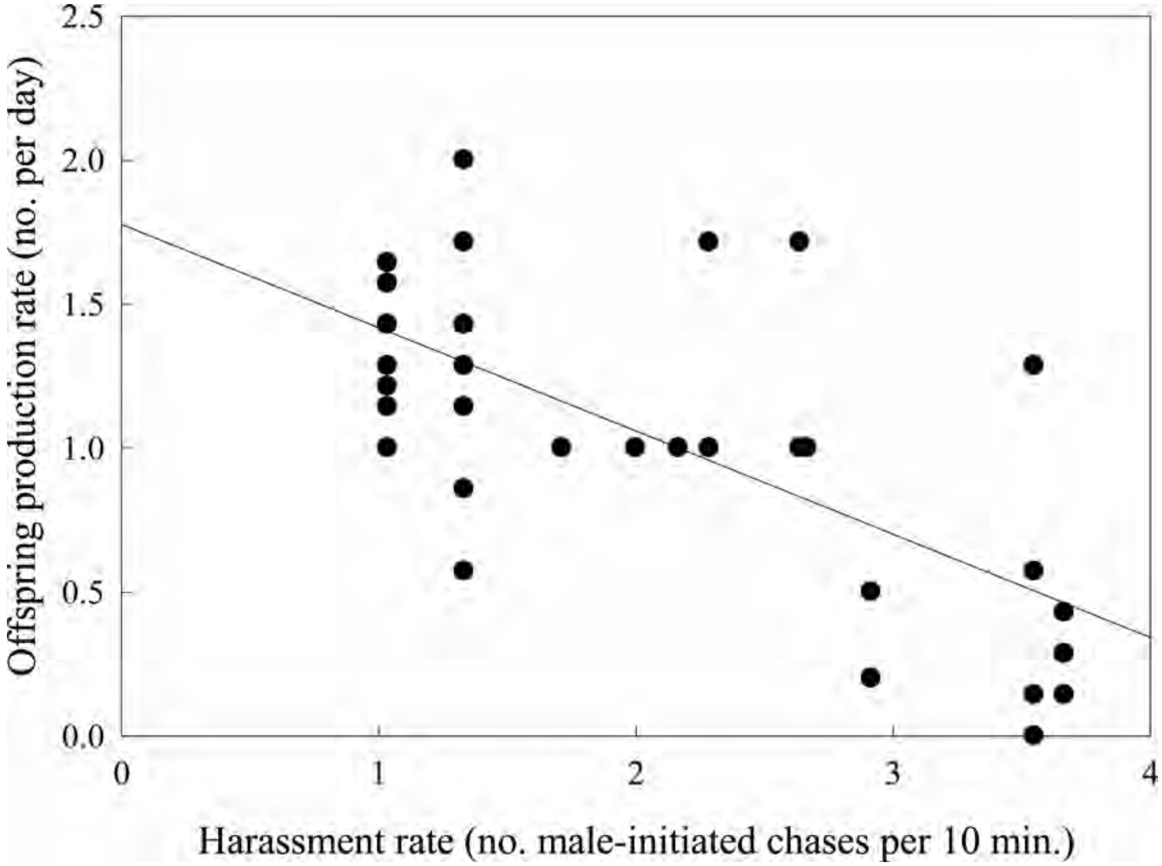

Figure 3.4 - Harassment rate reduced the offspring production rate per female (GLM: B+SE = -0.351+0.042, $R^2 = 0.442$, $p < 0.0001$).

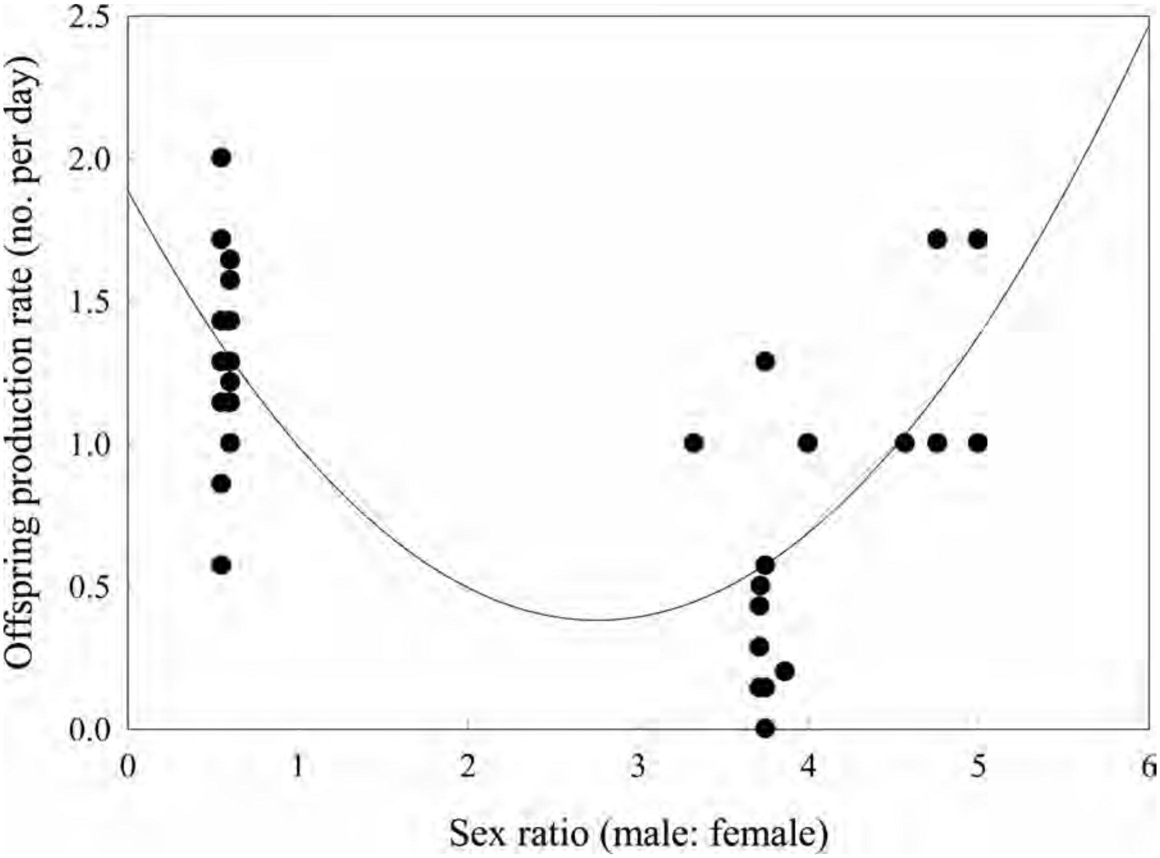

Figure 3.5 - Effect of sex ratio (males: females) on the offspring production rate per female. Offspring production rates were lowest at intermediate sex ratios (GLM of sex ratio and its second order term: $R^2 = 0.481$, $N = 34$; Table 3.2).

Table 3.1 - The types and numbers of each cage treatment.

Sex ratio (males:females)	Bee density (total number bees)	Number of trials with these treatments
0.5:1	12	2
3:1	8	2
3:1	12	2
3:1	16	1
4:1	10	1

Table 3.2 - Quadratic model for the effect of sex ratio on offspring production (GLM: $R^2 = 0.481$, $N = 34$). For this GLM analysis, our model uses wald test statistics to test the significance of the coefficient and control for possible cage effects.

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square.	df	Sig.
Intercept	1.963	0.062	1.841	2.086	988.885	1	< 0.0001
sex ratio	-1.183	0.121	-1.419	-0.946	95.951	1	< 0.0001
sex ratio^2	0.215	0.023	0.170	0.260	88.253	1	< 0.0001
Scale	0.153						

Dependent Variable: Offspring production rate

Table 3.3 - Eigenvalues from the principal components analysis of female body size measures. Only the first three principal components were used in analyses.

	Initial Eigenvalues			Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.690	67.25	67.25	2.69	67.25	67.25
2	0.623	15.58	82.83			
3	0.540	13.50	96.34			
4	0.147	3.67	100.00			

Extraction Method: Principal Component Analysis

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CHAPTER 4

SEXUAL HARASSMENT INDIRECTLY AFFECTS OFFSPRING DIAPAUSE

ABSTRACT

Sexual conflict can indirectly affect a female's offspring (e.g., influencing offspring reproductive success or quality). In many insect species such as the alfalfa leafcutting bee (*Megachile rotundata*), females determine which of their offspring undergo diapause (i.e., overwinter) or emerge as adults that same season. Because the incidence of diapause is affected by a number of environmental factors (e.g., food availability, temperature), sexual conflict could indirectly affect offspring diapause by influencing these factors. We tested between two hypotheses to explain the production of early-emerging offspring: (1) Early-emerging offspring are produced by females making the "best of a bad situation" under poor foraging conditions. Females must create early-emerging offspring or risk producing no offspring at all. (2) They are produced by females under favorable foraging conditions as additional, bonus (i.e., marginal) offspring in excess of their usual (i.e., core) offspring. We exposed female alfalfa leafcutting bees to varying degrees of sexual harassment by males, which is known to decrease females' foraging efficiency. We measured ambient temperature, food availability, photoperiod duration, and the production of overwintering and early-emerging offspring. Females in poor foraging conditions (e.g., high harassment, low temperature, and low food availability), produced more early-emerging offspring supporting the hypothesis that early-emerging offspring are the result of females making

the best of a bad situation. Increased harassment indirectly affected females' offspring and resulted in a higher proportion of early-emerging offspring.

INTRODUCTION

In many insect species, some developing offspring enter diapause (i.e., dormancy) and emerge as adults the following season, while others develop and emerge as adults that same season. The incidence of diapause is often influenced by environmental factors such as photoperiod and ambient temperature (Ryan 1965; Stephen 1965; Parrish & Davis 1978). It can also be maternally determined (Ryan 1965; Rockey & Denlinger 1986; Reznik et al. 2002), sometimes through the mother's response to environmental factors (Saunders 1987). To our knowledge, however, an effect of sexual coercion or female mating behavior on the incidence of offspring diapause has not been demonstrated. In many species, offspring that do not undergo diapause develop and emerge as adults in the same season that they were produced. It is not always clear why a female might create one of these early-emerging offspring instead of one that would undergo diapause and overwinter.

Two possible hypotheses to explain the production of early-emerging offspring are: (1) Early-emerging offspring are produced by females making the "best of a bad situation" under poor foraging conditions. Females must create early-emerging offspring or risk producing no offspring at all. (2) They are produced by females under favorable foraging conditions as additional, bonus (i.e., marginal) offspring in excess of their usual (i.e., core) offspring. Harmful male mating tactics observed in many species could create poorer foraging conditions for females. Thus, it is likely that sexual conflict can have an indirect impact on the production of early-emerging offspring.

Research on the consequences of sexual conflict has mostly focused on the direct genetic effects on female behavior and fitness (Arnqvist & Rowe 2005). However, non-genetic effects on the offspring could also result from sexual conflict. For example, sexual coercion by males, a form of sexual conflict (Clutton-Brock & Parker 1995), can cause females to mate multiply (Rowe et al. 1994), which can affect offspring quality or reproductive success (Tregenza et al. 2003; Ivy & Sakaluk 2005; Priest et al. 2008; Dunn et al. 2009). Sexual coercion can also cause a reduction in a female's foraging ability (Rowe et al. 1994; Stone et al. 1995; Schlupp et al. 2001). If offspring quality is related to maternal diet, sexual coercion would thus indirectly affect the offspring quality of the coerced females. This, in turn, could affect the life history of the offspring in substantial ways, such as how likely they are to enter diapause.

In the alfalfa leafcutting bee (*Megachile rotundata* Fabricius), male harassment reduces female foraging efficiency and fecundity (Chapter 3). Females build linear nests in pre-existing tunnels. The tunnels are lined with leaf-cuttings used to form individual brood cells, which are provisioned with nectar and pollen. Populations in the United States are bivoltine (Gerber & Klostermeyer 1972), so females produce two types of offspring: those that undergo diapause as pre-pupae and overwinter and those that develop directly into adults and emerge mid-summer of that same season (also known as 2nd generation offspring). Offspring diapause appears to be maternally controlled (Parker 1978; Parker & Tepedino 1982). Thus harassment could indirectly affect the life history of a female’s offspring by affecting the factors females use to determine whether an offspring will be overwintering or early emerging.

Early-emerging offspring are typically found only among the offspring produced during a female’s first week of nest-building and reproduction. However, there is variation across females in the number and proportions of early-emerging offspring (Tepedino & Parker 1988). A number of factors may interact to determine early-emerging offspring. These include photoperiod, temperature, and provisioning regimes.

Photoperiod is an unmistakable measure for the progression of the season. Once the day length falls below some threshold value, females may stop producing early-emerging and focus solely on overwintering offspring (Bitner 1976; Parker & Tepedino 1982). Indeed, females often initially produce both types of offspring and most early-emerging offspring are produced earlier in the season (Richards 1984).

Temperature may also be a reasonably reliable indicator of time of season. Bees exposed to low temperatures shortly before emerging as adults (suggesting that it is late in the season) produce proportionally fewer early-emerging offspring (Parker & Tepedino 1982). Low temperatures can also negatively influence activity levels and foraging in bees. Higher temperatures could allow bees to collect more nectar and pollen and provision more per offspring. Thus, if provision mass affects offspring type, temperature could be indirectly responsible by influencing foraging activity.

Finally, offspring may also react physiologically to the amount of nectar and pollen they receive. Brood that are provisioned more would be large and better able to survive the winter (Pitts-Singer, T. L. unpublished data). Such brood may, therefore, undergo diapause at higher rates. In contrast, smaller bees have an advantage in being able to develop faster. Thus, less provisioned bees could develop in time to reproduce before the season ends. Given that offspring size is under female control through the amount of provisions she places with the egg (Klostermeyer et al. 1973), female feeding behavior may closely regulate offspring life history.

Early emerging offspring as "best of a bad situation"

On first glance, it is unclear how a female could benefit from producing an early-emerging offspring instead of an overwintering offspring. Such offspring find floral resources on the decline and must either stay and produce few offspring or disperse long distances and attempt to find more plentiful resources (Parker & Tepedino 1982).

Alfalfa leafcutting bees run serious risks in having open cells that they are provisioning. An open cell is exposed to predators (Stephen et al. 1969), parasites (Stephen et al. 1969), and conspecifics that could usurp her nest tunnel (Stephen et al. 1969; McCorquodale & Owen 1994), and provisions could dry out. Therefore, although a well-provisioned cell may be desirable, keeping it open too long may incur excessive costs. The best of a bad situation would favor closing the cell and producing an early-emerging offspring over failing to produce an offspring at all.

The best of a bad situation would come into effect when food is scarce or more difficult to gather. For example, alfalfa leafcutting bee females are known to produce fewer offspring and provision less per offspring under low floral resource conditions (Richards 1996; Peterson & Roitberg 2006). There could also be situations where rewarding flowers are plentiful, but they cannot be exploited efficiently. For example, ambient temperature influences bee activity levels (Willmer & Stone 2004), such that lower ambient temperatures reduce foraging efficiency. Age could play a role, where younger females produce early-emerging offspring because of inexperience in foraging and provisioning. More interestingly, harassment from males could interfere with female foraging ability (Chapter 3). Potentially, females exposed to higher rates of harassment would expect to be able to bring back less food (or at a slower rate and therefore produce a higher proportion of early-emerging offspring.

If females produce early-emerging offspring to make the best of a bad situation, more will be produced when females are exposed to poor foraging conditions. Specifically, when ambient temperatures are low, floral resources are low, and harassment by males is high, females should produce fewer early-emerging offspring.

Early emerging offspring as marginal offspring

The types of offspring females produce can be thought of as falling into two categories: core and marginal (Mock & Forbes 1995). Core offspring are the minimum offspring females can expect to raise to independence under normal conditions. Marginal offspring are the additional offspring that females may produce when conditions are more favorable. In alfalfa leafcutting bees, overwintering offspring could be a female's core offspring. If foraging conditions are favorable, females could then produce additional, marginal offspring. This differs slightly from the traditional use of the core-marginal dichotomy in which females produce marginal offspring based on the expected, future conditions when females will be caring for and feeding live offspring (Mock & Forbes 1995). However, female alfalfa leafcutting bees care for young with their nest construction and provisions. Even though females are not predicting future conditions and are instead experiencing present conditions, the core-marginal dichotomy is still applicable.

Contrary to the best of a bad situation hypothesis, the core and marginal offspring hypothesis predicts that the proportion of early-emerging offspring a female produces should be higher when she is exposed to favorable foraging conditions. Specifically, when ambient temperatures are high, floral resources are high, and harassment by males is low, females should produce more early-emerging offspring.

We measured the number and proportion of early-emerging offspring produced under various levels of harassment, ambient temperature, floral resources, and photoperiod to determine whether early-emerging offspring are produced under poor foraging conditions (best of a bad situation hypothesis) or under favorable foraging conditions (marginal offspring hypothesis). We also determined whether sexual harassment by males can have indirect effects on offspring by influencing the type of offspring that are produced and the incidence of diapause in those offspring.

METHODS

In the summers of 2006 and 2007, eight 2 x 6 x 6m (H x W x L) outdoor screened cages were placed in a field of alfalfa (*Medicago sativa*) in Logan, UT, U.S.A. and each was equipped with a small domicile that housed a Polystyrene nest board with prefabricated tunnels. Nest tunnels were 10 cm deep and 6mm in diameter. We cut nest boards to size so that four nest tunnels were provided for each female and two nest tunnels per male to prevent overcrowding and provide space for both sexes to rest in tunnels at night (Stephen 1981). Paper straws were inserted in the available nest tunnels to allow progress of each nest to be monitored, as described below. Alfalfa leafcutting bees forage close to their nest (Richards 1984), so this cage setup resembled their natural conditions.

Bees were obtained from a commercial bee supplier (JWM Leafcutters, Inc., Nampa, ID, U.S.A.) as pre-pupae in leaf-covered cocoons. Alfalfa leafcutting bees overwinter as pre-pupae and are stored in this state over the winter season (Gerber & Klostermeyer 1972). Pre-pupae were incubated (in staggered batches of approximately 60 bees) individually in clear gelatin capsules (size 00, Capsuline Inc., Pompano Beach, FL, U.S.A.) at 30 degrees C for 2-3 weeks until they emerged as adults (Pankiw et al. 1979; Richards 1984). Only bees (males and females) of equal age were used in each cage for each trial.

We uniquely color-marked each female upon emergence and took four body size measures – fresh weight at emergence, head width, intertegular width (Cane 1987) and wing length. Virgin males and females were released into cages and allowed to freely interact, mate, examine nest tunnels, forage and build nests. Variations in sex ratio and bee density represented different treatment conditions (Table 3.1) and included possible sex ratios of 0.5:1, 3:1 and 4:1 (male: female) and bee densities of 8, 10, 12 and 16 total bees (males and females). This is similar to what has been done in studies of sexual harassment in water striders (Rowe et al. 1994). The bee density was varied to control for the possible effect of overcrowding in the cages.

we monitored the bees' activities at the nest box for 2-3 days until at least 75% of the females had initiated nests. Females do not initiate nests until after they have mated. Frequent chases and occasional mountings of females by males were observed, but it is unknown if these resulted in successful copulations. We then monitored the nesting females for 7 days. In total, we monitored 34 females in eight different cages. To measure male harassment, we counted the number of male-initiated chases of any females within 50 cm of the nest in 10 minute observations twice a day in each cage. Observations were made from 1000 to 1500 hours (MDT), the bees' active period (Klostermeyer & Gerber 1969). The exact time of observation was varied from day to day to represent every part of the active period in the data sets and ensure that each cage was observed during the same times of day. "Harassment rate" was defined as the mean number of male-initiated chases per day divided by the number of females (known to be present that day).

Ambient temperature and food availability (floral resources) positively affect bee activity levels (reviewed in Willmer & Stone 2004) and reproduction (Kim 1996; Richards 1996; Kim 1999; Peterson & Roitberg 2006). Ambient temperature was monitored using a Hobo data-logger (Onset Computer Corp., Pocasset, MA, U.S.A.) that was placed inside each domicile in each cage. For analyses, we used the mean temperature for each day during the bees' active period from 1000 to 1500 hours.

We estimated the floral resources by counting open, unvisited flowers every other day in four 0.25 m^2 quadrats placed in four evenly-spaced locations within each cage. When an alfalfa flower is visited by a bee, pressure on the keel petal causes the flower to "trip," meaning the sexual column is released. The bee can then collect both pollen and nectar from the flower (Larkin & Graumann 1954). Thus, we used untripped flowers, identified by the exposed sexual columns, as representative samples for the floral resources available to bees. We conducted the first flower survey before bees were introduced to ensure floral resources were adequate for nesting success to occur and determine the maximum floral resources available to bees.

Males and females were counted every night in nest tunnels. Though the numbers of males and females remained relatively stable, the sex ratios and bee densities did vary from the initial starting values. A mean sex ratio and bee density was calculated for each cage and each female (across the days she lived), and those values were used in our analyses. Also at night, we removed each nest tunnel's paper straw and measured the distance from the back end of the nest to the end of any nest construction to determine the progress made each day by each bee. We calculated the mean nest progression (mm) for each female from these data.

To determine total offspring production, we removed and x-radiographed each nest at the conclusion of trials. X-radiography has no significant, negative effects on developing offspring (Stephen & Undurraga 1976; Maki et al. 1990). In the x-ray images, developing offspring are clearly visible within the individual cells (Figure 3.2). The nest-building distances from each day were compared to the x-ray images to measure offspring production, which was defined as the number of offspring each female produced each day. An offspring (of either sex) was counted if it developed to at least the prepupal stage. It was not possible, from the x-radiographs, to identify offspring that died before developing to this stage because females will also produce cells without an egg (Pitts-Singer 2004). Overwintering offspring were identified as pre-pupae. Early-emerging offspring developed past the prepupal stage and could be identified as pupae. All adult bees were removed and frozen, so we could make additional morphological measurements that were not used in these analyses.

Data analysis

Our analyses were restricted to 30 females from seven cages where we had simultaneously collected data on floral resources.

To determine which factors affected variables measured at the cage (treatment) (e.g., harassment rate, temperature, food availability, and photoperiod), we conducted linear and nonlinear (when appropriate) regression analyses level. $N = 7$ unless otherwise indicated.

To determine the factors affecting variables measured at the individual female level (e.g., overwintering and early-emerging offspring production), we used a linear regression model, the Generalized Linear Model (GLM) (Laird 2004) with robust standard errors adjusting for possible cage effects (SPSS 15, SPSS Inc., Chicago, IL, U.S.A.). $N = 30$ unless otherwise indicated.

The values for sex ratio and bee density differed from their initial starting values (Table 3.1) due to disappearances of males or females and were calculated as means over the seven days. Five females in three of the cages were not present for the entire seven days, because they either died or escaped, so separate mean harassment rates, temperatures, floral resources, and photoperiods were calculated for each of them only using measures from days they were present.

All descriptive statistics are reported as mean (SD, range).

RESULTS

The mean harassment rate averaged 1.97 (1.02, 1.04 – 3.67) male-initiated chases per 10 min. per female. The mean temperature averaged 29.52 (5.31, 25.3 – 37.0) degrees C. There were an average of 16783.3 (9880.5, 4113.9 – 39287.7) untripped flowers per female per day. As flowers were tripped, they were regularly replenished by new untripped flowers. For all analyses and tables, mean untripped flowers was scaled by dividing by 1000. Mean photoperiod averaged 891.9 (20.3, 838.3 – 901.7) minutes, though only four females experienced a mean photoperiod less than 891 minutes.

The independent variables were not correlated with one another, with the exception of harassment and temperature. Mean temperature was positively correlated with mean harassment rate (Spearman correlation: $r = 0.811$, $p = 0.0269$). However, the two variables were not collinear (tolerance > 0.2). There was no significant correlation between mean temperature and mean number of untripped flowers (Spearman correlation: $r = -0.631$, $p = 0.1289$). There was no significant correlation between mean number of untripped flowers and mean harassment rate (Linear Spearman correlation: $r = -0.464$, $p = 0.2939$). There was also no significant correlation between mean photoperiod and mean temperature (Spearman correlation: $r = 0.527$, $p = 0.2239$), mean number of untripped flowers (Spearman correlation: $r = -0.3063$, $p = 0.5040$), or mean harassment rate (Spearman correlation: $r = -0.414$, $p = 0.3553$). A non-parametric test was used because variables were non-normal.

A model with mean harassment rate, mean temperature, mean untripped flowers, and mean photoperiod as main effects had a significant effect on the mean overwintering offspring produced per female per day (GLM: R2 = 0.675, Table 4.1). We also found an effect on the overwintering proportion of all offspring when photoperiod was removed from the model (GLM: R2 = 0.264, Table 4.2). As harassment increased, the number and proportion of overwintering offspring decreased. As temperature and flowers increased, the number and proportion of overwintering offspring increased. As photoperiod increased, only the number of overwintering offspring increased.

A model with mean harassment rate, mean temperature, mean untripped flowers, and mean photoperiod as main effects had a significant effect on the mean early-emerging offspring produced per female per day (GLM: R2 = 0.057, Table 4.3) and the proportion of all offspring produced that were early emerging (GLM: R2 = 0.145, Table 4.4). As harassment and photoperiod increased, the number and proportion of early-emerging offspring increased. As temperature and flowers increased, the number and proportion of early-emerging offspring decreased.

Overall, females that produced offspring earlier in the season produced a lower proportion of overwintering offspring (GLM: B+SE = 0.023+0.002, R2 = 0.453, p < 0.0001; Fig. 4.1) and a higher proportion of early-emerging offspring (GLM: B+SE = -0.014+0.003, R2 = 0.086, p < 0.0001).

DISCUSSION

Female alfalfa leafcutting bees exposed to unfavorable foraging conditions produce more early-emerging offspring and fewer overwintering offspring. This pattern is most consistent with females making the best of a bad situation (i.e., an early-emerging offspring is better than no offspring at all). This pattern does not support the alternative hypothesis of early-emerging females being marginal offspring that are most often produced when conditions are favorable.

The most interesting finding is that increased mean rates of sexual harassment correlate with a decrease in the number and proportion of offspring that undergo diapause. Harassment increases the duration of foraging trips because females spend more time fleeing from males. Females cannot make as many foraging trips given the time they have to forage resulting in lower overall offspring production (Chapter 3).

We also found effects of temperature and food availability on early-emerging offspring production that was consistent with previous research. Females exposed to lower mean temperatures have to wait longer for the temperature to reach the minimum temperature needed for flight and quit foraging earlier in the afternoon. Thus, the period of time during which females forage is shortened under lower temperatures. Also, because ambient temperature is correlated with bee activity levels, females in lower temperatures could not forage as quickly as females in higher temperatures. Females that experienced low food availability would have had to foraging longer and farther to gather enough resources for each cell. It is also possible, however, that these environmental factors somehow influenced offspring directly rather than indirectly through maternal behaviors such as foraging.

Photoperiod was negatively correlated with the proportion of offspring that were early-emerging. We conducted experiments in July and August, so photoperiod decreased as the season continued. Thus, our data were not completely consistent with the idea that females produce more of their early-emerging offspring early in the season. However, our dataset did not completely cover the alfalfa leafcutting bees' season, which can start as early as late May. This, in addition to missing the summer equinox when the change in photoperiod switches from increasing to decreasing, may have interfered with our ability to get a true picture of photoperiod's effect on offspring production. Also, while most of the early-emerging offspring are produced earlier in the season, this may not be due to a direct effect of photoperiod. There may be a more complex interaction between photoperiod and other environmental factors that results in more early-emerging offspring being produced earlier.

Interestingly, six females produced all overwintering and eight females produced all early-emerging offspring. Females that produce only early-emerging offspring may be the females least capable or skilled at foraging, avoiding and resisting male harassment, competing with other females. Recent work has shown that the number of early-emerging offspring increases when there is more competition among females for nesting space (Pitts-Singer, T. L. unpublished data). Alternatively, certain females may choose to specialize in producing only one type of offspring to avoid having early-emerging offspring that kill overwintering offspring as they chew their way out of the nest tunnel. When overwintering and early-emerging offspring both produced in a single nest tunnel, overwintering offspring can be killed as early-emerging offspring chew threw their cells on their way outside (Tepedino & Frohlich 1984).

Further research is needed to fully understand the extent that environmental and maternal factors affect production of early-emerging offspring. This study system is very conformable to larger scale studies as long as daily, behavioral monitoring is not necessary. Large numbers of bees could be released onto large alfalfa fields and followed over an entire season. X-radiography makes it possible to monitor the reproduction of each female including the size of provisions, while environmental factors are continuously measured. This more complete and long term data set would make it possible to untangle the complex relationship between the environmental conditions and early-emerging offspring production.

It is clear that sexual harassment by males has an impact on reproductive strategy and productivity within a season. Thus, harassment can have an indirect influence on offspring that is separate from the direct effects on female fecundity (Chapter 3). The degree to which this carries over across years in terms of population-level effects (e.g., population size and sex ratios) would be interesting to determine.

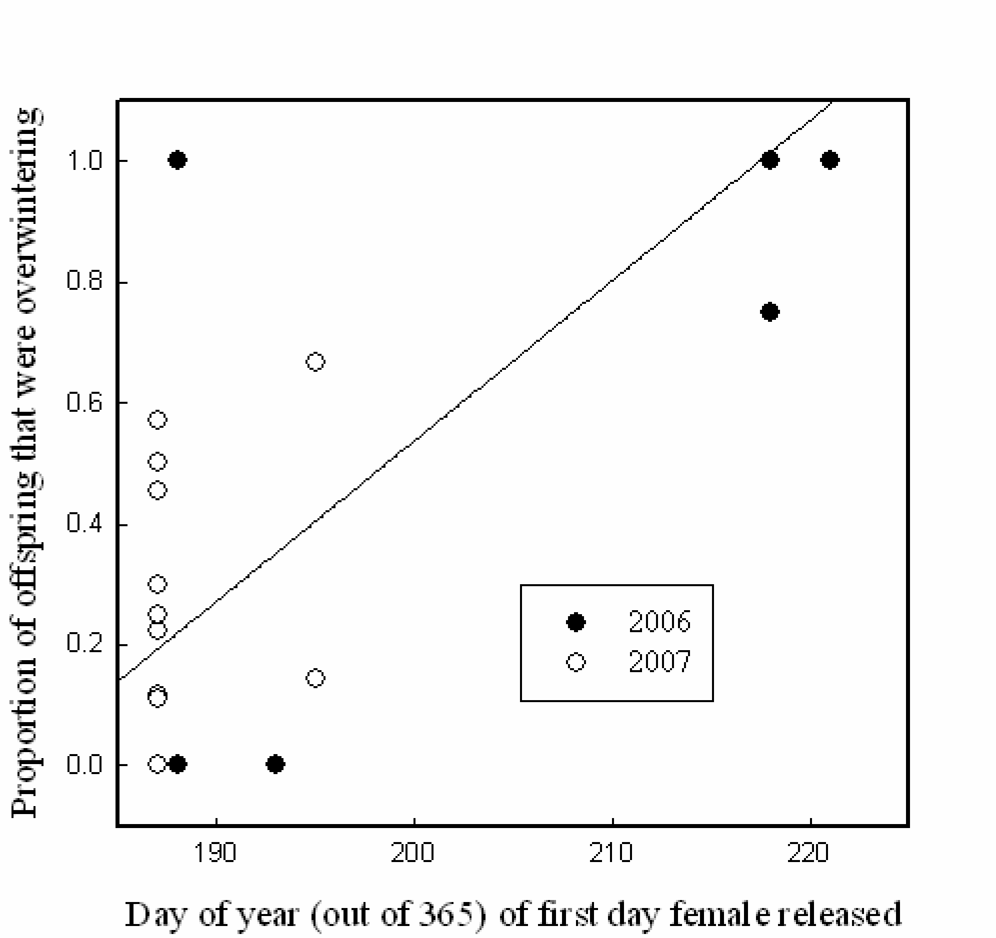

Figure 4.1 - Females that produced offspring earlier in the season produced a lower proportion of overwintering offspring (GLM: B+SE = 0.023+0.002, R2 = 0.453, $p < 0.0001$). Each female was given a day of year value based on the first day that she was released into the outdoor cage. For example, a female that was released on July 1, 2006 would be given a day of year of 182.

Table 4.1 - A generalized linear model with mean harassment rate, mean temperature, mean untripped flowers, and mean photoperiod as main effects had a significant effect on the mean overwintering offspring produced per female per day (GLM: $R^2 = 0.675$). All independent variables were means per female per day except photoperiod, which was a mean per day. The dependent variable was mean overwintering offspring produced per female per day.

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	B	SE	Lower	Upper	Wald Chi Square	df	Sig.
Intercept	6.487	1.692	3.170	9.804	14.695	1	0.0001
harassment rate	-0.473	0.019	-0.511	-0.436	614.945	1	< 0.0001
temperature	0.091	0.009	0.073	0.109	99.503	1	< 0.0001
untripped flowers	0.013	0.002	0.010	0.016	66.452	1	< 0.0001
photoperiod (min)	-0.009	0.002	-0.012	-0.006	31.635	1	< 0.0001
Scale	0.067						

Table 4.2 - A generalized linear model with mean harassment rate, mean temperature, and mean untripped flowers, as main effects had a significant effect on the mean proportion of offspring that were overwintering per female (GLM: $R^2 = 0.264$). Mean photoperiod did not have a significant effect. All independent variables were means per female per day except photoperiod, which was a mean per day. The dependent variable was mean proportion of offspring overwintering (arcsine-transformed).

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	B	SE	Lower	Upper	Wald Chi Square	df	Sig.
Intercept	-9.531	6.730	-22.72	3.659	2.006	1	0.1567
harassment rate	-0.716	0.226	-1.159	-0.272	10.007	1	< 0.0001
temperature	0.167	0.057	0.056	0.277	8.695	1	< 0.0001
untripped flowers	0.023	0.005	0.013	0.033	20.536	1	< 0.0001
photoperiod (min)	0.007	0.006	-0.005	0.019	1.214	1	0.2705
Scale	0.174						

Table 4.3 - A generalized linear model with mean harassment rate, mean temperature, mean untripped flowers, and mean photoperiod as main effects had a significant effect on the mean early-emerging offspring produced per female per day (GLM: $R^2 = 0.057$). All independent variables were means per female per day except photoperiod, which was a mean per day. The dependent variable was mean early-emerging offspring produced per female per day.

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	B	SE	Lower	Upper	Wald Chi Square	df	Sig.
Intercept	23.517	2.374	18.865	28.169	98.158	1	< 0.0001
harassment rate	0.717	0.031	0.655	0.778	523.757	1	< 0.0001
temperature	-0.231	0.013	-0.256	-0.206	336.980	1	< 0.0001
untripped flowers	-0.028	0.002	-0.032	-0.025	276.193	1	< 0.0001
photoperiod (min)	-0.019	0.002	-0.024	-0.015	70.303	1	< 0.0001
Scale	0.274						

Table 4.4 - A generalized linear model with mean harassment rate, mean temperature, mean untripped flowers, and mean photoperiod as main effects had a significant effect on the mean proportion of offspring that were early-emerging per female (GLM: $R^2 = 0.145$). All independent variables were means per female per day except photoperiod, which was a mean per day. The dependent variable was proportion of offspring early emerging (arcsine-transformed).

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	B	SE	Lower	Upper	Wald Chi Square	df	Sig.
Intercept	15.803	2.587	10.732	20.874	37.310	1	< 0.0001
harassment rate	0.576	0.090	0.400	0.752	41.091	1	< 0.0001
temperature	-0.180	0.022	-0.224	-0.136	64.794	1	< 0.0001
untripped flowers	-0.019	0.002	-0.023	-0.015	77.557	1	< 0.0001
photoperiod (min)	-0.012	0.002	-0.017	-0.008	27.466	1	< 0.0001
Scale	0.162						

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CHAPTER 5

UNEXPECTED MALE MATE PREFERENCE FOR PREVIOUSLY MATED FEMALES

ABSTRACT

Mate choice by males is expected if males invest in mating and thus maximize their reproductive success by being more selective. Previous studies have found that males often discriminate based on the mating status of females, usually preferring to mate with virgin over non-virgin females. Mate preferences also need not be universal and can vary based on the characteristics of the individual choosing male. We examined individual variation in male mate preference in alfalfa leafcutting bees (*Megachile rotundata*) in two laboratory experiments. For Experiment 1, male-female pairs were placed under a 10 cm Petri dish and left to interact for one hour. Larger males touched (i.e., were in physical contact) females for longer durations. For trials where males attempted to mate, larger males attempted to mate at a higher rate. Female body size had no significant effect on the rate or duration of interactions. For Experiment 2, males were presented with two females, a virgin and mated, and we recorded the time males spent near each female. Larger males spent more time near mated than near virgin females. The percent time spent near virgin females correlated with virgin and mated female body sizes. Neither female's body size correlated with percent time spent near mated females. Our results demonstrate that there is individual variation in male mate preferences (based on male body size) for female mating status with larger males preferring mated females.

We discuss reasons that might explain males preferring mated over virgin females and the possible impact of male mate choice on female fitness and sexual conflict.

INTRODUCTION

Determining the factors that affect male mating behavior is important for understanding how sexual conflict operates. While most of the research on intersexual selection has focused on female choice (Andersson 1994) or female resistance to mating (Cameron et al. 2003; Parker 2006), there are conditions under which male choosiness should evolve. Two such conditions are high variance in the quality of the opposite sex and costly mating investments by males. Mating investment is "investment in each mating that occurs at the cost of the male's ability to invest in future matings" (Bonduriansky 2001) and can include energetic investment, survival reduction (e.g. injuries), or resources (e.g., nuptial gifts).

Studies of male mate preferences have focused on a variety of female traits preferred by males (reviewed in Bonduriansky 2001). One factor found to affect male mate choice where males have preferences for virgin females (Aquiloni & Gherardi 2008; Martel et al. 2008; McCartney & Heller 2008; Zahradnik et al. 2008). To our knowledge, overall preferences for previously mated females have not been found. Males might prefer virgins to avoid sperm competition, at least until she mates again (Aquiloni & Gherardi 2008; Martel et al. 2008; Zahradnik et al. 2008). Virginity could also be an indicator of youth, which would mean more eggs to fertilize than would be found in an older, mated female (McCartney & Heller 2008).

Studies of solitary bees reveal that males are attracted to females through olfactory and visual cues (Cane & Tengö 1981; Smith & Ayasse 1987; Tengö et al. 1988; Paulmier et al. 1999). Males are especially attracted to newly emerged (and thus virgin) females (Shimron & Hefetz 1985; Wcislo 1992), though, to our knowledge, no study of solitary bees has directly compared the attraction of males to virgin versus mated females of the same age.

Male choosiness can also be costly because rejecting a female means losing out on a potential mate (Parker 1983). Thus, males are expected to be choosy only in the presence of significant mating investment costs. In species where mating opportunities may be relatively scarce because of high male-male competition, low female receptivity, low population sizes, or male-biased sex ratios, choosiness should be low or absent (Emlen & Oring 1977; Parker 1983). In addition, we would also expect individual variation in the degree of male choosiness. Low quality males, those least capable of successfully mating, should discriminate the least since their mating opportunities are rare (Parker 1983). Previous research has found evidence of individual variation in male mate preferences for female size (Foote 1988; Basolo 2004) and color (Pierotti et al. 2008).

We tested for the presence of individual variation in male mate preferences in a solitary bee, the alfalfa leafcutting bee (*Megachile rotundata* Fabricius; Hymenoptera: Megachilidae). Pronounced male mate choice is not necessarily expected in this species. It has a male-biased sex ratio, ranging from 1:1 – 5:1 males: females (Klostermeyer et al. 1973; Richards 1993; Pitts-Singer & James 2005), with sexual coercion by males

(Chapter 3) and strong competition for females, who typically mate only once providing them with a lifetime supply of sperm (Richards 1994). Males respond to any visual and olfactory cues that resemble those of females (Paulmier et al. 1999). All females appear intensely resistant to all mating attempts by males, with the intensity of resistance appearing to increase after mating (Gerber & Klostermeyer 1972).

There is variation in mating status among females as new virgins are continuously emerging and available throughout the season (Gerber & Klostermeyer 1972). Females will occasionally mate more than once (Blanchetot 1992), so any female could be a potential mate. Nevertheless, every pursuit of a female will cost time, energy, and possible injury (e.g. females of a related species *M. apicalis* were observed stinging and chewing the legs off of males, Kim, Jong-yoon, personal communication) Therefore, although still potentially costly to pursue, virgin females may be more attractive to choosy males than mated females. Females live approximately 30 days, and males live approximately 15-23 days, though many individuals of either sex live longer. Therefore, males are present throughout most of the females' lifespans.

Males may discriminate between females using olfactory cues, visual cues, or both. Visual stimuli increases the activity level and sexual interest of males (Paulmier et al. 1999). Cuticular hydrocarbons are known sexual pheromones in this species, and the composition of these hydrocarbons differs across young females, old females, and males (Paulmier et al. 1999), though it is unknown whether mating, per se, alters the composition of a female's cuticular hydrocarbons.

Male body size should affect male mate preferences. Wittman & Blochtein (1995) suggested that larger males might be a better "fit" for a wider variety of female sizes and, thus, be better able to subdue females. If this is indeed the case, small males should be less choosy because mating opportunities are rarer. However, we could also predict that larger males should be less choosy because their mating opportunities are common and, being better able to subdue females, their mating investment costs of time and energy would be lower. Similarly, female size could alter preferences in opposing directions. Males could prefer to mate with smaller females that are easier and less risky to subdue. Alternatively, males could prefer larger females because of higher potential fecundity (Klostermeyer et al. 1973).

We examined male mate preferences for virgin females and individual variation in the strength of the preference based on male body size with two experiments. For Experiment 1 – Male-Female Pairs we placed males and females in pairs to test for individual variation in male behavior. We monitored the frequency and duration of physical contact between the male and female and predicted that male body size would affect the time spent touching and attempting to mate with the female. For Experiment 2 – Dichotomous Choice Test, males were given a choice of two females, one virgin and one mated. We monitored the time spent near each female. We predicted that males should prefer virgin over mated females and that male body size would affect the strength of the preference.

GENERAL METHODS

Study species

The mating system of alfalfa leafcutting bees appears to be a form of scramble competition (Gerber & Klostermeyer 1972), and the male mating strategy resembles sexual coercion through sexual harassment with apparent attempts at forced copulations (Clutton-Brock & Parker 1995). Males patrol and chase females near nesting and foraging sites, and they pounce on females found resting, foraging at flowers, entering nest tunnels, or flying about. This harassment appears to interfere with females' nesting activities (Gerber & Klostermeyer 1972). Once a male captures a female, he moves to dorsally mount her and copulate (Wittmann & Blochtein 1995).

Mating status would often correlate with female age. Females usually mate within the first few days post-eclosion (Gerber & Klostermeyer 1972; Richards 1984), so the average mated female is likely to be older than a virgin female. Younger females would be alive longer and have more eggs available to fertilize than older females.

All experiments

Bees were obtained from a commercial bee supplier (JWM Leafcutters, Inc., Nampa, ID, U.S.A.) as pre-pupae in leaf-covered cocoons. Leafcutting bees overwinter as pre-pupae and are stored in this state over the winter season (Gerber & Klostermeyer 1972). Pre-pupae were incubated (in staggered batches of approximately 60 bees) individually in clear gelatin capsules (Capsuline brand size 00) at 30 degrees C for 2-3 weeks until they emerged as adults (Pankiw et al. 1979; Richards 1984). Each bee was incubated separately from other bees to make sure they remained virgins after emergence. Following each experiment, the bees were killed via hypothermia and their intertegular widths were measured to determine body size (Cane 1987). From this point forth, intertegular width will be referred to as “body size.” Only bees (males and females) of equal age were used in each trial.

Data were analyzed using SPSS (Version 15, SPSS Inc., Chicago, IL, U.S.A.). All descriptive statistics reported as mean (SD, range).

EXPERIMENT 1 – MALE-FEMALE PAIR

Methods

For 16 trials, virgin males and virgin females were paired randomly with respect to size on a mating arena consisting of white poster board and a 10 cm Petri dish cover (Figure 5.1a). We scored one hour of interactions in two categories: (1) *Touch*, a male and female in physical contact, and (2) *Mating attempt*, a struggle where the male tried to dorsally mount the female. For each interaction, the initiating individual and the duration were recorded.

Results

The average male body size was 2.59 mm (0.15, 2.37 – 2.92). Female body size averaged 2.69 mm (0.16, 2.38 – 2.99). Pairs spent an average of 19.5% (25, 0.7 – 92) of their time interacting and an average of 15.5% (25, 0.1 – 92) of their time touching. Of the males that attempted to mate, they spent 5.5% (13, 0.01 – 43) of their time in mating attempts.

For all interactions combined, touches alone, and mating attempts alone we calculated the following time variables (both total and male-initiated versions of each): proportion of time interacting, mean time per interaction, and rate of interaction. Since all mating attempts were male-initiated, only total versions of each variable were calculated. Several variables were log-transformed due to non-normal distributions of errors (Table 5.1). The distributions of errors were normally distributed after transformation.

Effect on total interactions

Male body size affected the total interactions between the paired males and females, but female body size did not. As male body size increased, there was an increase in the proportion of time interacting (Linear Regression: $F_{1,14} = 9.392$, $R^2 = 0.36$, $p = 0.0084$; Figure 5.2a) and mean time per interaction (Linear Regression: $F_{1,14} = 9.760$, $R^2 = 0.37$, $p = 0.0075$), but not the rate of interactions (Linear Regression: $F_{1,14} = 0.167$, $R^2 < 0.01$, $p = 0.6890$). Also, as male body size increased, there was an increase in the proportion of time spent in male-initiated interactions (Linear Regression: $F_{1,14} = 9.364$, $R^2 = 0.36$, $p = 0.0085$), mean time per male-initiated interaction (Linear Regression: $F_{1,14} = 5.770$, $R^2 = 0.24$, $p = 0.0307$), and the rate of male-initiated interactions (Linear Regression: $F_{1,14} = 6.137$, $R^2 = 0.26$, $p = 0.0266$). Female body size had no significant effect on any interaction time variables.

When male and female body sizes were included in the same model, pairs of large males and females spent a higher proportion of time interacting (Linear Regression: $F_{2,13} = 10.945$, $R^2 = 0.57$, $p = 0.0016$; Table 5.2) and a higher mean time per interaction (Linear Regression: $F_{2,13} = 13.115$, $R^2 = 0.62$, $p = 0.0008$; Table 5.3).

Effect on touches

Touches between males and females were affected by the male body size, but not by the female body size. There was a significant increase in the mean time per touch (Linear Regression: $F_{1,14} = 5.545$, $R^2 = 0.23$, $p = 0.0337$), proportion of time spent in male-initiated touches (Linear Regression: $F_{1,14} = 11.193$, $R^2 = 0.41$, $p = 0.0048$), and mean time per male-initiated touch (Linear Regression: $F_{1,14} = 5.212$, $R^2 = 0.22$, $p = 0.0386$) as male body size increased. The proportion of time spent touching also increased with male body size at a marginally significant rate (Linear Regression: $F_{1,14} = 4.506$, $R^2 = 0.19$, $p = 0.0520$). Female body size had no significant effect on any touch time variables.

Effect on mating attempts

Among the trials where mating attempts were observed (10 out of the 16 trials), larger males attempted to mate at a higher rate (Linear Regression: $F_{1,8} = 15.369$, $R^2 = 0.62$, $p = 0.0044$; Figure 5.2b). Female body size had no significant effect on any mating attempt time variables.

There was no significant difference in male or female body sizes between trials where mating attempts did and did not occur (male: T-test: $t_{14} = -0.437$, $p = 0.6591$; female: T-test: $t_{14} = -0.742$, $p = 0.4770$).

EXPERIMENT 2 – DICHOTOMOUS CHOICE TEST

Methods

We placed each bee into one of three cages – virgin males, virgin females, and mated bees (males and females). Virgin males and virgin females were only housed with other virgin males or females, respectively. To create mated females, some of the newly emerged females were placed in a cage with males at a ratio of 3 males to 1 female, which is the average sex ratio of this species in the wild (Gerber & Klostermeyer 1972). The mating status of each female was determined randomly with respect to female emergence. All bees were fed a 10% honey water solution at 30 degrees C and held for at least 24 hours before being used for the experiments.

We conducted 24 choice tests using a Y-shaped glass tube. Male mate preference was indicated by time spent in close proximity to a female. Males were initially placed in a smaller glass cylinder at the bottom end of the “Y” and given 30 minutes to enter the tube after which they had 30 minutes to explore. At either end of the “Y” were females that varied in their mating status. The females were separated from the male by fiberglass window screening to allow for visual and olfactory (tactile and volatile) contact and prevent possible coercive interactions (Figure 5.1b). Males visited both ends of the tube in every trial, so they did have opportunities to observe and compare both females. All trials were recorded using 8mm camcorders. Glassware was regularly cleaned using Multi-Terge detergent and acetone to remove any pheromones or other residues, and fresh screening was regularly replaced for each trial. A similar experimental design demonstrated female preference for scents in nesting materials (Pitts-Singer 2007). Thus bees were known to be able to navigate through the tubes.

We scored the videotapes by measuring how much time males spent in the furthest two-thirds of each branch of the tube. From this we calculated the percent time that males spent near each female. We also recorded the first branch the male traveled to and the time until this occurred. Relative body sizes were calculated by dividing the female's body size by the male's body size. To calculate an index of male mate preference, we divided the proportion of time spent near the mated female by the proportion of time spent near the virgin female.

Results

The average male body size was 2.93 mm (0.24, 2.46-3.54). Female body size averaged 3.10 mm (0.24, 2.63 – 3.58) with virgin female body size averaging 3.14 mm (0.23, 2.72 – 3.58) and mated female body size averaging 3.06 mm (0.24, 2.63 – 3.45).

Males spent an average of 75% (15, 44 – 97) of their time near females. Males did not spend a significantly higher proportion of time near one female than with the other (Paired t-test: t = -0.524, p = 0.6056).

There was no difference in the percent time spent near virgin or mated females (Paired T-test: $t = 0.058$, $p = 0.9545$), the number of visits to each female (Paired T-test: t

= -0.524, p = 0.6056), or the mean time spent per visit (Paired T-test: t = -0.553, p = 0.5856) by the male.

There was also no difference in the percent time spent near the larger or smaller female (Paired t-test: t = -0.013, p = 0.9894), the number of visits to each female (Paired t-test: t = 1.547, p = 0.1354), or the mean time spent per visit (Paired t-test: t = 0.023, p = 0.9822).

Male body size

Male body size correlated with the time spent near each female as well as the male mate preference index. The percent time spent near virgin females decreased as male body size increased (Linear regression: $F_{1,22}$ = 6.941, R^2 = 0.21, p = 0.0151; Figure 5.3a). Percent time spent near virgins was log-transformed due to the skewed distribution of the residuals. The percent time spent near mated females increased with male body size, though the trend was marginally significant (Linear regression: $F_{1,22}$ = 4.118, R^2 = 0.12, p = 0.0547; Figure 5.3b). The male mate preference index increased with male body size (Linear regression: $F_{1,22}$ = 11.790, R^2 = 0.32, p = 0.0024). We log-transformed the first male mate preference index due to a single outlier (Cook's distance = 3.01). When reanalyzed, the effect was only close to significant (Linear regression: $F_{1,22}$ = 3.934, R^2 = 0.11, p = 0.0599; Figure 5.3c).

Female body size

The time males spent with virgin females correlated with the relative body sizes of both females. The percent time spent near virgin females increased with the relative body size of mated females (Linear regression: $F_{1,22} = 4.988$, $R^2 = 0.15$, $p = 0.0360$; Figure 5.4a). Percent time near virgins also increased with relative virgin size, though the trend is not significant (Linear regression: $F_{1,22} = 3.609$, $R^2 = 0.10$, $p = 0.0707$; Figure 5.4b). For these two analyses, percent time spent near virgins was log-transformed due to the non-normal distribution of errors. There was no effect of absolute virgin or mated body sizes (virgin: Linear regression: $F_{1,22} = 0.172$, $R^2 < 0.01$, $p = 6820$; mated: Linear regression: $F_{1,22} = 1.879$, $R^2 = 0.04$, $p = 0.1843$) on percent time near the virgin female.

The time that males spent with mated females was not correlated with either female's body size. The percent time spent near mated females was not affected by the absolute virgin body size (Linear regression: $F_{1,22} = 0.005$, $R^2 < 0.01$, $p = 0.9462$), relative virgin body size (Linear regression: $F_{1,22} = 1.200$, $R^2 = 0.01$, $p = 0.2853$), absolute mated body size (Linear regression: $F_{1,22} = 1.056$, $R^2 < 0.01$, $p = 0.3153$), or relative mated body size (Linear regression: $F_{1,22} = 2.676$, $R^2 = 0.07$, $p = 0.1161$.

The relative difference in female body sizes (calculated by dividing the body size of the mated female by the virgin female) showed no effect on the time spent near either the virgin female (Linear regression: $F_{1,22} = 1.054$, $R^2 = 0.06$ $p = 0.3157$) or the mated female (Linear regression: $F_{1,22} = 0.631$, $R^2 < 0.01$ $p = 0.4354$) nor on the strength of male mate preference (Linear regression: $F_{1,22} = 0.009$, $R^2 < 0.01$, $p = 0.9236$).

DISCUSSION

In *M. rotundata*, there is individual, size-based variation in male mate preferences for mating status that is unaffected by female size. Larger males are also more likely to attempt to mate. Males, on average, did not prefer virgin to mated females, but males did show individual preferences based on male body size -- only the smaller males spent significantly more time with virgin than mated females. Mated females are known anecdotally to be more resistant. Larger males may have an advantage over small males at subduing females. Thus, larger males may not need to spend as much time pursuing the easier-to-subdue virgin females and are able to devote time with the harder targets (mated females). Yet it is unclear why males, large or small, would spend more time near mated females rather than virgins, since only a fraction of her eggs may be eventually fertilized.

The difference in preferences could be due to differences in competitive abilities of males. Elgar et al. (2003) found that male orb-web spiders, *Nephila plumipes*, that were heavier preferred to mate with virgin females, while lighter males preferred mated females. They suggested this was due to the heavier males' ability to exclude other males from access to the more desirable, virgin females. Studies of male competition in solitary bees have found that smaller males are at a disadvantage when competing for access to females (Stone et al. 1995). On the other hand, there could be an advantage to small size if smaller males are somehow harder to dislodge from females by competitors. Also, if females prefer mating with smaller males, they may reduce their resistance to these males, so larger males would do better by focusing some of their time on the less desirable, mated females. It is also possible that the mated females were somehow communicating a lower degree of resistance (or higher degree of receptivity) due to their prior mating experience. To create mated females, virgins were housed with young, virgin males. If females have a preference for older (Jones & Elgar 2004) or previously mated males (Wedell & Ritchie 2004; Krupke et al. 2008; Iyengar 2009) over young, virgin males, the mated females in our experiment may have sought a second mating with a more experienced male. More research is needed to understand the degree of female choice in this species and the factors that cause a female to mate a second time, which is rare.

A preference for mated females could also be a form of mate copying where mated females appear easier to subdue because they have already been subdued by at least one other male. However, if this were the case, males of any size should spend more time near the mated female.

A preference for mated females could occur due to last male precedence. However, studies of several species of honeybees have found no evidence for a last male advantage, and the probability of paternity for males mating with multiply mated females is equal to the proportion of each male's sperm that is present in the female (Franck et al. 2002; Schluns et al. 2004; Schluns et al. 2005). Sperm precedence can vary greatly between species (Singh et al. 2002) and even between (Clark & Begun 1998) and within individuals (Mack et al. 2003), so further study is needed to determine the degree of sperm precedence in the alfalfa leafcutting bee.

The preferences we found are not likely to be due to rejection by females. In our Experiment 2, females were confined and could not flee from or otherwise reject males. Males may detect signs that rejection is more or less likely, but we expect the probability of rejection to contribute to a male's particular preferences. Because female resistance increases after her first mating, mated females would be expected to exhibit more of these rejection signs. If there is, in fact, a large male advantage, it is possible that large males ignore these signs and small males respond to these signs by spending less time near the mated females.

The result that the females' body sizes affected time spent near the virgin female but not near the mated female was unexpected. Also, the body size of the females relative to the males had this effect, while absolute body sizes did not. Males may be using female body size relative to their own, rather than absolute female body size, to judge the chances of success in subduing or mating with a given female.

The larger the mated female, the more time that was spent with the virgin with no change in time spent with the mated female. Larger females may be more difficult for males to subdue, and mated females are already more resistant to mating than virgins. A male presented with a large, mated female may do better by spending more time near the possibly easier-to-subdue virgin female and ensure that he has access to her. Also, if the mated female is large, there might be greater competition for access to the virgin female (under normal conditions), and males would do better to remain close to the virgin to secure access to her.

The proximate mechanism by which males distinguish between virgin and mated females is unknown. Our experimental design allowed males to able to use visual as well as olfactory cues, which are known to play a role in male sexual behavior (Paulmier et al. 1999). Because the composition of cuticular hydrocarbons in males and females differs, it is possible that males transfer hydrocarbons to females during mating that alters the females' scent (Thornhill & Alcock 1983; Arnaud & Haubruge 1999; Paxton et al. 1999). Studies by Paulmier et al. only compared the composition of young, virgin females to old, mated females, and did not compare virgin and mated females of the same age. It is possible that the physiology of egg production alters the composition. If males are able to visually assess mating status, it is unclear what cues they are using to do this. Female alfalfa leafcutting bees are known to use both olfactory (Raw 1992; Guédot et al. 2005, 2006) and visual (Campan et al. 1993; Campan & Lehrer 2002) cues in nest-building, so it is not surprising that males would also be able to use these types of cues.

Much of what we are calling male mate preferences comes from males simply being close to particular females. Though we observed mating attempts, as far as we could tell, we observed no actual copulations. However, proximity to males is frequently used as a proxy for female mate preference in dichotomous choice experiments (Houde 1997), and we believe it is appropriate for male mate preference in this species for several reasons. First, copulations are difficult to observe in this species and can be hard to distinguish from failed mating attempts where males are dislodged from females. Therefore, it would not have been possible to examine male preferences by directly measuring copulations. Females may also have some control over which males copulate by dislodging males with rapid abdominal thrusts (Wittmann & Blochtein 1995) and leg kicks (Rossi, personal observation). Thus, mating attempts alone inaccurately represent male mate preferences.

Second, an approach by males can be considered a “courtship” behavior. Males pursue and chase females. If they successfully capture and subdue a female, they mount her and attempt copulation. Therefore, an approach by a male can be viewed as analogous to a more traditional courtship behavior in a species with more traditional male courtship and female choice.

Finally, In Experiment 1, the fact that larger males were both more likely to touch females and more likely to attempt to mate suggests that a male’s proximity to a female is a suitable proxy for preference. Previous research has also found that male proximity to females is an indicator of male sexual receptivity and interest (Paulmier et al. 1999).

It is possible that the degree of choosiness we detected is different from what would normally be expressed in nature. Under natural conditions, males may not often be presented with two females that they are free to observe and investigate. Choosiness may be lower than that observed in our experiments. However, males’ behavior consists of frequent chases and tackling of females (and other males) at the nest and foraging sites where females are abundant. This may be a way to gain more information about the females to determine how much additional effort should be made towards them before moving on to another, nearby female. Thus, the choosiness measured here may be an accurate reflection of their natural levels after all. Even if it is not, the fact that males possess this ability to discriminate between females and express a preference at all indicates that males must exhibit choosiness at least some of the time.

Our results have broader implications for impact of sexual conflict on females. There is sexual harassment in this system, and it is important to understand how the economic costs of harassment could be affected by the states of the interacting individuals (Fricke et al. 2009). If females usually mate only once and would usually be older than virgins, one might expect that once a female mates, she is free from harassment. Because all nest-building and egg-laying takes place after a female has reproduced, mated females may be ignored by harassing males due to being the less desirable mate. However, larger males are spending more time with mated females. Thus, mated females would still be subjected to harassment by males in nature and would continue to pay any costs of that harassment as long as large males are present. In addition, since choosiness should be influenced by sex ratio (Emlen & Oring 1977; Parker 1983), so the costs of harassment to virgin and mated females may vary with the sex ratio. Future work should test for additional female traits males may base mate choices on such as female age. In addition, there may be individual variation in male mate preferences based on traits other than body size. For example, older males may have different preferences from young males possibly becoming less choosy as the time they have left to mate shrinks.

Studies that test for male mate preferences tend to focus on overall preferences at the group level and leave out details about individual variation in preferences (e.g., Markow 1985; Simmons et al. 1994). For example, Zahradnik et al. (2008) found that, overall, male gastropods preferred to mate with virgin females when given a choice of a virgin and a mated female. However, some males chose to mate with the mated females before mating with virgins. Whether males that chose mated females over virgins differed from other males in traits like body size is not known. Future studies should look for variation in preferences among males even when there are strong preferences overall.

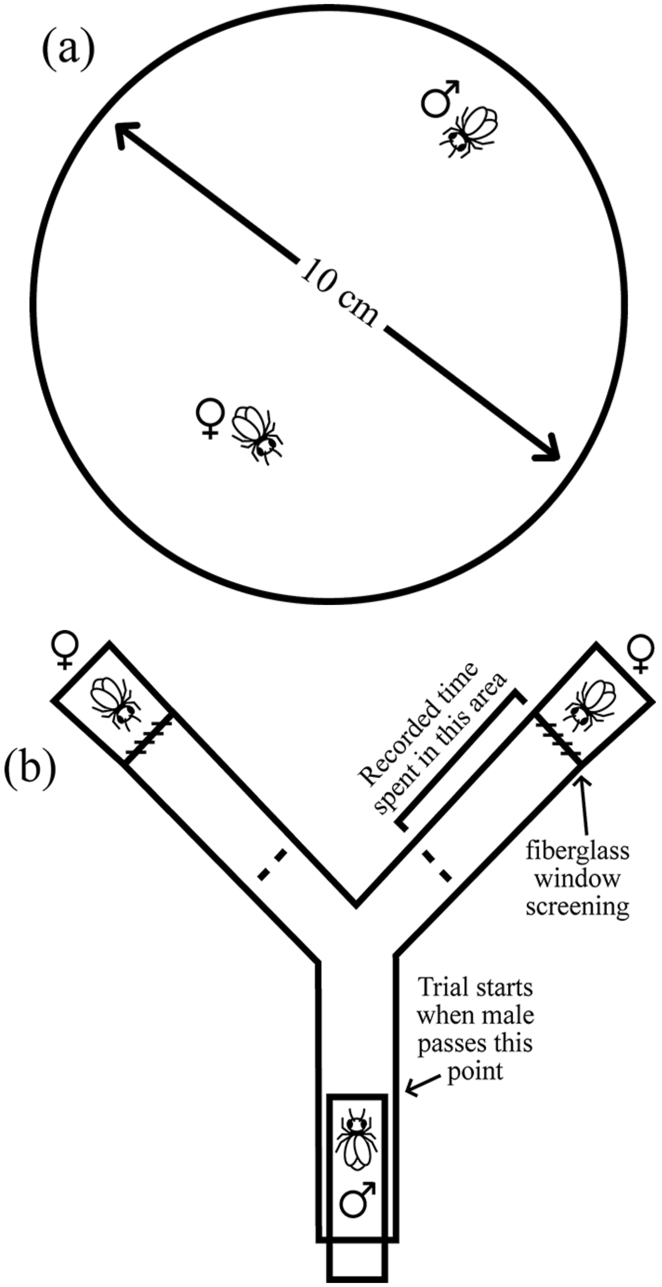

Figure 5.1 - (a) Testing arena for Experiment 1. (b) Testing arena for Experiment 2. Diagrams are not to scale.

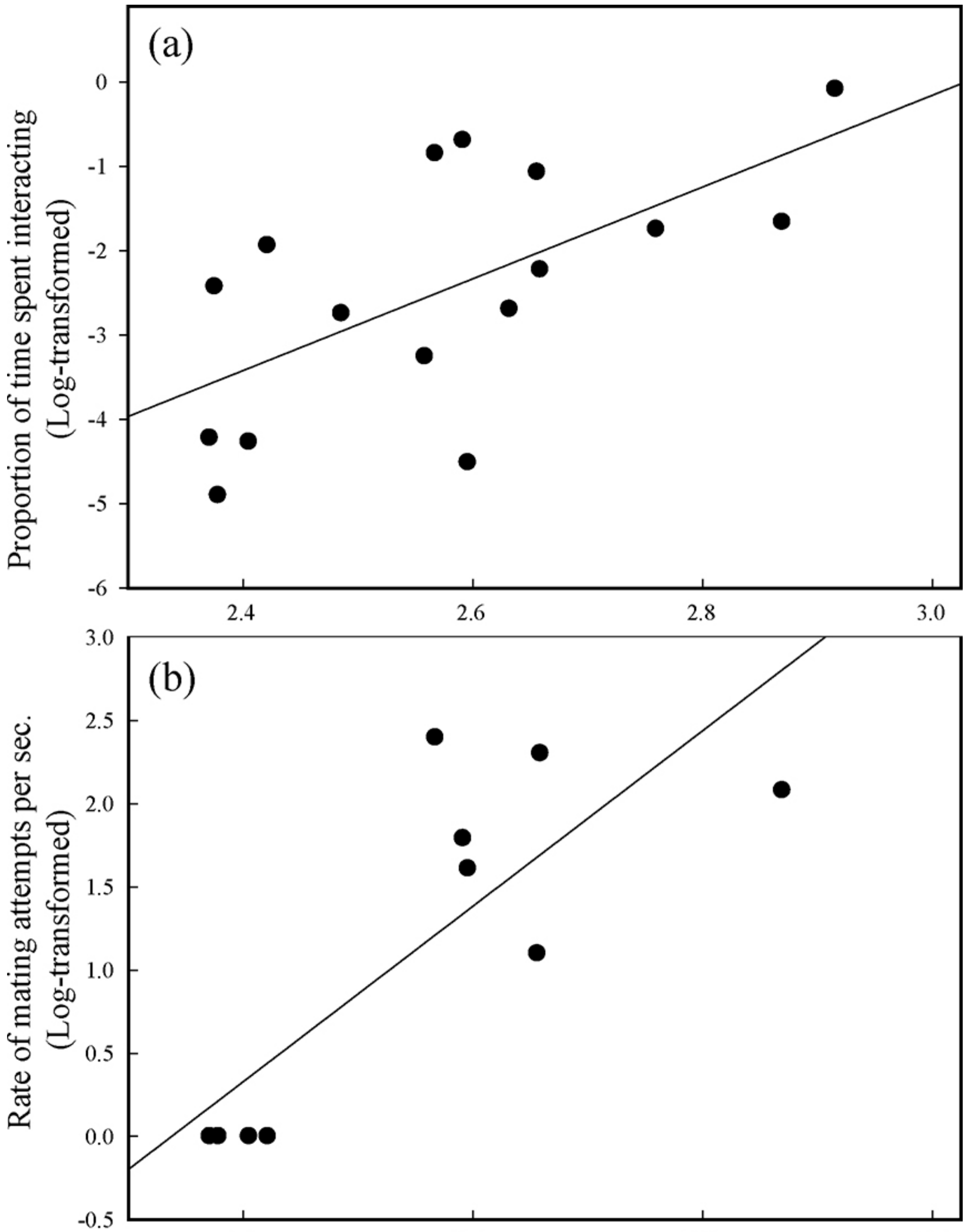

Figure 5.2 - (a) Larger males spent a greater proportion of time interacting (Linear Regression: $F_{1,14} = 9.392$, $R^2 = 0.36$, $p = 0.0084$). (b) In trials where males attempted to mate, larger males made attempts at a higher rate (Linear Regression: $F_{1,8} = 15.369$, $R^2 = 0.62$, $p = 0.0044$).

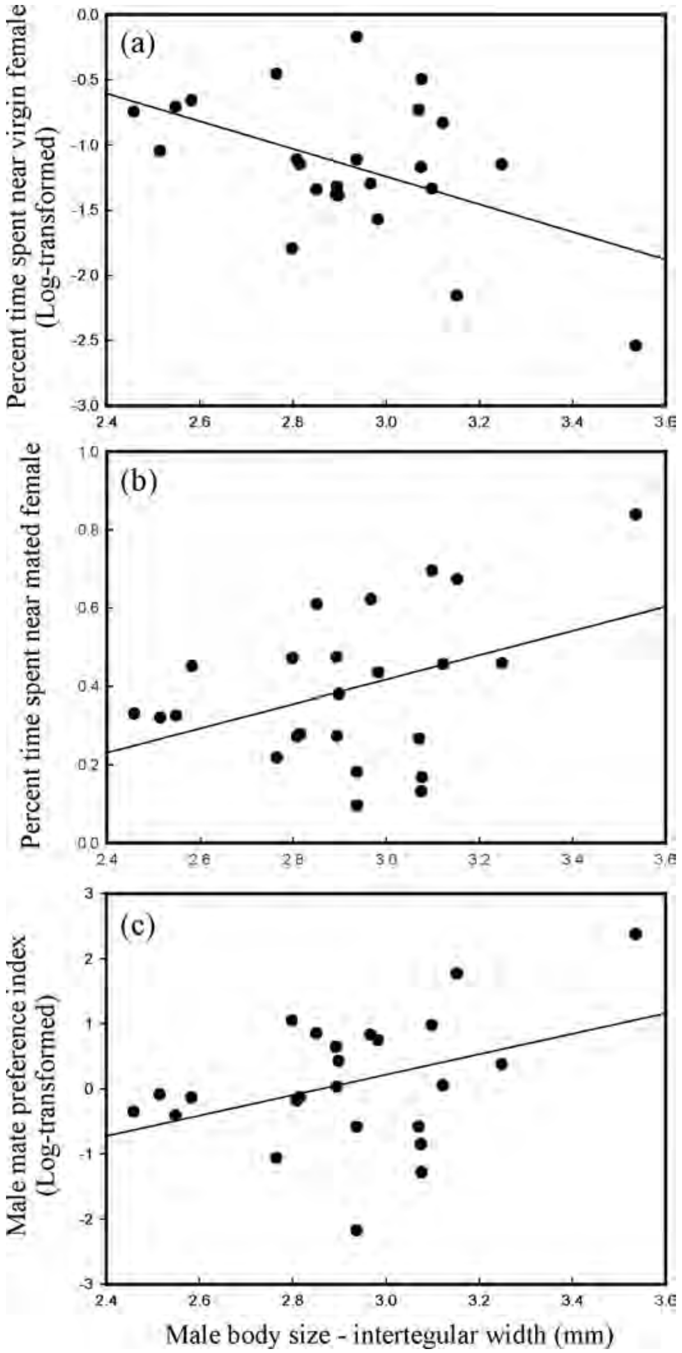

Figure 5.3 - (a) Larger males spent a lower percent of their time near virgin females (Linear regression: $F_{1,22} = 6.941$, $R^2 = 0.21$, $p = 0.0151$). (b) The percent time spent near mated females increases with male body size, though the trend is marginally significant (Linear regression: $F_{1,22} = 4.118$, $R^2 = 0.12$, $p = 0.0547$). (c) The male mate preference index increases with male body size (Linear regression: $F_{1,22} = 3.934$, $R^2 = 0.11$, $p = 0.0599$). Larger males spent more time with mated than virgin females.

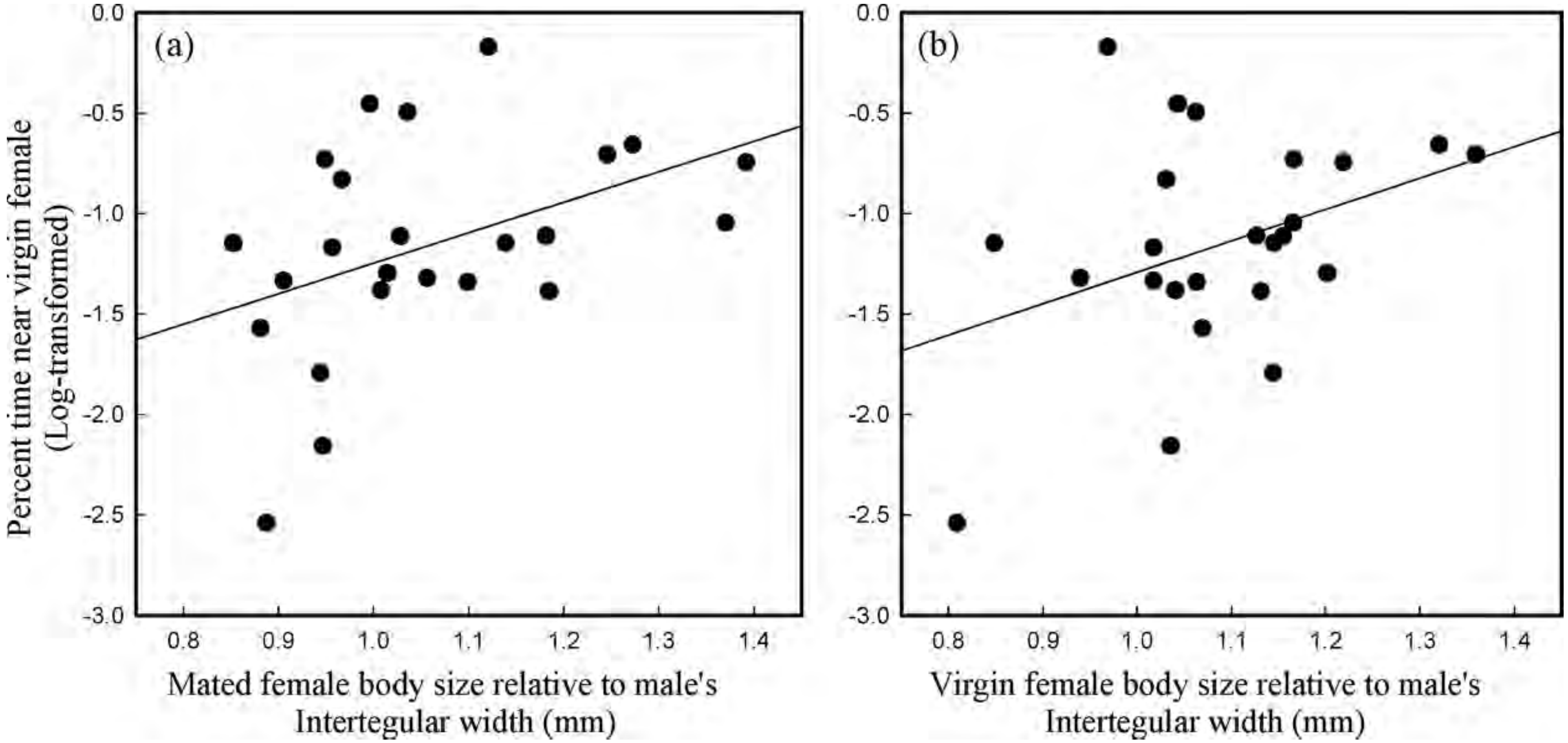

Figure 5.4 - (a) As the body size of the mated female relative to the male's body size increases, males spent a greater percent of their time near virgin females (Linear regression: $F_{1,22} = 4.988$, $R^2 = 0.15$, $p = 0.0360$). (b) As the body size of the virgin female relative to the male's body size increases, males spent a greater percent of their time near virgin females, though the trend is not significant (Linear regression: $F_{1,22} = 3.609$, $R^2 = 0.10$, $p = 0.0707$).

Table 5.1 - Regression results for the effects of male body size on interaction, touch, and mating attempt time variables.

	male intertegular width		
All interactions	$F_{1,14}$	R^2	p-value
Proportion of time spent interacting[a]	9.392	0.36	0.0084
Proportion of time spent in male-intiated interactions[a]	9.364	0.36	0.0085
Mean time per interaction[a]	9.760	0.37	0.0075
Mean time per male-intiated interactions[a]	5.770	0.24	0.0307
Rate of interactions[a]	0.167	< 0.01	0.6890
Rate of male-intiated interactions[a]	6.137	0.26	0.0266
Touches			
Proportion of time spent touching[b]	4.506	0.19	0.0520
Proportion of time spent in male-intiated touches	11.193	0.41	0.0048
Mean time per touch[b]	5.545	0.23	0.0337
Mean time per male-intiated touches[a]	5.212	0.22	0.0386
Rate of touches	0.989	< 0.01	0.3370
Rate of male-intiated touches	0.973	< 0.01	0.3406
Mating attempts			
Proportion of time spent in mating attempts	0.094[c]	< 0.01	0.7672
Mean time per mating attempt	0.142[c]	< 0.01	0.7162
Rate of mating attempts[b]	15.369	0.62	0.0044

[a] Variables log-transformed due to non-normal distributions of error

[b] Variables log-transformed due to outliers (Cook's distance > 1.0)

[c] $F_{1,8}$

Table 5.2 - Pairs of large males and females spend a higher proportion of time interacting. Male and female body size is measured as intertegular width (mm) ($R^2 = 0.57$).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	20.1	2	10.07	10.945	0.0016
Intercept	22.2	1	22.25	24.184	0.0003
male body size	15.3	1	15.28	16.616	0.0013
female body size	7.2	1	7.25	7.881	0.0148
Error	12.0	13	0.92		
Total	128.6	16			
Corrected Total	32.1	15			

Table 5.3 - Pairs of large males and females had a higher mean time per interaction. Male and female body size is measured as intertegular width (mm) ($R^2 = 0.62$).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10369.5	2	5184.77	13.115	0.0008
Intercept	9600.8	1	9600.83	24.286	0.0003
male body size	5497.9	1	5497.88	13.907	0.0025
female body size	6217.4	1	6217.36	15.727	0.0016
Error	5139.2	13	395.33		
Total	24542.9	16			
Corrected Total	15508.8	15			

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