Effects of multiple mating and male harassment on female fecundity and longevity in the seed beetle *Callosobruchus maculatus*.

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Abstract

Polyandry is a common mating system in many species, however females may experience considerable costs related to multiple mating including male harassment, energy use and in some cases physical damage. Male harassment of females may occur when there is sexual conflict related to mate choice or mating frequency. Females may, however, benefit from polyandry either indirectly by having more genetically viable offspring, or by increasing the genetic diversity among offspring. She may also gain direct benefits when the males provide resources, e.g. in the form of nuptial gifts like spermatophores, containing nutrients and water. These costs and benefits create a trade-off in multiple matings, with the optimum number of matings often differing between males and females. In the seed beetle *Callosobruchus maculatus*, males invest resources in spermatophores benefiting females, but females also might experience costs due to mating harassment and direct harm during copulations. By manipulating the female's exposure to males, either by varying the sex ratio or the period of time exposed, we test the effect of multiple mating on female longevity and fecundity. Female life span and fecundity (number of eggs laid) were higher in the group that was exposed to males for a short-term compared to females permanently exposed to males. For the permanently exposed females, life span decreased with the number of males, but there was no such effect on fecundity (eggs laid). These results indicate that there is both benefits and costs related to multiple mating. Females in the group with permanent exposure seemed to pass a threshold where the costs of male harassment outweighed the benefits of multiple mating resulting in a negative effect on longevity.

Contents

Introduction

The life-history trade-off between investing in current reproduction or future survival impacts an individual's lifetime reproductive success (Johns et al., 2018). Several factors can affect the longevity and fecundity of an individual, including access to nutrients, competition, and exposure to predators. For instance, more resources expended on reproduction are traded off with resources allocated to other processes like survival (Stearns, 1989) which in turn can reduce longevity (Arnqvist & Nilsson, 2000). Other strategies can involve having fewer offspring, but multiple times, both with and without parental investment after mating. More resources invested in parental care reduces resources for the next reproductive attempt. Factors like egg size, number of eggs, number of hatched eggs, and size of offspring may all affect an individual's reproductive output.

Males and females often have different investment in reproduction (Trivers, 1972). An example of this difference is gamete size, with females producing larger gametes (eggs) than males. Because females use more resources per gamete, males can produce more gametes than females. This gives the females a reason to be choosy when selecting a male, because the female is limited by the number of gametes she can produce (Bleu, Bessa-Gomes, & Laloi, 2012). Males on the other hand are limited by the number of females they can fertilize, leading to male competition for fertilizations. This difference leads to sexual selection, where females are more selective when choosing a mate, while males attempt to mate with as many females as possible (Bateman, 1948; Bleu et al., 2012).

Even though one mating should in theory be enough to fertilize all the female´s eggs, females of many species still mate with multiple males (Arnqvist & Nilsson, 2000). There are multiple benefits to females from mating with several males such as increased genetic diversity in sperm and offspring, parental investment from several males, and in species with nuptial gifts, the female may receive nutrients from the males. There are also costs associated with multiple mating, for example increased competition among males, more energy expended on mating and increased harassment of the females. All these factors can influence both longevity and fecundity.

Sexually antagonistic coevolution can be seen as an evolutionary arms race between the sexes where one sex evolves traits that are beneficial for itself, but costly for the opposite sex (Rönn, Katvala, & Arnqvist, 2007), which may lead to sexual conflict. Species with multiple mating often have high levels of male-male competition and females often experience costs

from male harassment that can have negative effects on both longevity and fecundity (Li, Fail, & Shelton, 2015). An example of sexual antagonistic coevolution is the seed beetle *Callosobruchus maculatus*, where the males evolved spikes on the reproductive organ that can injure the females during mating (Eady, Hamilton, & Lyons, 2006). This is an adaptation towards the male-male competition with these spines having multiple benefits for the males (Rönn et al., 2007). With strong sperm competition, it might be beneficial to harm the female during mating in order to increase the time before the females remate. This limits the total number of matings of the female and more of the eggs may be fertilized by the focal male. The female *C. maculatus* then evolve counter-adaptations against this e.g. kicking to either prevent or reduce the time of copulation, leading to an evolutionary arms race (Rönn et al., 2007). Male harassment often occurs when there is conflict between the sexes due to a sexually antagonistic coevolution, and is quite common in species that reproduce sexually (Darden & Watts, 2012). There can be differences in evolutionary interest between the sexes of a species, without it leading to behavioural conflict (Parker, 2006). It is firstly in cases where mating optimums cannot be reached simultaneously by both the that sexual conflict occurs e.g. males that are limited by the number of matings will have a higher optimum number of matings than females. In these situations, male harassment is common.

Fecundity is a measure of an individual's relative breeding success (Hine, 2008). Several factors may influence fecundity, including age, size, mating strategy, and environmental factors such as access to nutrients and water (Bradshaw & McMahon, 2008). In some species seminal fluids can also affect fecundity. Many of these factors may also affect longevity, but the effect may be different. In *C. maculatus*, females with access to more males or seeds laid more eggs than females with access to fewer males or seeds, but the beetles that laid fewer eggs lived longer (Messina & Fry, 2003). This indicates that mating with fewer males might have a negative effect on fecundity, but a positive effect on longevity.

Females receiving nuptial gifts benefit from multiple matings. Such benefits are for instance an increase in longevity (Snook, 2014) or increased egg production (Arnqvist & Nilsson, 2000). These benefits of multiple mating select for polyandry in many species (Arnqvist, Nilsson, & Katvala, 2005). One of the most important factors determining polyandry is the operational sex ratio. The operational sex ratio is the ratio between sexually active males and females (Weir, Grant, & Hutchings, 2011).

Callosobruchus maculatus is an example of a polyandrous species. Here the females receive nuptial gifts from males through the male ejaculate (spermatophores). From these nuptial gifts, females may gain nutritional and genetic benefits (Moya-Laraño & Fox, 2006). Virgin males ejaculate larger spermatophores (Fox, Hickman, Raleigh, & Mousseau, 1995) and spermatophore size is correlated negatively with the number of matings (Eady et al., 2006). These nuptial gifts might make the females inclined to engage in multiple matings for a greater chance of mating with several virgins in order to get more nuptial gifts per mating. Fox (1993a) found that females which had mated with several males lived longer than females mating with only one male. In addition to this, multiple matings resulted in females laying and an increased number of eggs (Wasserman and Asami, 1985; Credland and Wright, 1989; Fox, 1993a) and Fox (1993b) found that multiple mating and feeding resulted in larger eggs.

The objective of the study was to look at how mating interactions and opportunities for multiple male matings affected longevity and fecundity in terms of the number of days lived and the number of eggs laid in female *Callosobruchus maculatus*.

In an experiment, we tested whether female longevity (duration of the adult life stage) or fecundity (number of eggs laid) was affected by

1. the number of potential mates she was exposed to, and/or
2. the duration of exposure to a single or multiple males.

Methods

Study species

Callosobruchus maculatus Fabricius (Chrysomelidae), commonly known as the seed beetle or cowpea weevil, is an agricultural pest living of legumes (Fox, Bush, Roff, & Wallin, 2004). The beetles are widespread and can be found on most continents (Fabricius, 1775). The female seed beetle lays her eggs on the outside of beans. After 4-5 days the egg hatches and the larva burrows into the bean, and after another 3-4 weeks the adult beetle emerges from the bean (Beck & Blumer, 2014).

The *C. maculatus* is suitable for experiments due to the fact it needs no food or water after hatching, the sexual dimorphism makes it easy to determine each sex, plus the short generation time of ca 5-6 weeks makes it suitable for multi generation research (Fox & Moya-Larano, 2009). In addition to this one female can lay more than 100 eggs over its lifetime (Beck & Blumer, 2019), making it easy to start and maintain populations of beetles in the lab.

The population used in the experiments were imported to the University of Bergen from Carolina Biological Supply in 2016. In our lab, the beetles have given rise to several populations, with separate strains being kept on mung beans, adzuki beans or black-eyed peas. All beetles used in this experiment were taken from populations kept on mung beans. Before the experiment started, the beetle populations were kept in room temperature (20°C), whilst the beetles in the experiment were kept in a heating incubator holding 28°C, and in constant light.

Study design and experiments

To test the effect of multiple mating on female longevity and fecundity, we manipulated the sex ratios and the duration that females were exposed to males. The four different ratios of female(F):male(M) used in the experiments were 1F:1M, 1F:2M, 1F:4M and 1F:8M. For each sex ratios, we varied the time period that females were exposed to males: Females where either constantly together with males, *permanent exposure*, or only exposed to males for one hour each day, *short-term exposure*. This resulted in eight different treatment groups initiated in randomized order (n = 5-7 replicates per treatment).

To accurately estimate the total reproductive output in the females, the beetles used in the experiment needed to be virgins. Before the adult beetles emerged from the beans, single beans were isolated in 24-well plates until the start of the experiment. We ensured that each bean had one visible egg on its surface. All in all, 28 well plates were prepared with a total of 672 beans. Because each experiment involved 1-8 males for each female, the number of males was the limiting factor determining the number of experiments that we started. With an expected sex ratio of 50/50 roughly 336 males were expected, and 6 of each experiment were planned.

To get an accurate measure of individual longevity, we checked the beans daily and recorded the day of emergence. Beetles were randomly assigned to each treatment group, and we ensured that all the individuals in a replicate/dish had emerged from beans within a 24 hours interval. To ensure we had enough males, no more than five replicates of any treatment were started before all the treatment groups had five replicates. We used a random number generator to determine which treatment to start next.

Immediately after emergence the male seed beetle are not fully sexually mature, with the ejaculate not being fully developed (Fox et al., 1995). Therefore, each treatment was started the day after the day of emergence. This was done to ensure that all the beetles were at least 24, but no more than 48 hours old and sexually mature when the experiment started. In each experiment males that emerged the same day as the females were used.

The beetles were kept in petri dishes (120mm diameter) on mung beans with enough beans for the female to lay her eggs on individual beans (ca. 130 beans). A single female was placed in each petri dish with the number of males prescribed by the treatment group. The petri dish where kept in heating cabinets at a constant temperature of 28°C. To keep track of the life length, each dish was checked daily at approximately the same time every day. The date of death for both males and females where recorded.

For the group with constant exposure one petri dish were prepared with 7.5g of mung beans (ca. 130 beans). In the short-term exposure group, we had one petri dish containing mung beans (same as the permanent exposure group) and one empty dish for the males. In each experiment the petri dishes where paired and marked. Males where kept in the same petri dish to keep each experiment separate, whilst ensuring that the same males were paired with the same female every day. Each day at approximately the same time, males were moved from

their petri dish to the matching dish containing the female. After one-hour exposure the males were moved back to their petri dish. While handling the beetles, deaths were noted for the males and females to measure adult life span.

Weighing

To account for some of the variance associated with the weight of the females affecting the amount of eggs being laid, the females were weighed before the experiment started. The weighing was done with a Sartorius M3P microbalance (0,001mg precision). Each female where placed in a container, and to ensure they did not escape a small cover glass was placed on top of the weighing boat. The combined weight of the female, container and glass cover were measured by gently placing it on the scale using tweezers and waiting 20 seconds to allow the weight to settle before reading the result.

Fecundity

We used the number of eggs laid as a measure of female fecundity. Reproductive output may also depend on other factors, including egg size and number of eggs laid on a single bean. We provided enough beans for the female to spread her eggs among individual beans, but did not consider emergence rate of offspring form beans. Instead, we froze the beans with eggs attached to prevent further development of offspring. In each dish, all beans were examined, and we counted the number of eggs laid. This was done to determine if either the ratio of males or the exposure time affected the amount of eggs laid by the females.

Statistical analysis

To determine differences in average adult female life span and number of eggs laid between the different treatment groups, two-way ANOVAs were used. Adult female life span and number of eggs laid were used as the response variables, while Exposure type (permanent or short-term) and number of males per female (sex ratio) were used as explanatory variables. Female initial weight may affect fecundity positively and could have therefore been included as an additional explanatory variable. However, due to the small sample size, this may have led to unreliable p-values. Therefore, we refer to the model without weight in the results below.

Results

Adult female life span

Adult lifespan was lower for females exposed permanently to males compared to females that only encountered males for a short-term every day (Fig 1). The number of males per female (sex ratio) had a negative impact on female adult life span in treatment groups where females were permanently exposed to males, but not in the group with shorter exposure time (Fig. 1a), as shown by a statistically significant interaction term (ANOVA: $F_3 = 3.19$, $p = 0.033$).

The number of males per female (sex ratio) had a statistically significant influence on the average life expectancy in the female beetles (ANOVA: $F_3 = 2.90$, $p = 0.046$). Female beetles exposed to more males lived shorter than females exposed to fewer males. Females under short-term exposure (1 hour daily) lived on average longer (mean = 12.00 days; SE +/- 0.27; Fig1a) than females under permanent exposure (mean = 9.19 days; SE +/- 0.42; Fig1a). Exposure time had a significant effect on average adult female life span (ANOVA: $F_1 = 38.23$, $p < 0.001$).

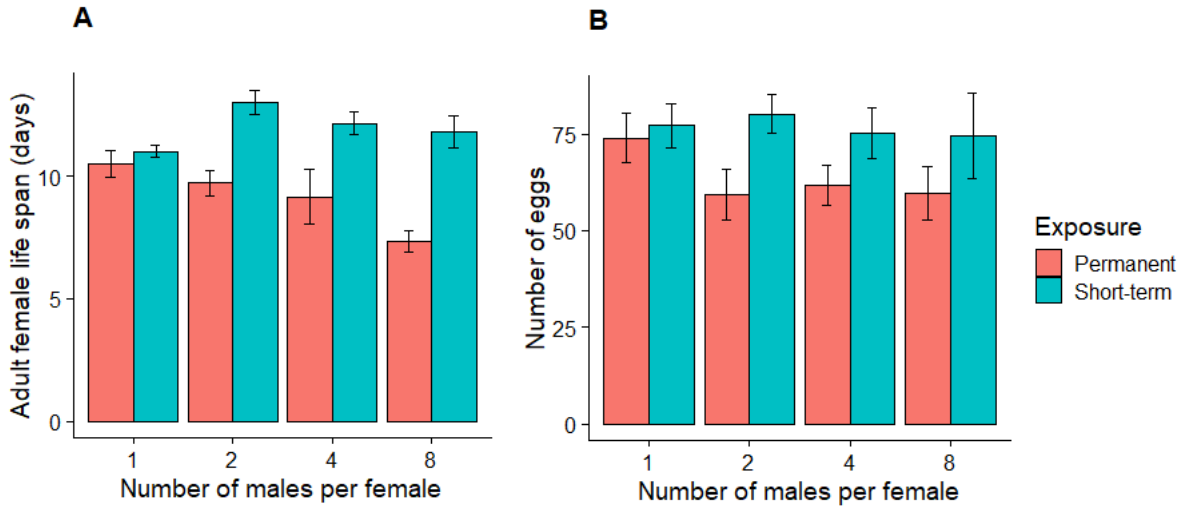

Figure 1: Average adult female life span (A) measured in days after emergence, and average female fecundity (B) measured in number of eggs laid. Red bars indicate females with permanent exposure to the males, while the green bars indicate females with a short-term exposure to the males (one hour every day). Error bars indicate ± standard error. $N_{total} = 50$ this is the number of females, not males

Neither exposure time (ANOVA: $F_1 = 1.04$, $p = 0.31$) nor the number of males per female (ANOVA: $F_3 = 0.33$, $p = 0.80$) had a statistically significant impact on the average male adult life span (mean = 9.73 days; SE +/- 0.16;Fig. 2). The interaction of the factors was not statistically significant either (ANOVA: $F_3 = 0.71$, $p = 0.55$).

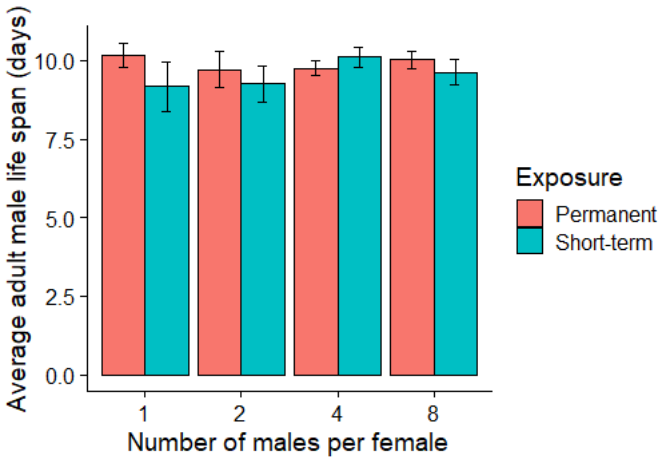

Figure 2: Average adult male life span (in days) in the different treatments. Red colour indicates the group of females with permanent exposure to the males, while the green colour indicates the group of females with a short-term exposure (one-hour) to the males every day. Error bars indicate ± standard error. N = 50 -> this is the number of females, not males

Fecundity

Exposure time had a statistically significant effect on the number of eggs the females laid (ANOVA: $F_1 = 7.87$, $p = 0.008$). Females under short-term exposure laid more eggs (mean = 76.8 eggs; SE +/- 3.3; Fig 1b) than females under permanent exposure (mean = 63.5; SE +/- 3.2; Fig1b). Neither the number of males per female (ANOVA: $F_3 = 0.670$, $p = 0.56$) nor the interaction between exposure time and number of males (ANOVA: $F_3 = 0.604$, $p = 0.62$) showed a statistically significant effect on the number of eggs the female laid (Fig. 1b). This shows that even though the females exposed to more males live shorter, that does not translate to them laying fewer eggs.

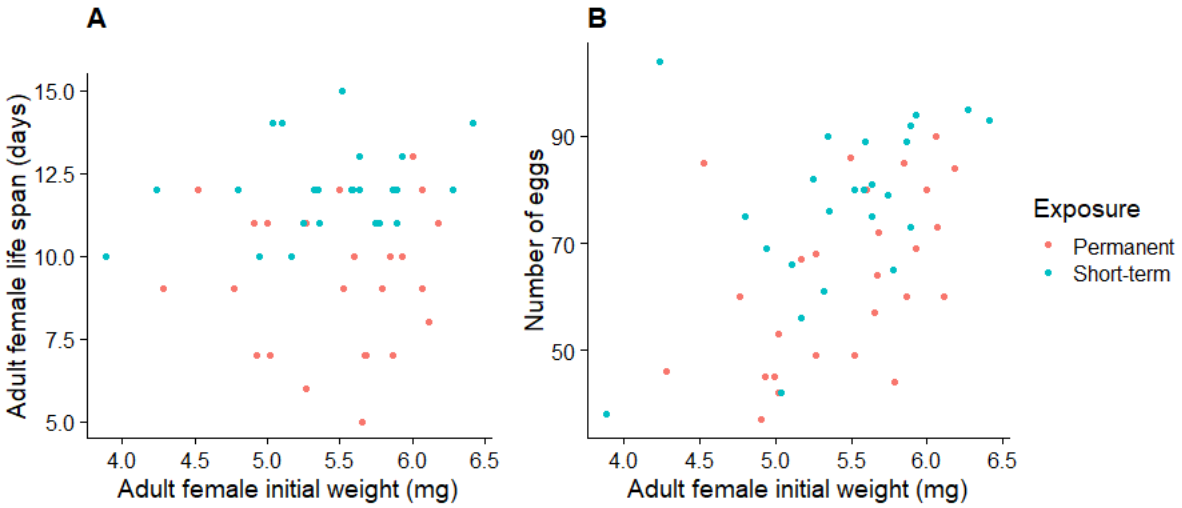

Figure 3: Adult female life span (A), and the amount of eggs laid (B) as a function of female initial weight. No significant correlation between adult female life span and female initial weight. Red colour indicates the group of females with permanent exposure to the males, while the green colour indicates the group of females with a short-term exposure (one-hour) to the males every day. N = 50 this is the number of females, not males

Female weight

Female initial weight did not explain a significant amount of variation in female adult life span ($R^2 = 0.310$, $p = 0.508$) (figure 3a). Females were evenly distributed in weight over the two different exposure times, and the females with the short-term exposure (indicated by green colour) generally lived longer than the females with permanent exposure (indicated by red colour). Female initial weight explained a significant amount of variation in the number of eggs laid ($R^2 = 0.659$, $p = 0.002$), indicating that heavy females tend to lay more eggs than light females (Fig. 3b). There is also a significant positive correlation between adult female life span and number of eggs laid ($R = 0.496$; $N = 50$; $p < 0.001$; Figure 4). Although adult female life span and adult female initial weight were not significantly correlated, they both show a statistically significant positive effect on number of eggs laid.

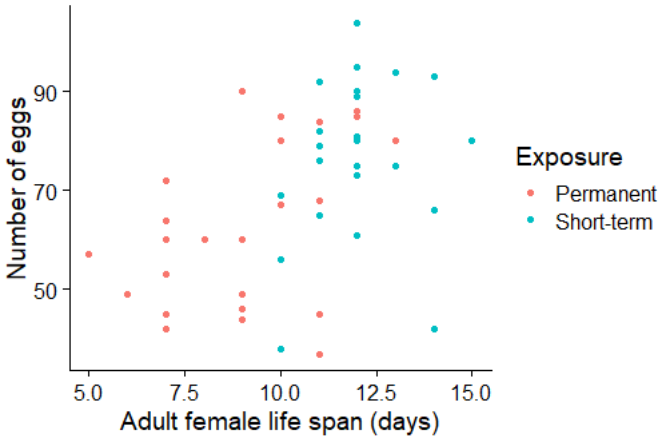

Figure 4: Number of eggs laid as a function of adult female lifespan. Red colour indicates the group of females with permanent exposure to the males, while the green colour indicates the group of females with a short-term exposure (one-hour) to the males every day. N = 50 this is the number of females, not males

Discussion

In the present study, male exposure had a negative effect on the longevity of adult female beetles, and the effect was particularily pronounced in male biased treatments with permanent exposure (Fig.1a).

When females were permanently exposed to males, their life spans decreased when the number of males in the dish (sex ratio) increase. This indicate that even though females get benefit from multiple mating, there are also costs associated with multiple matings. For females *C. maculatus,* one of the major costs is damage during copulations caused by the spikes on the reproductive organ of males (Crudgington & Siva-Jothy, 2000). Unlike many other insect species, these spikes are not used to scrape out sperm from other males, instead the spikes harm the reproductive tract of females and likely serve to delay remating (Simmons, 2001). Females may also perceive the damage to genitalia as a threat to survival and increase current reproduction by increasing egg production and egg laying (Crudgington & Siva-Jothy, 2000). Edvardsson and Tregenza (2005) however, found that spikes may have evolved for other reasons than harming the female, and that the harm is only a side effect.

For the number of eggs laid, the trend differed somewhat from what we observed for longevity. Females that were exposed permanently to males laid fewer eggs than the group with short-term exposure to males (Fig. 1b). Even though the females with the more skewed sex ratio lived shorter than the females with more balanced ratios, this had no significant effect on the number of eggs laid. This indicates that even though female life span may be reduced when exposed to more male harrasment, this does not necessarily translate to lower fecundity. Similarly, Liddle et al. (1995) found that receiving seminal fluid products had a negative effect on female longevity, but induced an increase in the rate of egg laying.

We found a positive correlation between female adult life span and number of eggs laid across all treatment groups (fig 3), which may indicate a cost to short lived individuals. This is mainly explained by the fact that the females in short-term exposure, both, lived longer and laid more eggs, than the females exposed permanently. This indicates that the group with short-term exposure may have experienced less costs of multiple mating due to reduced exposure to male harrassment. When females spend less time with males, it is likely that they mate less frequently (Weir et al., 2011), then females received fewer spermatophores which can mean less nutrients from males (Fox, 1993a), but also less costs. However, we found no positive relationship between longevity and fecundity within either of the treatment groups.

Other studies have found a negative relatitonship between longevity and fecundity (Eady et al., 2006; Messina & Fry, 2003; Savalli & Fox, 1999a). Savalli and Fox (1999a) found that females mated once with a virgin male had longer lifespan and lower lifetime fecundity than females mated one time with four virgin males mated with one male every 48 hours. And even whith the amount of femal genetal damage beeing negatvely correlelated with how manny times the male have mated earlyer. Eady et al. (2006) found that females mated with a male that have mated four or five times earlyer have higher logevity, and lower fecundity compared to females mated to a male mating for the first or second time. This is most likely due to the fact that the spermatophore size decreases with each successive mating while the cost of mating stays the same, or it might even increase (Eady et al., 2006). Hence mating with a virgin male or one that have mated few times might be more beneficial. This is supported by the study Savalli & Fox (1999a) which find that females mated with several virgins had an increase in fecundity. They also found that females mated with non-virgin males were more likely to mate again sooner than females that mated with virgins. However, in another study, females that mated only once showed no difference in lifetime fecundity compared to females exposed with males throughout life. And females mated at 48-hour intervals laid more eggs then females that had mated once (Fox, 1993a). Messina & Fry (2003) found that depriving females of seeds caused a 70 percent increase in longevity as well as a three-fold decrease in fecundity.

Studies that have looked at how female fecundity and longevity of *C. maculatus* are affected by multiple matings show no clear consensus on what the optimal female mating rates are (Arnqvist & Nilsson, 2000). In general, it is difficult to separate the costs of multiple mating from the benefits of receiving more nutrients in spermatophores (Eady et al., 2006). Some studies show a decrease in both fecundity and longevity with multiple mating (Li et al., 2015) whereas most studies find a negative relation between longevity and fecundity, where females that mate multiply experience increased fecundity, but decreased longevity (Eady et al., 2006; Messina & Fry, 2003; Savalli & Fox, 1999a). Fox (1993b) however found that multiple matings increased longevity for females living in starvation condition, while no such increase was found when females had unlimited access to nutrients and water.

In our study, longevity decreased with male exposure time and sex ratio. With short exposure to multiple males, females may receive the potential benefits of multiple matings without the cost of constant harassment. This may explain why fecundity is higher in long-lived females in our study, as these females are only exposed to males for a short-term period.

The differences in longevity and fecundity between different male exposure treatments could then in large be explained by the permanent exposure group experience more negative effects (costs) than the group with short-term exposure. If given the chance, male beetles attempt to mate more often than what is optimal for females, hence females experiences more harassment from males. Sakurai & Kasuya (2008) found that females living with males whose aedeagus (penis) were ablated (they could harass but not mate with the female) had lower longevity (18,6%) than females receiving no harassment or copulations. This indicates that females suffer great costs from male harassment. Females also try to reduce the number of matings and the duration of mating by kicking the males. When females are prevented from kicking, matings last longer and the females receiving more damage This suggests that it is beneficial for females to limit the number of matings due to the cost of damage to the sexual organs.

We found no correlation between the initial weight of females and longevity. This result differs from some other studies, including Savalli & Fox (1999b) that found a correlation between size and longevity for both male and female beetles. Note however, that life span was affected by treatment group which might have confounded this relationship. In our study there was a positive correlation between female weight and number of eggs laid. This supports the findings of previous studies (Edvardsson & Tregenza, 2005)

We used number of eggs as a measure of female fecundity, similarly to what previous studies have done (Eady et al., 2006; Messina & Fry, 2003). Other studies have used factors like the number of fertile eggs (Fox, 1993a; Savalli & Fox, 1999a), number of surviving offspring (Edvardsson & Tregenza, 2005), or egg size. Credland and Wright (1989) found that differences in sex ratio did not affect number of eggs laid, but it did have a negative effect on number of eggs hatched. In their study van Lieshout, McNamara and Simmons, (2014) found that the length of mating did not affect the number of eggs laid, but it did have an effect on the number of adult offspring. These studies show that different ways of measuring fecundity can lead to different results. Female reproductive success can therefore be influenced by egg size, choice of seed and larval competition, hence further studies should go further than counting eggs e.g. include number of surviving offspring to determine the effect of multiple male mating and harassment on reproductive success.

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Appendix

All R code used for statistical testing:

```
#### Correlation test
cor.test(StartingWeight, LifeLengthF)
cor.test(Eggs, StartingWeight)
cor.test(Males, Eggs)
cor.test(LifeLengthF,Eggs)

#### Anova: Average female adult life span
AnovaLifeLengthF <- lm(LifeLengthF~factor(Males)*factor(Exposure), data= oppset)
hist(residuals(AnovaLifeLengthF))
plot(AnovaLifeLengthF)
summary(AnovaLifeLengthF)
anova(AnovaLifeLengthF)

#### Anova: Number of eggs laid
AnovaEggs <- lm(Eggs~factor(Males)*factor(Exposure), data= oppset)
hist(residuals(AnovaEggs))
plot(AnovaEggs)
summary(AnovaEggs)
anova(AnovaEggs)

#### Anova: Average male adult life span
AnovaLifeLengthM <- lm(GjennomsnittLivslengdeM~factor(Males)*factor(Exposure), data= oppset)
hist(residuals(AnovaLifeLengthF))
plot(AnovaLifeLengthF)
summary(AnovaLifeLengthF)
anova(AnovaLifeLengthF)
```