Male mate choice and selectivity in relation to female mating status in the seed beetle *Callosobruchus maculatus*

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by
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Abstract

Sexual selection plays a role in evolution and often follows the pattern where males are fighting to mate, while females are choosy due to their higher gamete investment. Males can also be selective if they have high costs of mating or if females vary a lot in quality. By being cryptic and less common than female selection, male mate choice is often underestimated. This study investigates male choice in relation to female prior mating status in the seed beetle *Callosobruchus maculatus*. I also study the dynamics between male and female mating preference and rejection behaviour. The results showed that males were more likely to mate with virgin females than non-virgins. This difference was not due to female size nor female repelling behaviours or female handling. Prior mating status is the major factor determining male choice and is assessed by males after the first encounter with females. Even if males are selective, females could refuse to mate by kicking off males and running away or accept mating, showing that male and female choices are linked to each other and can be challenging to discern.

Acknowledgement

I would like to express my gratitude towards my supervisor Sigrunn Eliassen for guiding me through all the process of the master thesis. Thank you for your constructive feedbacks and for always being responsive and available to help me. I also feel thankful for the advices from Richard Telford and Beatrice Diaz Pauli, particularly for the statistical analysis. I would also like thank my fellow students during these years at UiB. Finally, I extend a huge thank to my family and my friends who supported and motivated me to complete my degree.

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Introduction

Mate choice is characterized by individuals of one sex preferring to mate with certain individuals while rejecting others, suppressing randomization in mating (Andersson, 1994; Darwin, 1871; Halliday, 1983). Selectivity in mates has consequences on evolution, by varying the reproductive success of individuals through different mating behavior and sense shuffle gene frequencies in the next generation (Halliday, 1983).

Individuals can be selective when the cost of mating is high and quality difference among mates are substantial (Bonduriansky, 2001). The degree of choosiness in mate choice depend on reproductive investment in gametes and parental care (Goubault & Burlaud, 2018; Kokko et al., 2006; Trivers, 1972). As females invest more in each gamete, her reproductive success is more limited by resource while males are limited in access to mates (Bateman, 1948; Parker et al., 1972; Trivers, 1972).

Female mate selection is well studied as this pattern of male competing for access to females and females choosing their optimal mate is widespread (Andersson, 1994; Darwin, 1871). Darwin mentioned some anomalous cases that did not fit with the traditional sex roles, as in the case where males are selective (Darwin, 1871). Male mate choice is often described as acceptance or rejection of certain females or inequality in number of mating attempts (Bonduriansky, 2001). In systems with reversed sex roles, females may compete for males to gain parental care benefits (Gwynne, 1991), but male selectivity is also observed in cases where males do not provide care of offspring (Bonduriansky, 2001). Thus, males have also evolved to mate selectively and it has been observed through many species (Bonduriansky, 2001; Edward & Chapman, 2011; Gowaty et al., 2003; Gwynne, 1991)

The evolution of mate choice is affected by the cost and investment in mating effort (Edward & Chapman, 2011). Just as females, males are paying reproductive costs such as spermatogenesis, mature sperm maintenance but also costs and risks of finding a mate (Dewsbury, 1982; Parker, 1983). The cost of male courtship has been illustrated in a study on *Drosophila melanogaster* where males that copulated more often died earlier (Cordts & Patridge, 1996). Thus, there is a positive correlation between mating cost and male selection, as shown in the study on scorpion fly (Engqvist & Sauer, 2001). There are also other costs that both sexes are paying, for example mating increases the risk of predation (Arnqvist, 1989; Katvala & Kaitala, 2001) and sexually transmitted diseases (Hurst et al., 1995). Additionally, in many insect species, males are producing a nutrient mixture called spermatophore, representing between 5 and 10 % of total male weight in certain species (Savalli & Fox, 1999; Wedell, 1998; Wedell et al., 2002). The spermatophore is either eaten by females or injected in the female genital track during copulation (Ortíz-Jiménez & Castillo, 2015; Vahed, 1998). Females can store sperm from several matings creating an internal sperm competition and it is optimal to be the last male to mate with the largest quantity of sperm (Parker & Pizzari, 2010; Ridley, 1989). By giving this nuptial gift, males assure their paternity by copulating long enough to inseminate a worthwhile amount of sperm into females and avoid sperm competition (Dickinson, 1986; Martin et al., 1974; Simmons, 2001).

Given these mating costs, males are expected to be more selective when encountering females that are varying in quality in order to optimize their reproductive success (Bonduriansky, 2001; Gwynne, 1991; Parker, 1983; Simmons, 1992; Wedell et al., 2002). Indeed, females can vary in size, fecundity, prior mating status, age or even receptivity. These factors are known to affect male choice in several species , including the common dung fly *Sepsis cynipsea* (Martin & Hosken, 2002), *Drosophila hibisci* (Polak et al., 1998), and the bushcricket *Requena verticalis* (Simmons et al., 1993). Males can adapt their investment and selectively reduce sperm allocation in one copulation to conserve it for later (Reinhold et al., 2002). This adaptation of sperm allocation to female quality is demonstrated in many studies (Byrne & Rice, 2006; Gage & Barnard, 1996; Simmons & Kvarnemo, 1997; Wedell & Cook, 1999). Males tend to choose females with high fecundity, represented through big body size, by different mechanisms of recognition such as direct visual, olfactive cues or tactile assessment (Bonduriansky, 2001; Thomas, 2011).This tendency is illustrated in the study on the lekking moth (Goubault & Burlaud, 2018), in bugs (Katvala & Kaitala, 2001) and drosophila (Byrne & Rice, 2006) where virgins males tend to mate with the heavier females that lay more eggs. Another male preference is towards virgin rather than non-virgin females. In a study on the moth *Plodia interpunctella*, males preferred to mate with virgins and if they copulated with mated virgins their reproductive investment were lowered by injecting less sperm than with virgins (Cook & Gage, 1995).

Male choice may be hard to observe due to its cryptic nature or the expectations that males would not invest in parental care and thus not be choosy (Martel et al., 2008; Reinhold et al., 2002). Another reason to explain that male choice has been less in focus is that, compared to female selection, male preference does not impact as much females evolution (Schlupp, 2018). Male choice is underestimated but is still having a role in mating that needs further investigations (Goubault & Burlaud, 2018). Male choice is considerable in evolution in various ways, for example by affecting the rate of adaptation in the environment (Lorch et al., 2003; Whitlock & Agrawal, 2009) or by influencing species speciation (Ritchie, 2007). Male decision has consequences on population fitness by influencing male investment such as parental care and nuptial gift towards preferred females (Parker, 1983; Schlupp, 2018). In addition, male preference can respond to female selectivity, leading to a mutual choice. For example, in a study on mutual mate choice in parthenium beetle, larger males would prefer larger females and vice versa, and both sexes would benefit from being choosy (Afaq, 2013). Understanding the dynamics between male and female choice is hence important.

In this study, I investigate mate choice in the seed beetle *Callosobruchus maculatus.* It is a convenient model system because of their ease of sampling, storage and maintenance but also due to their short generation time (Messina & Slade, 1999). *C. maculatus* are capital breeders, which means that they store resources during larval stage and then do not feed for survival and reproduction (Messina & Slade, 1999). Both male and female invest a large amount of resources in breeding which may select for mutual choice. For females, mating is costly due egg production, but they also suffer from male harassment and injury due to the spiny intromittent organs of the males (Crudgington & Siva-Jothy, 2000). When mating, males deposit a spermatophore that represents a relatively large energetic investment on their part (Fox et al., 1995). This parental investment might lead males to show sexual selective behaviors (Trivers, 1972). Females also vary in quality and fecundity and therefore males would benefit from being demanding, although virgin males do not appear to be selective in response to the size of virgin females (Holme, 2019). Female choice has been largely explored (Fox et al., 2004; Fricke & Arnqvist, 2007; Nojima et al., 2007; Savalli & Fox, 1999) while male selection still need further investigations (Fox et al., 1995; Holme, 2019).

The aim of this study was to investigate whether males are selective depending on female mating status and how female response may affect mating patterns in the seed beetle *Callosobruchus maculatus*. This was done by giving virgin males a choice between a non-virgin and a virgin female and recording their respective behaviors. The prediction was that 1) males would preferer to mate with virgins, 2) and approach these females more often.

Materials and methods

Study organism

Callosobruchus maculatus (F.) (Bruchidae) is a cosmopolitan seed parasite and a challenging storage pest of cowpea seed *Vigna unguiculata*, responsible for quantitative losses in legume crop (Caswell, 1981; Oluwafemi, 2012; Tiroesele et al., 2014; Tiroeselea et al., 2019). Adult females lay eggs on the surface of seed (Fabaceae) and after 4 to 5 days (at ca.28°C), the eggs hatch and the larvae burrow into the seed to feed from it (Fox et al., 2004; Messina, 1991). Adults emerge after 3 to 4 weeks depending on the type of seed and live for 2 to 3 weeks in the lab. The beetle can amass all the resource required for its entire lifespan during larval development in the seed, hence can be categorized as a "capital" breeder (Messina & Slade, 1999). These special traits about female oviposition and reproduction make *C. maculatus* easy to maintain in the laboratory and convenient for our study.

Rearing and preparation of beetles

The population used in the present study was imported from Carolina Biological Supply, USA to the University of Bergen, Norway in 2016. The culture has been maintained at 28°C with constant light and overlapping generations representing at least 500 individuals in Petri dishes with access to abundance of cowpea, *Vigna unguiculata*.

To avoid bias effect from age or mating status, I isolated each beetle prior to emergence from the bean to ensure their virginity. Since females are laying eggs on the surface of beans, it is easy to isolate the beetles by placing one bean presenting one larva into separate chambers in a 6 by 4 well plate. The beetle boxes were checked every day to collect males and females that hatched on the same day. Newly emerged adult beetles were placed into 35mm Petri dishes and allowed to mature in an incubator at 28°C. Though males may directly mate after emerging, the ejaculate is not fully formed (Fox et al., 1995). Therefore, I waited at least 24h after emergence before exposing males to potential mates.

One day after emergence, half the virgin females were exposed to two males each from the main population. The selection was done randomly with respect to female size even though males are not known to show size preferences for their first mate (Holme, 2019). Females were left with the males in a 60mm Petri dish until mated, which usually took between 30-60 minutes. Note that this is longer than the average time until mating for virgins, but males from the main populations may have already mated and needed time to get ready for remating (Fox et al., 1995). Mated females were given 24 hours to recover due to the injuries of mating referred as "traumatic penetration", causing damage on the epithelial surface on the female's genital tract (Crudgington & Siva-Jothy, 2000; Lange et al., 2013).

I acknowledged the treatment differences between non-virgin and virgin females' preparation. Although half of the collected females were moved from their boxes and mated, the other half virgin females remained undisturbed until the experiment. Thus, to control for potential effects of handling, I conducted 29 experiments where each virgin females were removed from their 35mm Petri dishes and placed individually in another Petri dish for 10 minutes, then returned to their original Petri dishes. These additional trials represented a control for the effect of handling on virgin female behavior prior to the experiment.

Body weight

All females and males were weighed less than one hour prior to the start of the experiment. The beetle was placed in a 1cm^3 small container covered with a 1cm^2 piece of wax paper. The lid was pressed and sealed on the top of the container to prevent beetle escaping before placing it on the weight. Weight readings were recorded after 40 seconds to avoid bias from beetle movement on the balance. A sartorius® M3P micro balance (0,001mg precision) was used for the weightings.

Male mate choice experiments

I tested male mating preference depending on female mating status by giving virgin males a choice between one virgin and one already mated female. I weighed and paired virgin and non-virgin females with a sufficient weight difference (>0,031mg) to be able to distinguish them. I randomly selected virgin males, weighted them, and assigned them to a pair of females. The males were placed in a 60mm Petri dish that already contained the two females, and the timer was started. I observed the beetles continuously until mating. The female that was not selected was removed and weighted for identification. At the end of mating, the male and female were also weighted for control. If 15 minutes passed without any mating, I categorized the trial as unmated. This method is comparable to the protocol used in similar experiments of mate choice only that our study focuses on male selectivity instead of female choice (Savalli & Fox, 1999). Additional data were recorded such as time until mating, time until the females started kicking off the male, and duration of mating. Females kick males with their back legs to end copulation, this may be due to the genital damage caused during the penetration, leading to a reproductive conflict in *C. maculatus* (Crudgington & Siva-Jothy, 2000). In total, 135 mating trial were conducted.

Male and female mating behaviours experiments

In the second experiment, I observed and recorded male and female behaviors following mate encounters. Each virgin males were assigned a pair of females as described in the first experiment. The timer started once the male had been placed in the 60mm Petri dish box containing the virgin and mated females. I observed and recorded behaviors and times from females and males until mating. The females that were not selected were removed from the box and weighted for identification. The behaviors recorded correspond to beetle reactions immediately after a male had physical contact with a female. In case of consecutive encounters, each behaviour was recorded following each physical contact even in case of repetitive behaviours. The recordings were stopped when the male was copulating with one of the females and she was not kicking off the male, demonstrating a successful mating. In total, 82 behavioral trials were conducted. I categorized the responses of male and females in the following types of behavior:

Male behaviors:

A. **Running away:** the male runs away from the female.

B. **Inactive:** either the male does not move or the female runs away which does not let time for the male to make any mating attempt.

C. **Chasing:** the female runs away and the male chases her by running after her.

D. **Attempt mating:** the male goes on the back of the female and she either accepts the mating or kicks back and runs away.

Female behaviors:

A. **Running away:** the female walks or runs away from the male also when the male is chasing her.

B. **Remain passive:** the male runs away before the female moves.

C. **Refuse mating:** the male climbs on the female's back, but she kicks him with her hind legs and runs away.

D. **Accept mating:** the female is not moving and accepts the male's mating attempt.

Statistical analysis

Data were analyzed using R studio version 1.3.959 (R Core Team, 2020). Since the data are following a Poisson distribution and the days when the beetles emerged may have an effect on my experiments, I compared generalized linear models (GLM) to generalized linear mixed effects models (GLMM) with the days as random effects. I picked the models with the lowest Akaike information criterion AIC (Akaike, 1974)(See the appendix for coding and comparison of AIC). Generalized linear models (GLM) with Poisson distribution were performed to investigate male mate choice and male behavior on first and second encounters. GLM were analyzed by a Chi squared test through ANOVA and for deeper analysis of each factors' effect, pairwise comparisons with "emmeans" package were applied. The same statistical method was used with females to examine the effect of handling virgin females prior the experiment, the visited females and their behaviors, and the effect of female size and behaviors on male choice. A Kruskal-Wallis test, also known as one-way ANOVA on ranks, was used to test whether male takes same mean time until first visit between the two types of females.

Results

Male mate choice

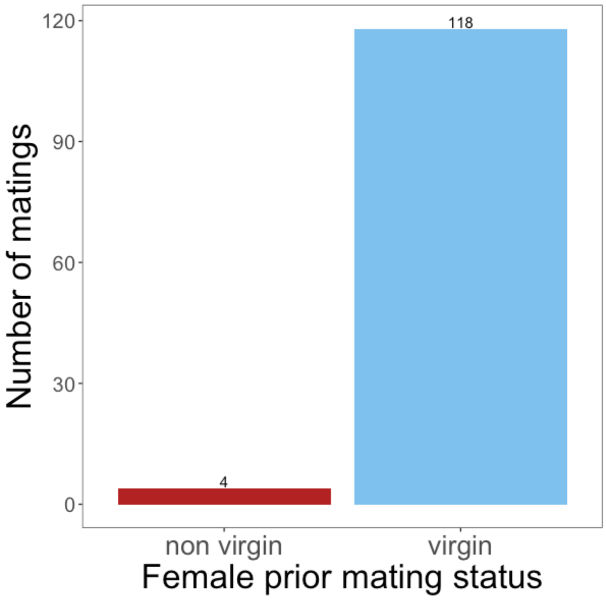

Figure 1: Males mating with females of different prior mating status. N= 122 (13 cases with no mating within 15 min are removed from the 135 trails).

Virgin males copulated predominately with virgin females and seldom with females that were previously mated (GLM: Dev=133.92, N=122, df= 1, p<0.001).

Male and female mating behaviours

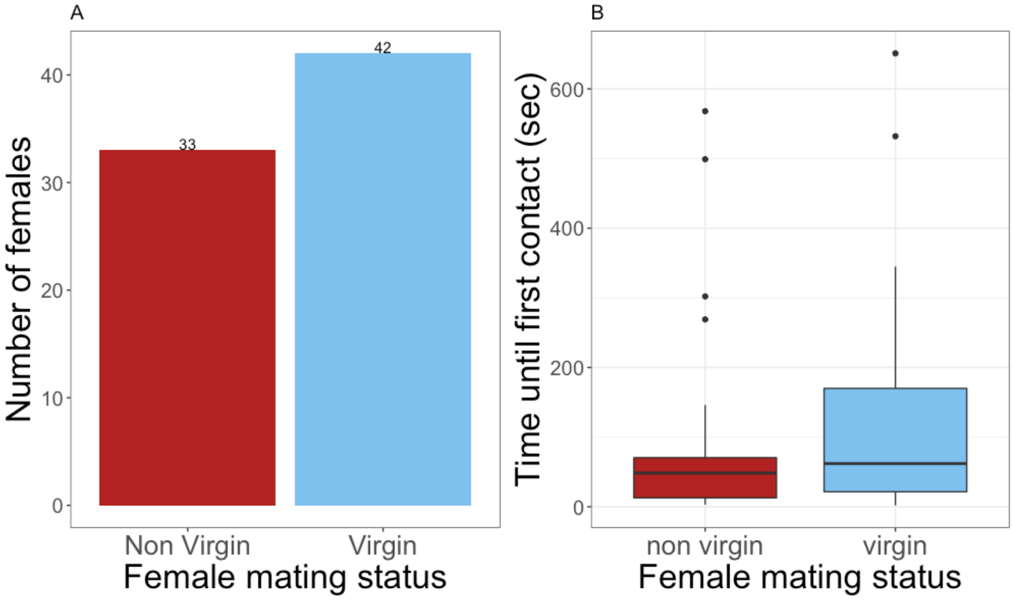

Figure 2: Male choice on the first visit. **A**: Males first visit depending on female mating status. N= 75 **B**: Time until first male visit depending on female mating status. N=75.

The male's first close encounter with a female did not depend on whether she was virgin or previously mated (GLM: Dev= 1.0826, N=75, df= 1, p= 0.3) as shown on Figure 2A. Behavioural trials (N = 82 in total) where the male remained unmated after 15 minutes (N=7) were not included in the analysis. Time until the first encounter between a male and a female did not significantly differ between females of the two mating types (Figure 2B; Kruskal-Wallis test: chi-squared = 1.3145, N= 75, df = 1, p= 0.25).

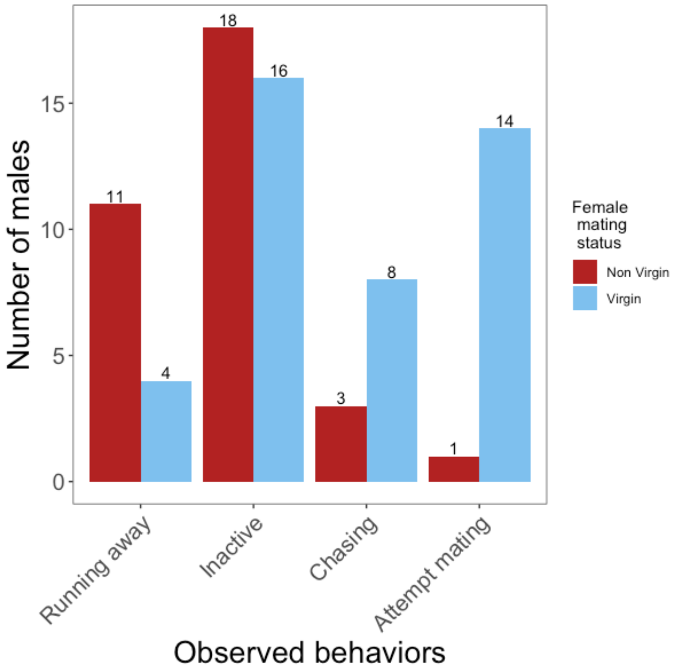

Figure 3: Male behaviours after first encounter with a female depending on her mating status. All behaviours described are observed after the male had a first physical contact with the female. See method section for description of behaviours categories. N = 75.

Males may not be able to assess females mating status at a distance but once they have close contact with females, their subsequent behavior are significantly different depending on female mating status (GLM: Dev= 18.2369, N=75, df=3, P< 0.001). On the first close contact with a female, males mated more often with virgins than non-virgin females (P= 0.01) but there were no significant differences in how often he ran away (P= 0.08), remained inactive (P= 0.73) or chased the females (P= 0.14).

Female behaviours are different depending on female mating status (Figure 4; GLM: Dev= 86.226, N=75, Df=3, P<0.001). Only virgin females accepted to mate on the first visit and they also accepted more to mate compared to non-virgin females in general (P <0.001). There was no difference in the frequency of females kicking off the male (P= 0.49) and both virgins and non-virgins were frequently running away from the males (P= 0.58).

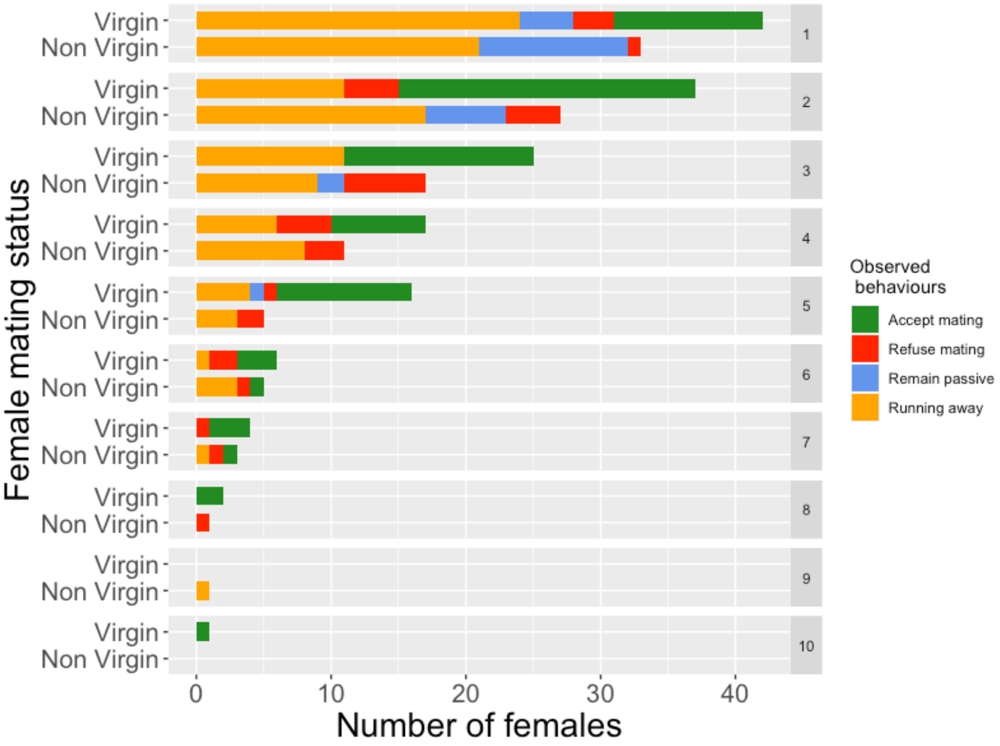

Figure 4: Female passive/active behavior depending on each encounter. Encounters are numbered from 1 to 10 on the y axis. All behaviours described are observed after the male had a first physical contact with the female. See method section for description of behaviours categories. N = 75.

In Figure 2 and 3, I showed that the likelihood of approaching a female of a certain mating status were equal, but that males behaved differently towards females after the encounter. This tendency is also observed in subsequent encounters as showed on Figure 4, males are not significantly visiting one female type more than the other on each encounter (GLM: Dev= 3.261, N=75, df=7, P= 0.85)

After observing that females reacted differently to male contact depending on their mating status, I investigated male's choice from first to second encounter. Male choice was significantly different depending on the female mating status (Figure 5; GLM; Dev= 6.1896, N=22, Df=1, P= 0.012) and males were more likely to switch to the other female after encountering a non-virgin compared to when encountering a virgin female (P=0.04).

Male tendency to change female on the second encounter was not dependent on female behaviour on the first visit (GLM: Dev= 0.04734, N=64, Df=1, P= 0.82)

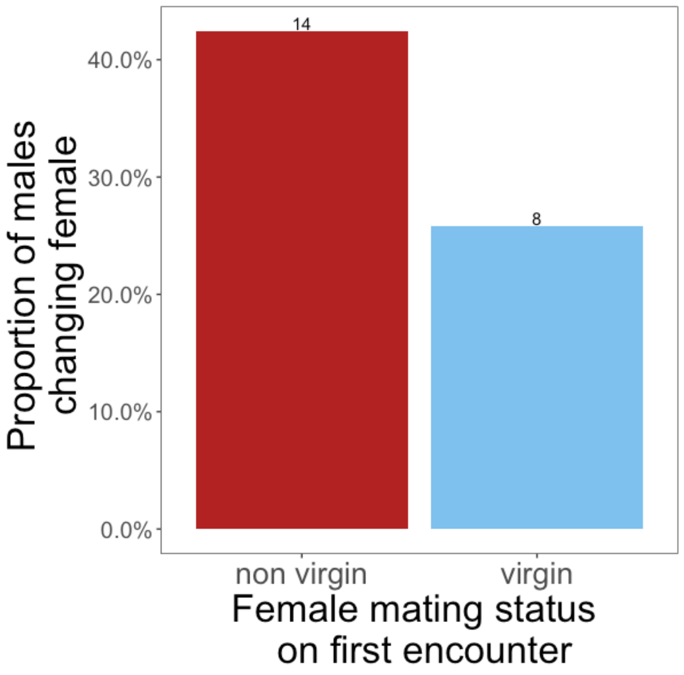

Figure 5: Proportion of males changing female on second encounter depending on female mating status on the first encounter. N= 64 as 8 females mated on the first visit where N=75.

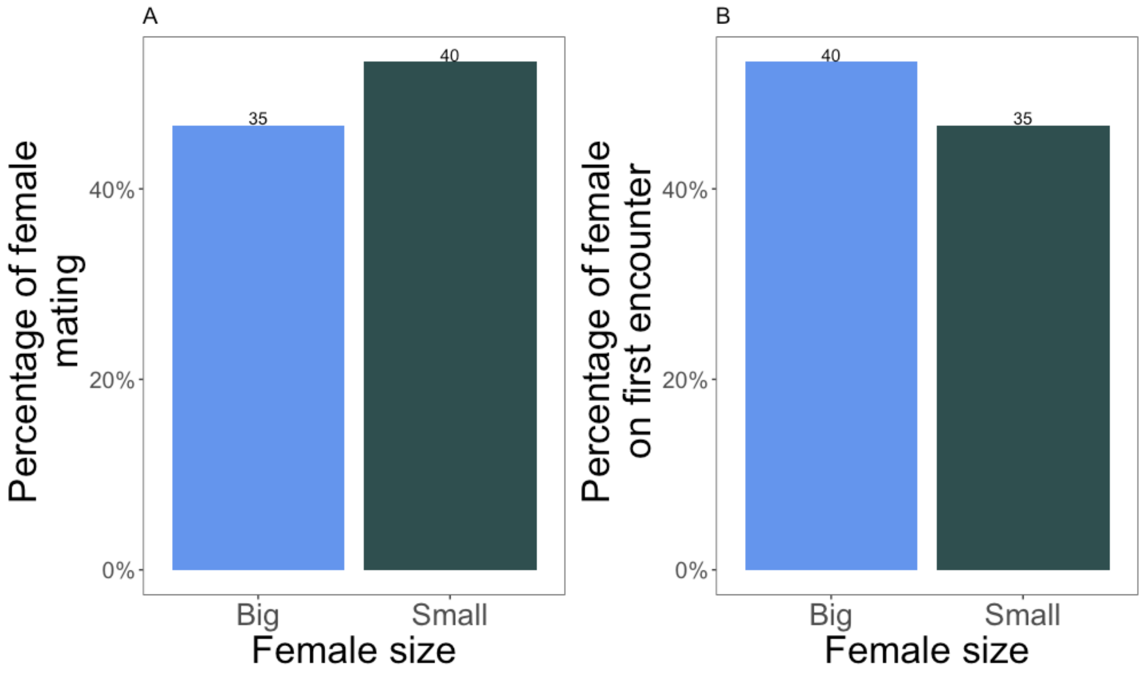

Figure 6: Male mating preference and encounter rate with females of different size. A: Male mating choice depending on female size**. B:** Male first encounter choice depending on female size. N=75.

Males did not show a mating preference depending on female size (GLM: Dev= 0.33358, N= 75, df=1, P=0.56) and male's first encounter with a female did not depend on her body size (GLM: Dev= 0.33358, N= 75, df=1, P=0.56). Since size does not affect male choice on mating and first encounter, I did not consider it as a random effect in generalized linear models (GLMER).

As mentioned in the method, all non-virgin females and some virgin females were handled while other virgins remained undisturbed during the preparation of the experiment. I am testing then if handling has an effect on female subsequent behaviour after male encounter.

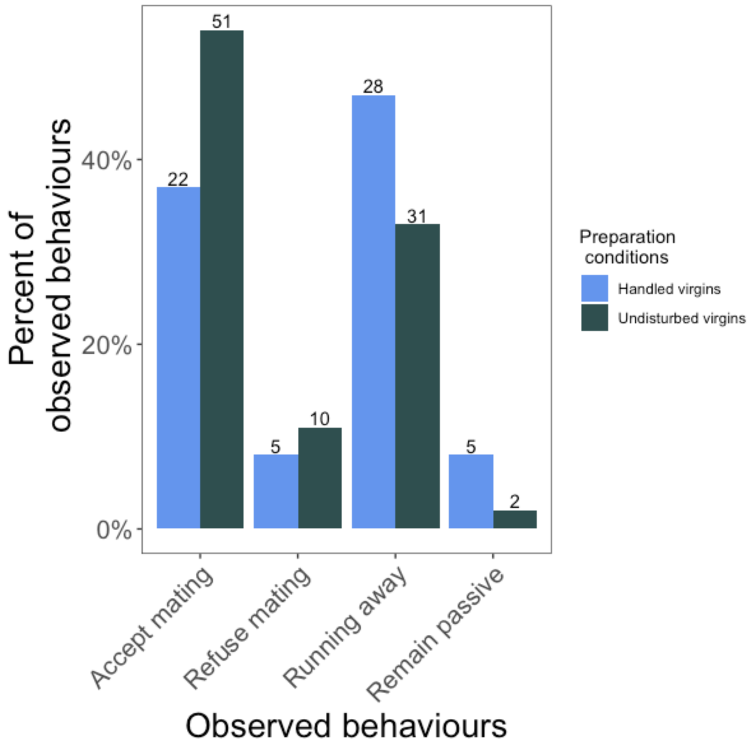

Figure 7: Handling effect on virgin female behaviour. The virgin females remained undisturbed in 53 trials while 29 trials were conducted after displacing the virgin females into another Petri dish for 10 minutes prior the experiment. N= 154.

I found no significant difference in female behaviour between virgin females that were undisturbed and those that were handled prior to the mating trial (GLM: Dev= 1.13399, df=3, N=154, P=0.76)

For R codes used in statistical analyses, see Appendix.

Discussion

General results: Males copulate more often with virgin females than females that were already mated. On the first encounter with a female, males were approaching females independent of mating status. After the first physical contact with a female, males were actively attempting to mate with virgin females but such attitude difference was not observed in running away, chasing and inactive behaviours. The behavioural response of females following close contact with a male was different depending on the female mating status: only virgins accepted to mate on the first encounter but females of both mating types often refused to mate and ran away. Males were more likely to continue following virgin females once they had first encountered her and this was not due to female size nor due to female repelling behaviours or female handling prior the experiment.

My results show that males tend to avoid mating with already mated females, which may be a way to avoid sperm competition with other males. This preference for virgins has also been found in other species including the common bed bug *Cimex lectularius* (Wang et al., 2016), the flour beetles (Haubruge & Arnaud, 1999; Lewis & Iannini, 1995), the moth *Plodia interpunctella* (Cook & Gage, 1995) or the parasitoid *Trichogramma turkestanica* (Martel et al., 2008). The preference is explained by males attaining low fertilization success when mating with already mated females (Bonduriansky, 2001; King et al., 2005). In *C. maculatus*, the low fertilization success might outweigh the benefit of investing a large amount of sperm or being the last male copulating (Dickinson, 1986; Martin et al., 1974). Differences in sperm investment has also been observed in other species where males copulate with both virgins and non-virgins (Bukowski & Christenson, 1997; Lüpold et al., 2011; Wedell, 1998; Wedell & Cook, 1999). In these examples, males copulated longer and invested more sperm with non-virgin females to maximizing fertilization success (Wedell et al., 2002).

Only in a few trials, did mating occur on the first close encounter. The behaviour of males and females following the first contact may hence be important for understanding the mate selection and mating result. At first, males were equally likely to approach any female independent of mating status. This may be due to males lacking information about female mating status before physical contact. Here, it is hard to know if males were aware that females of different mating status were available. Males in the experiment had never been in contact with other beetles, which are known to affect their behavior and investment towards females (Kelly & Jennions, 2011; Parker & Pizzari, 2010; Van Lieshout et al., 2014).

Males can recognize females visually but more often by touching them with their antennae that receive female sex pheromones (Battaglia et al., 2002; Bonduriansky, 2001; Carazo et al., 2004; Santos et al., 2017; Vuts et al., 2015). In many insect species, there are two types of female sex pheromones: sex attractant pheromones that are volatiles with a long-distance effect and contact sex pheromones that are effective in short distance (Birch, 1974; McNeil & Brodeur, 1995; Tanaka et al., 1986). For example, this mechanism is observed in the parasitoids *Aphidius ervi* (McClure et al., 2007) and *Aphidius nigripes* (McNeil & Brodeur, 1995), in *Drosophila melanogaster* (Tompkins & Hall, 1981) and in *Argynnis* butterflies (Vane-Wright & Boppre, 1993). In *Callosobruchus maculatus*, females emit sex pheromones with low volatility after emergence, then the amount of pheromones decreases after mating while male attraction to pheromones stays constant (Qi & Burkholder, 1982). Sex pheromones play important roles in *Callosobruchus* reproduction by improving copulation activity, affecting behavioral responses and recognition of other individuals and influence pheromonal communication in general (Howard, 1993; Lextrait et al., 1994; Nojima et al., 2007; Qi & Burkholder, 1982; Tanaka et al., 1986).

The presence of higher sex attractant volatile pheromones emission by virgin females, suggests that males would tend to visit virgin females first. However, I did not observe such discrimination on the first visit. A reason could be that female pheromones emission declines with age and may be affected by temperature, humidity or even atmospheric pressure which could bias male detection and choice (McClure et al., 2007; McNeil & Brodeur, 1995). I also used a different Petri dish every day but not every experiment and this may have affected the distribution and volatility of female sex pheromones. Long distance pheromones may be more equally distributed within the dish, hence not readily linked to any type of female and so, confusing male detection. Furthermore, in a study with *C. maculatus* pheromone release was coupled with calling behaviours where the females put their heads down and elevate the back of their bodies (Qi & Burkholder, 1982). These behaviours were not observed during my experiments and may also have altered the released of pheromones and attraction of males.

Another possible reason that I did not observed male preference on the first visit is due to the low volatility sex attractant pheromones of *C. maculatus*, favouriting antennae contact to obtain information about females (Qi & Burkholder, 1982). Female contact sex pheromones are produced from the abdomen and spread on the whole female body surface which could explain why males need a physical contact to get information about female sexual receptivity (McClure et al., 2007; McNeil & Brodeur, 1995). For example, it has been proved that antennae contact is fundamental in recognition of mates and mating in the parasitic wasp *Trichopria drosophila*, even if females emit long distance sex attractive pheromones (Romani et al., 2008). Antennae contact is also necessary in species where the male produced sex pheromones that females use in male recognition and lead to mate acceptance (Barrass, 1960; Battaglia et al., 2002). In other species, antenna contact is not necessary, as in the parasitic wasp *Mormoniella vitripennis* where males with antennae removed could still court and copulated (Barrass, 1960). Antennae contact may hence have different roles with diverse gradient of importance in mating and mate discrimination depending on the species. In *Callosobruchus maculatus*, a physical contact by touching females with their antennae might be required in male mate choice.

Not all males visiting virgin females attempted to mate, and this has also have been observed in ladybird beetles (Santos et al., 2017). Savalli and Fox observed that *C. maculatus* males present a variety of courtship persistencies that is not affecting mating success, this could explain why males may not attempt to copulate on first female encounter (Savalli & Fox, 1999).

My results show that some males tried to mate with the virgin female immediately, some ran away from them, remained inactive or did not have time to make any attempt due to the female escaping. Where males ran away or remained inactive, I can assume that this is an active male choice. In the other scenarios where the female ran away, it is hard to make any conclusion about male choice. To get a better understanding of the effect of female choice on male choice, I observed female reaction after the antennae contact to investigate its effect on male decisions. The variety of female behaviours observed, have also been described in other species, where females refusing mating, move their hint legs to kick off the male, run away or accept to mate (McClure et al., 2007; McNeil & Brodeur, 1995; Ortíz-Jiménez & Castillo, 2015; Santos et al., 2017).

Non-virgin females actively avoiding males could be explained by the risk of a second mating. *C. maculatus* mating is defined as «traumatic penetration» which causes damage on the epithelial surface on the female's genital tract (Crudgington & Siva-Jothy, 2000; Lange et al., 2013). Mated females need a recovery time until remating depending on the species. For example, in the study of the neotropical katydid, the females that copulated a second time before full recovery from the first mating died (Ortíz-Jiménez & Castillo, 2015). It would be interesting to follow the evolution of the few non-virgin females that remated. Many non-virgin females refused mating, and the risk of a second mating must be weighed against the benefits from obtaining more nutrient from spermatophores (Savalli & Fox, 1998), increasing fertility (Martin et al., 2004) or accumulating genetic diversity and get fitter offspring (Hosken & Stockley, 2003).

Not all virgin females accepted to mate on the first visit from males. Similar results have been observed in others studies where virgin females remained passive or walked away from males and this can be due to female receptivity variation (Byrne & Rice, 2006; McClure et al., 2007; Santos et al., 2017; Singh & Singh, 1999). Another reason could simply be female sexual selection. As shown in the study of female mate choice in *Callosobruchus maculatus* females would benefit from mating with larger males by obtaining a larger amount of sperm and increase their fertility (Savalli & Fox, 1999). They did not find clear evidences for female choice towards big males, suggesting that virgins may not be selective in this respect or may be limited by short lifespan (Savalli & Fox, 1999). I can also mention that in my method, virgin females were isolated until the experiment, which could create a stress and affect their behaviours after male encountering.

As mentioned by Martel, after a physical contact between males and females, it is challenging to discern male choice from female choice (Martel et al., 2008). Male and females *C. maculatus* have different reasons to be selective depending on their mating investment but both males and females would benefit from being choosy (Bonduriansky, 2001; Engqvist & Sauer, 2001; Fox et al., 1995; Savalli & Fox, 1999). In this study, I have investigated male choice following female reaction, to have a better understand of male decision making. Males continue to encounter non-virgin females even if they showed repelling and non-receptive behaviors. The male tendency of running away and avoiding mating attempt observed after visiting non-virgin females does not align with assumptions that males would inseminating all females and rather adapt their sperm investment during copulation. They rather select only virgin females to mate and avoid sperm competition.

My results show that males are selective, but that female behavior is also affecting mating results. This idea that males can be the first sex choosing is also discussed in a study on red flour beetles (Haubruge & Arnaud, 1999) where males are the ones interacting first by encountering females and trigger mating and the same conclusions were found by other studies focusing on the role of male choice (Arnqvist, 1988; Hemptinne et al., 1996). The female´s confirmation is also discussed in a bumblebee study where males cannot mate after only one interaction with the female (Sauter & Brown, 2001). A recent study on *Drosophila melanogaster* highlighted that in mating where females have a lot of control, such as refusing to mate by running away or kicking, males are observed to not be harmful while the inverse is observed with low male competition and female control (Yun et al., 2021). Following this theory, *C. maculatus* mating is described as harmful (Crudgington & Siva-Jothy, 2000), suggesting that males have some power and choice in mating.

My results confirm a previous study testing *C. maculatus* male decision given big and small virgin females, concluding that virgin males were not selective based on female size (Holme, 2019). Instead mating status is a major factor in male mating decision, as noticed in several other species including bumblebee *Bonbus terrestris* (Sauter & Brown, 2001). Other studies also concluded that mating status is determinant in male choice and highlighted that females are still taking the final decision by accepting to mate or not (Alonso-Pimentel & Tobin, 1992; Birch et al., 1989; Boake & Hoikkala, 1995).

Conclusions

The results of this study highlight the importance of understanding the dynamics of male and female mate selection. *Callosobruchus maculatus* males experience relatively large reproductive costs leading them to be selective. I showed that males are more often mating with virgin females. They had encountered females of both mating status on the first close contact, but behaved differently towards them. This suggest that males require a physical contact to obtain information about female receptivity and mating status. From this point, I highlighted the challenge of discerning male choice from female choice after a physical interaction between the two sexes. Virgin females are more likely to accept mating, but not all after a first mating attempt, manifesting their choice in mating. I finally demonstrated that mating status is a principal factor influencing male mating decision by testing the effect of others factors as female repelling behaviours, female handling and female size.

Appendix

R Code:

Males mating choice:

```
F1.glm  <- glm(counts ~ Female_chosen_to_mate , family=poisson, data= TotalFemale)
anova(F1.glm, test="Chi")
summary(F1.glm) #AIC= 13.876
emmeans(F1.glm, pairwise~Female_chosen_to_mate, type="response", adjust="none")

F1.glmer <- glmer(counts ~ Female_chosen_to_mate +(1|Day), family=poisson, data=
GLMER1)
summary(F1.glmer) #AIC = 125
```

Male choice on the first visit:

```
#A:
F2.glm  <- glm(counts ~ Who , family=poisson, data= FirstFemale)
anova(F2.glm, test="Chi")
summary(F2.glm)  #AIC = 14.919
emmeans(F2.glm, pairwise~Who, type="response", adjust="none")

F2.glmer <- glmer(counts ~ Who+(1|Day), family=poisson, data= GLMER2)
summary(F2.glmer) #AIC =  103.2

#B
#Mann Witney U test / Kruskal Test:
kruskal.test(Time_in_second~First_female_visited, data=Time1Visit)
```

Male behaviours after first encounter:

```
F3.glm  <- glm(counts ~ Observation + Who + Observation:Who, family=poisson, data=
Malebehavior.df)
anova(F3.glm, test="Chi")
summary(F3.glm) #AIC= 46.294
emmeans(F3.glm, pairwise~Who|Observation, type="response", adjust="none")

F3.glmer  <- glmer(counts ~ Observation + Who + Observation:Who + (1|Day),
family=poisson, data= GLMER3)
summary(F3.glmer) #AIC = 137.3
```

Female passive/active behavior:

```
#per observation
F4.glm  <- glm(counts ~ Observation + Who + Observation:Who, family=poisson, data=
GLM4)
anova(F4.glm, test="Chi")
summary(F4.glm) #AIC =92.87
emmeans(F4.glm, pairwise~Who|Observation, type="response", adjust="none")

F4.glmer  <- glmer(counts ~ Observation + Who + Observation:Who + (1|Day),
family=poisson, data= GLMER4)
summary(F4.glmer) #AIC = 358.4
```

```
#per number of visits
F4b.glm  <- glm(counts ~ Nb_of_interaction + Who + Nb_of_interaction:Who,
family=poisson, data= Visit)
anova(F4b.glm , test="Chi")
summary(F4b.glm) #AIC = 106
emmeans(F4b.glm, pairwise~Who|Nb_of_interaction, type="response", adjust="none")

F4b.glmer  <- glmer(counts ~ Nb_of_interaction + Who + Nb_of_interaction:Who + (1|Day),
family=poisson, data= Visit)
summary(F4b.glmer) #AIC =  381.8
```

Male changing female tendency

```
F5.glm  <- glm(percent ~ V1 + Behaviour + V1:Behaviour, family=poisson, data= Switch.df)
anova(F5.glm, test="Chi")
summary(F5.glm)#AIC = 26.199
emmeans(F5.glm, pairwise~Behaviour|V1, type="response", adjust="none")

F5.glmer  <- glmer(counts ~ FirstVisit + SecondVisit + FirstVisit:SecondVisit + (1|Day.x),
family=poisson, data= GLMER5)
summary(F5.glmer) #AIC= 111
```

Female behaviour effect on male choice

```
F6.glm  <- glm(percent ~ Observation + Who + Observation:Who, family=poisson, data=
EffectBehav)
anova(F6.glm, test = "Chisq")
summary(F6.glm) #AIC= 39.725
emmeans(F6.glm, pairwise~Who|Observation, type="response", adjust="none")

F6.glmer  <- glmer(percent ~ Observation + Who + Observation:Who + (1|Day),
family=poisson, data= GLMER6) #percent
summary(F6.glmer) #AIC= 105
```

Female size effect on male choice:

```
#Effect of size on male mating choice
F7.glm  <- glm(counts ~ TendencytoMate, family=poisson, data= MateTendency)
anova(F7.glm, test = "Chisq")
summary(F7.glm) #AIC= 14,929
emmeans(F7.glm, pairwise~TendencytoMate, type="response", adjust="none")

F7.glmer  <- glmer(counts ~ Matetendency + (1|Day), family=poisson, data= GLMER7)
summary(F7.glmer) #AIC= 102.7

#Effect of Size effect on first encounter choice
F7b.glm  <- glm(counts ~ TendencytoVisit, family=poisson, data= VisitTendency)
anova(F7b.glm, test = "Chisq")
summary(F7b.glm) #AIC= 14,929.
emmeans(F7b.glm, pairwise~TendencytoVisit, type="response", adjust="none")

F7b.glmer  <- glmer(counts ~ Matetendency + (1|Day), family=poisson, data= GLMER7b)
summary(F7b.glmer) #AIC= 119.5
```

Handling effect on female behaviour

```
F8.glm  <- glm(Percent ~ Observation + Group + Observation:Group, family=poisson, data= GroupVS)
anova(F8.glm, test = "Chi")
summary(F8.glm) #AIC = 53
emmeans(F8.glm, pairwise~Group|Observation, type="response", adjust="none")
```

```
F8.glmer  <- glmer(Percent ~ Observation + Group + Observation:Group  + (1|Day), family=poisson, data= GLMER8)
summary(F8.glmer) #AIC = 254.7
```

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