Male mate choice and selectivity in relation to female body size, in the seed beetle *Callosobruchus maculatus*

Thesis for the degree of Master of Science in Biology

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Finally, I have to thank my parents for always supporting me and teaching me critical thinking, as well as encouragement during the writing of this thesis.

Front page: A *Callosobruchus maculatus* male (right) copulating with a female (left).

Photo credit: Ivain Martinossi-Allibert.

Abstract

Selectivity in mate choice and mating behaviour is a central element of sexual selection, and an important component of evolution in a number of species across the animal kingdom. Mate choice relates to how individuals respond to the phenotypic traits of potential mates in order to maximize their own fitness. As most studies on sexual selection concentrate on female mate choice, it is interesting to investigate to what extent male choice may play a role in evolution. In the seed beetle *Callosobruchus maculatus* males are paying a significant cost when mating, by inseminating the female with a large spermatophore. There is also differences in fitness consequences when choosing mates, as larger females generally show higher fecundity. These factors may select for male mate choice, but whether male *C. maculatus* are able to discriminate between females of different size and be selective in their mating behaviour, is currently unknown. This study takes an experimental approach to investigate whether males discriminate between females of different weight, both when choosing mates and in their energy investment during copulation. Results indicate that males do not show any mating preference in their first mating, and do not transfer bigger spermatophores when copulating with larger females. However, as this study only focuses on the male's first mating after isolation, it is still possible that male choice becomes more important later when they are exposed to more mates.

Table of Contents

Introduction

Sexual selection contributes to differences in the reproductive success among individuals, where factors such as mating competition and mate choice determine the distribution of matings in a population (Halliday, 1983). Mate choice can be defined as a behavioural pattern, shown by members of one sex, leading to it being more probable to mate with certain members of the opposing sex than with others (Halliday, 1983, p. 4). Such discrimination towards potential mating partners, can be based on selective responsiveness to both behavioural and morphological traits. This typically consists of accepting some mates, while rejecting others. Any selectivity in mate choice, involves a trade-off between the number of mates and the quality of mates (Andersson & Iwasa, 1996).

An individual's relative breeding success is measured in fitness (Hine, 2008). If mate choice has implications for fitness and the cost of mating is high, we can generally expect that the species is more selective in its mating behaviour. In addition, the cost of mate search and assessment of mate quality should be low for such behaviour to evolve (Bonduriansky, 2001). Ultimately due to the imbalance in gamete investment, females are usually paying a considerably higher cost than males when mating, they are generally associated with a greater parental investment, and subsequently tend to exhibit a more selective behaviour (A. J. Bateman, 1948; Bonduriansky & Brooks, 1998; Jones & Ratterman, 2009; Trivers, 1972). In general, a female's reproductive success is expected to be limited by her ability to produce eggs, while a male is limited by the number of eggs he can fertilize (A. J. Bateman, 1948).

In several animal species, males invest little energy into a single mating and do not provide much effort in raising offspring. Therefore, they are often oriented towards quantity rather than quality when looking for mating opportunities (Pischedda & Stewart, 2016; Trivers, 1972). While mate choice in females has been extensively studied, much less empirical attention has been devoted to male preference. However, several studies suggest that males may discriminate between females in a wide range of species, including insects, fish and mammals (Bonduriansky, 2001; Edward & Chapman, 2011; Herbert, 1968; Rosenqvist, 1990).

In species with substantial male parental investment males may be selective in their choice of mate (Trivers, 1972). Indeed, male choice has been observed in several species where males contribute to parental care (Gwynne, 1991; Monaghan, Metcalfe, & Houston, 1996; Rosenqvist, 1990). However, this does not seem to be a necessity for male choice to evolve, as choosiness

among males has also been observed in species where they invest little in the care of offspring (Amundsen & Forsgren, 2001; Bondurianky, 2001; Edward & Chapman, 2011).

Variation in parental investment between the sexes can affect the operational sex ratio (OSR); the average ratio of sexually active males to receptive females in a population at any given time (Emlen & Oring, 1977). Recent theory suggest that OSR, rather than parental investment, is the causal factor for evolution of sex roles (Clutton-Brock, 2007; Owens & Thompson, 1994). Mating competition is predicted to be most intense among members of the sex in which OSR is skewed, whereas the other sex will then be more selective in their mate choice (Owens & Thompson, 1994; but see Hayes, Callander, Booksmythe, Jennions, & Backwell, 2016; Janicke & Morrow, 2018). If the tertiary sex ratio, the ratio of sexually mature individuals, is equal, OSR-theory predicts that the OSR will be skewed in the direction of the sex with the highest potential reproductive rate (PRR) (Clutton-Brock & Vincent, 1991; Owens & Thompson, 1994). There are studies suggesting that variation in mate quality may disrupt this pattern, as it potentially leads to a greater relative fitness payoff by choosing a high-quality mate. Owens and Thompson (1994) argue that if there is sufficiently high variation in quality amongst individuals of the least competitive sex, the opposing sex may also be the most choosy. Several studies include mate quality in their models and conclude that mate choice is influenced by an interaction of OSR and the variance of mate quality (P. W. Bateman, 2006; Clutton-Brock, 2007; Edward & Chapman, 2011; Kvarnemo & Simmons, 1999; Reinhold, Kurtz, & Engqvist, 2002).

The investment in mating effort might also constitute a significant cost and serve as an additional key factor in the evolution of mate choice (Edward & Chapman, 2011). In certain insect species copulations are associated with an increase in predation risk (Arnqvist, 1989; Wing, 1988) and sexually transmitted diseases (Hurst et al., 1995), thus copulations can be costly for both sexes. An increase in sperm competition could also lead to a greater investment in mating effort as males produce larger ejaculates (Edward & Chapman, 2011). By increasing the number of sperm in an ejaculate, the male can increase his fertilisation probability (Parker & Pizzari, 2010), but only at a certain cost. Even if males invest little in a single sperm, a single ejaculate or spermatophore can include millions of sperm, and sperm production is generally limited in several taxa (Dewsbury, 1982). If the number of sperm a male can produce is limited, one should expect some discrimination by males in the allocation of their reproductive investment (Dewsbury, 1982). In some insects, males are known to strategically adjust their ejaculation size depending on the socio-sexual situation (Gage, 1991; Gage & Baker, 1991;

Martin & Hosken, 2002). Ejaculations must be costly for males for such facultative ejaculate adjustment to be advantageous.

The ability for males to discriminate between females have been found in various taxa, and males often transfer larger ejaculates to higher quality females (Kelly & Jennions, 2011). In species where ejaculations are associated with the production of nuptial gifts, common in many arthropods, copulations could prove extra costly for males (Gwynne, 2008; Vahed, 2007). Such males could obtain additional fitness benefits by displaying phenotypic plasticity in response to female traits and allocate their ejaculates strategically. Accordingly, some optimal sperm allocation models predict that in species where sperm transfer depends on the size of nuptial gift, males should invest more when copulating with high quality females (Reinhold et al., 2002). Such cryptic male choice has been proven to exist in some species of insects where males produce nuptial gifts. For instance, several cricket species are known to discriminate between female traits and not only adjust their courtship behaviour, but also the size and composition of their spermatophore depending on these traits (P. W. Bateman & Fleming, 2006; Jarrige, Body, Giron, Greenfield, & Goubault, 2015; L. W. Simmons, Craig, Llorens, Schinzig, & Hosken, 1993).

Male cowpea weevils, *Callosobruchus maculatus*, similarly invest much energy in their spermatophores, but still little has been done to explore whether a similar degree of choosiness exists in this species. Several studies have examined various aspects of the mating behaviour of seed beetles, and a number of them focus on *C. maculatus*. While females of another seed beetle, *Stator limbatus*, prefer large males (Savalli & Fox, 1998b), there is scarce documentation of intersexual selection in *C. maculatus*.

Both male and female *C. maculatus* usually mate multiple times during their lifetime (Martinossi-Allibert, Arnqvist, & Berger, 2017). Males frequently attempts to mate with females, while females regularly kick with their hind legs, seemingly to dislodge males and end copulations (Edvardsson & Tregenza, 2005; Martinossi-Allibert et al., 2017; Rönn & Hotzy, 2012). Males inseminate females with large ejaculates, often constituting more than 5%, and sometimes more than 10%, of their body weight when mating for the first time (Fox, Hickman, Raleigh, & Mousseau, 1995; Savalli & Fox, 1999a). Spermatophore size generally decreases with subsequent copulations (Fox et al., 1995; Rönn, Katvala, & Arnqvist, 2008; Savalli & Fox, 1999a). The transfer of such large spermatophores may function as a nuptial gift (Fox & Moya-Laraño, 2009; Fox, Stillwell, Wallin, & Hitchcock, 2006), as it contains considerably more sperm than female *C. maculatus* are able to store in their spermatheca (Eady, 1994, 1995). More

specifically, there are evidence suggesting that this excess sperm can be metabolized by the female (Edvardsson & Canal, 2006; Fox, 1993a; Ofuya, 1995; Savalli & Fox, 1999a). Thus, the large investment in a single spermatophore could increase paternity, but may also inhibit the male's reproduction rate (Lewis & South, 2012). Both fecundity and fertility of female *C. maculatus* have shown to be negatively correlated with number of matings the male had performed that day (Ofuya, 1995). Ofuya (1995) showed that virgin females laid fewer and less viable eggs when experiencing a male's fifth or sixth mating, compared to the male's first two matings.

Studies of ejaculation depletion as reported above, emphasise the cost connected to the male's first mating. Savalli and Fox (1998a) found that the size of ejaculation in *C. maculatus* is a form of male investment where there is genetic variation, some of which are independent to body size. Furthermore, they found it plausible that some of the loci influencing ejaculation size may be located on the X-chromosome, indicating that ejaculation size may respond rapidly to selection (Charlesworth, Coyne, & Barton, 2002; Meisel & Connallon, 2013). The transfer of large ejaculations does not necessarily make males more successful in sperm competition (Edvardsson & Canal, 2006; but see: Eady, 1995), but may provide benefits to males by prolonging the female trajectory period or by delaying female remating. Several studies report that females are more reluctant to remate when mating with virgin males, which produce larger ejaculates (Rönn et al., 2008), than when mating with non-virgins (Eady, 1995; Savalli & Fox, 1999a).

In addition, reproduction is trading off with longevity as engaging in mating behaviour is shown to reduce the lifespan of male *C. maculatus* (Brown et al., 2009; Paukku & Kotiaho, 2005). Some findings indicate that it is the duration of copulation, rather than the size of spermatophore transfer, that has a negative effect on longevity. Brown and colleagues (2009) reported that the duration of a male's first copulation was negatively associated with their longevity, indicating that there is a trade-off between male reproductive behaviour and longevity in this species. Long copulations could also constitute a cost by increasing a period of immobility and consequently there are opportunity costs and potentially an increase in predation risks associated with this activity (Arnqvist, 1989; Wing, 1988). This could still be adaptive for male *C. maculatus* if copulation duration is associated with an increase in ejaculate transfer (Edvardsson & Canal, 2006) or a delay in female remating. In many insects copulating males or their ejaculates can provide stimuli which trigger a decrease in female receptivity to courting males (Cook, 1992; Fuchs, Craig, & Despommier, 1969). For some species, selection favours long copulations in

males even though most sperm is transferred at the beginning of copulation, as the later stages is associated with formation of copulatory plugs or transfer of other manipulative seminal fluids (Duvoisin, Baer, & Schmid-Hempel, 1999; Merritt, 1989). Thus, several factors influence the optimal copulation time, including sperm competition and the availability of mates (Parker, 1974).

For mate choice to evolve, there has to be variation in the net benefit gained by choosing different mates (Edward & Chapman, 2011; Parker, 1983). In *C. maculatus* females vary greatly in body weight and the genetic variation explaining these differences are known to be heritable (Fox, Bush, Roff, & Wallin, 2004). Female size is positively correlated with fecundity in this species (Credland, Dick, & Wright, 1986; Edvardsson & Tregenza, 2005; Fox, 1993a, 1993b; Katvala, Rönn, & Arnqvist, 2008), hence males could potentially benefit from discrimination between females of different size.

In species where there is high variation in female quality and where mating is costly for males, males could potentially show a degree of selectivity in their mating behaviour (Andersson, 1994; Kvarnemo & Simmons, 1999). As such conditions are met in the seed beetle species *C. maculatus*, this proves a good candidate for a species in which males are selective when choosing females. Establishing whether male choice has an impact on mating, could be an important contribution to the understanding of the mechanisms behind sexual selection in this species. Additionally, this knowledge could provide a basis for future research on sexual selection in other closely related species.

The objective of this study was to explore how variation in female size can influence male mating selectivity. This has been done by investigating if female body weight influence mating behaviour in male cowpea weevils, *Callosobruchus maculatus*. A number of experiments were conducted by presenting males with females of different weight.

The main hypotheses were that virgin *C. maculatus* males when choosing a mate:
i) show a preference for larger over smaller females and/or ii) have greater reproductive investment when mating with larger compared to smaller females.

The following predictions were made:

1. Virgin males will show a tendency to mate with the larger female when given a choice between two females of different weight.

2. Virgin males will be more eager to mate with larger females, measured as reduced time before start of copulation.

3. Virgin males will copulate for longer when mating with larger females.

4. Virgin males will transfer a larger spermatophore when mating with larger females.

Method

Study organism and rearing

The cowpea weevil, *Callosobruchus maculatus,* are seed parasites considered to be agricultural pest insects. They are of significant importance due to their cosmopolitan distribution and their role as a pest of stored legumes (Fabaceae) (Fox et al., 2004; Lopes, Sousa, Santos, Silva, & Abreu, 2018). Several aspects of the *C. maculatus* life history makes this a species well suited for laboratory experiments. Female oviposition takes place on the surface of certain bean types, particularly of the genus *Vigna* (Fox et al., 2004). Within 4-5 days (at 26-28°C), the egg hatches as the larvae burrows into the bottom of the egg and enters the bean by penetrating the seed coat (Fox, Bush, & Wallin, 2003; Souza et al., 2011). The larval stage occurs within the cotyledon of the bean, where the larvae goes through four instars (Devereau, Gudrups, Appleby, & Credland, 2003). Here it collects all necessary energy reserves to complete its life cycle and reproduce successfully (Savalli & Fox, 1999a). The larval stage lasts around three-four weeks, depending on the species of bean, before they eclose as adult beetles (Devereau et al., 2003; Rönn, Katvala, & Arnqvist, 2006). Average adult life span is approximately two-three weeks (Fox, Bush, et al., 2003; Fox, Dublin, & Pollitt, 2003). The adult beetles are facultative aphagous, which makes it possible to quantify an individual's investment in metabolism, behaviour and reproduction during the adult phase, by measuring energy expenditure over time. In the experiments, male beetle weight loss has been registered and used as a proxy for energy investment, in line with methods used in earlier experiments (Wagner & Bakare, 2017).

The *C. maculatus* population used in the present study was imported from Carolina Biological Supply, USA, to the University of Bergen, Norway, in February 2016. The imported line has given rise to several generations and cultures has been maintained alternately between room temperature and a 28-28.5°C incubator with a 24-hour light access. Populations were kept in petri dishes exclusively containing mung beans, *Vigna radiata*. The generation used in experiments derived from several bean layered petri dishes containing 50 males and 50 females each.

Experiments

Through a series of experiments, the influence of the weight of potential mates on male mating behaviour were examined. To ensure that the male and females had not previously been mated, all larvae containing beans were isolated prior to emergence of the beetle. They were kept isolated in a 24-well plate until onset of the experiment. To ensure complete and undisturbed larvae development, only beans containing a single larva was used in experiments. All beans were controlled once every day, and any emergence was registered. Every mating trial consisted

of beetles of the same age, emergence within no more than 24 hours from each other. Male *C. maculatus* is ready to mate immediately upon emergence, but their ejaculate are not fully formed (Fox et al., 1995). All beetles were therefore allowed to mature for at least 24 hours prior to the experiments, and no beetles for more than 96 hours. After emergence from the bean, adult beetle does not depend on additional water nor nutrition, hence in the experiments no such supplements were provided. Any beetles suffering from visible injury or birth effects was removed from experiments. All experiments were performed at 20°C, but beetles kept at 28°C.

Choice of mate. Male mate preference was examined by presenting virgin males with a choice between two virgin females of different weight, serving as a proxy for female size. Based on their relative weight, females were sorted into two groups; one consisting of the heaviest half, and the other of the lightest half. The female with the lowest weight in the heavy group, was paired with the lightest female in the light weight group. Similarly, the second lightest females from each group formed a pair and this pairing was repeated for all females. To ensure a sufficient weight difference in each pair of females, a requirement was set that that the two females in each pair were visually distinct from one another in respect to their body size. If one pair failed to fulfil this requirement, or if total number of females was odd, the female with the median weight was removed from the experiment. This method ensured a substantial weight difference in each pair and made easy to detect which female that mated, without marking them. The larger female in each pair were on average 24% (± 5,3% SD, range 16-40%) heavier than the smaller female, and all pairs had > 0.78 mg weight difference. A similar method has been used by Savalli and Fox (1998b, 1999b) in two studies investigating female choice of two males in both *C. maculatus* and *Stator limbatus*.

Time before copulation. All males were randomly selected and assigned to a random pair of females after weighing. The three beetles, two females and one male, were confined in a 60 mm petri dish and observed continuously until copulation. In total, 39 experiments were conducted. Estimates were made of the time prior to mount, or attempted mounting of the female, after which the female not participating in mating behaviour was immediately removed and reweighed for identification. If 15 minutes passed without any copulation attempt, the male was classified as "unmated".

Duration of copulation. The length of copulation was recorded to the nearest second, starting from the moment the female was mounted and ending when the genitalia were physically separated.

8

Male relative weight loss. Males where reweighed immediately after copulation, and male weight loss was used as an estimate of ejaculate size. Some of the mated females were reweighed as well, to serve as a control for the validity of this method. The mean difference between male weight loss and female weight gain was 0.07 mg ($\pm$ 0.02). There was a positive correlation between male weight lost during copulation and female weight gained, but this is inconclusive due to a very low sample size (linear model, p = 0.02; N = 9). However, previous studies have shown a strong correlation between male weight lost and female weight gained, indicating that this is a reliable method of measuring ejaculation size in this species (Edvardsson & Tregenza, 2005; Savalli & Fox, 1998a).

Female kicking behaviour. Any kicking-events made by females to ostensibly attempt to end or reject copulation were recorded. Time before kicking was measured from the moment the female was mounted until kicking was first observed, and the duration of kicking was measured up until end of copulation.

Weighing routines

All weighing was performed using a Sartorius® M3P micro balance, a six-decimal balance. The measurement uncertainty of this instrument was set to 1,7 µg + 0,0409 µg/mg * R, where R represents the net load indication. The measurement uncertainty was calculated to < 0.05% for all females and $\leq$ 0.06% for all male weights. Due to the sensitive nature of such balances and instability caused by beetle movement, weight readings were performed after 40 seconds of continuously stable weight or after two minutes. All females were weighed less than 12 hours prior to experiment. All males were weighed at least 30 minutes and no more than one hour prior to experiment.

Statistical analysis

A two-sided binomial test was performed to determine whether males showed a tendency to copulate with either a smaller or a larger female. A paired t-test was used to detect any differences in weight between the group of females that mated and the group that did not. To include male weight as a factor, a binary logistic regression, a form of generalized linear method (GLM), was also performed. In this test, the two potential outcomes were copulating with a smaller female versus a larger female.

The effect of mating with a smaller or a larger female on the time before copulation, was tested in a survival analysis, which estimates a survival function based on the Kaplan–Meier estimator (Therneau, 2015). The survival distributions of the two samples were compared in a log-rank test. This type of analysis was chosen to appropriately handle the "time to event data" and to

avoid any bias caused by censoring. Censored data occurred in trials where no copulation took place within the 15 minutes of observations. Several survival models were made with different error distributions, and compared to each other through an analysis of variance, ANOVA. Type of error distribution was chosen based on the model with the lowest unexplained variability.

Linear regression models were used to analyse the effect of male weight and relative weight difference on the duration of copulation. The effect of mating with a smaller or a larger female on duration, was tested in a survival analysis. For analysis on male relative weight loss, linear regression was used to test the effect of weight and the duration of copulation. Linear regression was similarly used to detect the effect of weight on both time before kicking and duration of kicking. All statistical analyses were carried out using R version 3.5.1 (R Core Team, 2018). Values are given as means ± SE unless otherwise stated.

Results

Choice of mate

Virgin males did not show any preference for heavier females over lighter females, when given a choice (binomial test, two-sided, p = 0.86; N = 34). Males chose to mate with the smaller female in 46.2% of the trials and the larger in 41.0%. In the remaining 12.8% trials, no copulation occurred. Weight of mated females in relation to male weight, is shown in Figure 1. The initial weight of the mated females (5.10 ± 0.12 mg; N = 34) did not differ from the weight of the unmated females (5.15 ± 0.11 mg; N = 34) (paired t-test, t = 0.25, 33 d.f., p = 0.80). The binary logistic regression showed no effect of male weight on male preference (Effect ± SE: 0.83 ± 0.88, $Z_{1,32}$ = 0.95, p = 0.34).
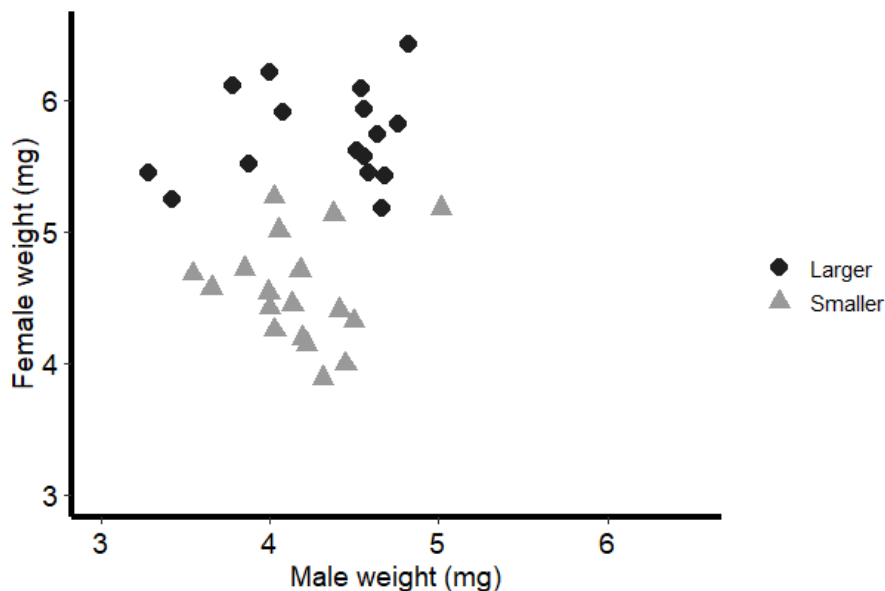

Figure 1: Weight of females selected as mates as a function of male weight (mg). All weights were measured prior to copulation. Males had a choice of copulating with two females of different weight. Triangles indicate that the male copulated with the smaller female, whereas diamonds indicate copulations with the larger of the two females. N = 34.

Time before copulation

The time until start of copulation did not significantly differ between the males that mated with the larger female (1.94 ± 0.87 minutes; N = 16) compared to the smaller female (2.53 ± 0.83 minutes; N = 18) (log-rank test, distribution = "lognormal", p = 0.39). The majority of the copulations started within the first minute and 88% of the matings started within 3.4 minutes. The relationship between time before copulation and female weight is illustrated in Figure 2.
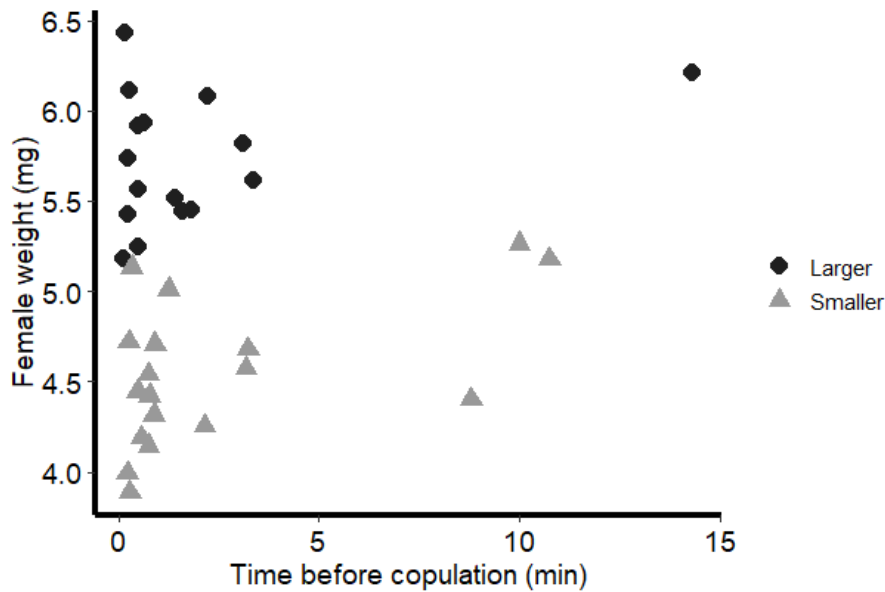
Figure 2: Weight of females selected as mates (mg) as a function of time before copulation (minutes). All weights were measured prior to copulation. Males had a choice of copulating with two females of different weight. Triangles indicate that the male copulated with the smaller female, whereas diamonds indicate copulations with the larger of the two females. N = 34.

Duration of copulation

The linear regression showed that male weight did not have any significant effect on the duration of copulation (Effect $\pm$ SE: -1.95 $\pm$ 1.48, $T_{1, 32}$ = -1.32, p = 0.20). Neither did the relative weight difference between the male and the female (4.70 $\pm$ 3.10, $T_{1, 32}$ = 1.52, p = 0.14). The duration of copulation did not significantly differ between the males that mated with the larger female (6.99 $\pm$ 0.78 minutes; n=16) compared to the smaller female (6.22 $\pm$ 0.96 minutes; n=18) (Log-rank test, distribution = "extreme", p = 0.93; Figure 3).
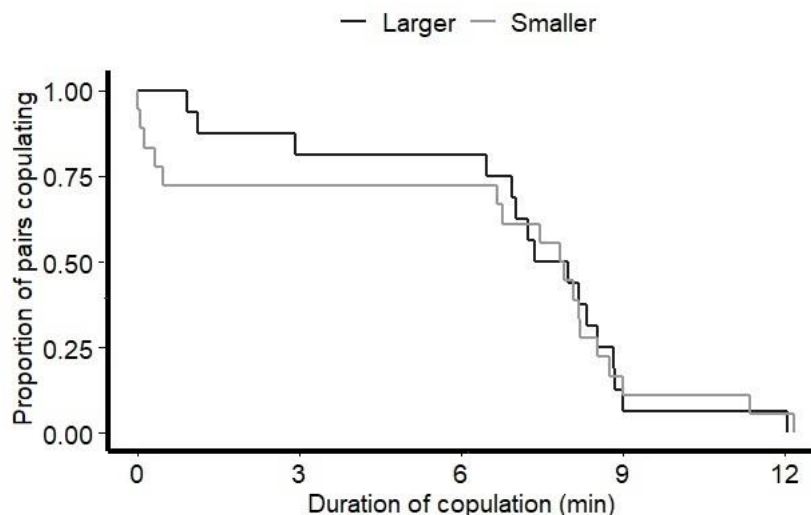
Figure 3: Survival plot of the duration of copulation (minutes). Males had a choice of copulating with two females of different weight. The two survival curves represent males copulating with a smaller (grey) and a larger (black) female. The proportion of individuals who are copulating is given as a function of the duration of copulation. Log-rank test, error distribution = "extreme", p = 0.93. N = 34.

Male relative weight loss

Male relative weight loss was not significantly affected by the duration of copulation, female and male weight, nor their interaction (Table 1). The relationship between male weight loss, duration of copulation and weight of female mate is shown in Figure 3. Six of the observations were removed due to very low duration (< 1 minute) and no weight loss (≤ 0 mg), indicating that copulation was unsuccessful. Another two observations were treated as methodological outliers and removed prior to analysis. One of the outliers yielding a negative weight loss and the other a very high relative weight loss (> 16%). Males had an average relative weight loss of 6.1% (± 0.4 SE, range 3-11%), which constitutes a mean absolute weight loss of 0.25 mg (± 0.02 SE).

Table 1: Test of linear model effects of duration (minutes) and body weight (mg) on male relative weight loss (mg). "Relative weight difference" measured as $\frac{female\ weight}{male\ weight}$. N = 26.

RESPONSE VARIABLE	PREDICTOR VARIABLE	EFFECT	SE	$T_{1,\ 24}$	P
Male relative weight loss	Duration	0.28	0.29	0.98	0.34
	Female weight	0.70	0.61	1.14	0.26
	Male weight	0.15	1.09	0.13	0.89
	Relative weight difference	1.92	2.16	0.89	0.38

13

Female kicking behaviour

The time before female kicking and the duration of copulation was closely related (Figure 4). More specifically, most copulations ended less than one minute after start of kicking (1.06 ± 0.24 minutes; N = 34; Figure 4) and only three copulations lasted longer than two minutes after the female started kicking. Neither male or female weight, nor the weight difference between mates, had any effect on the time before kicking or the duration of kicking (see Appendix A).
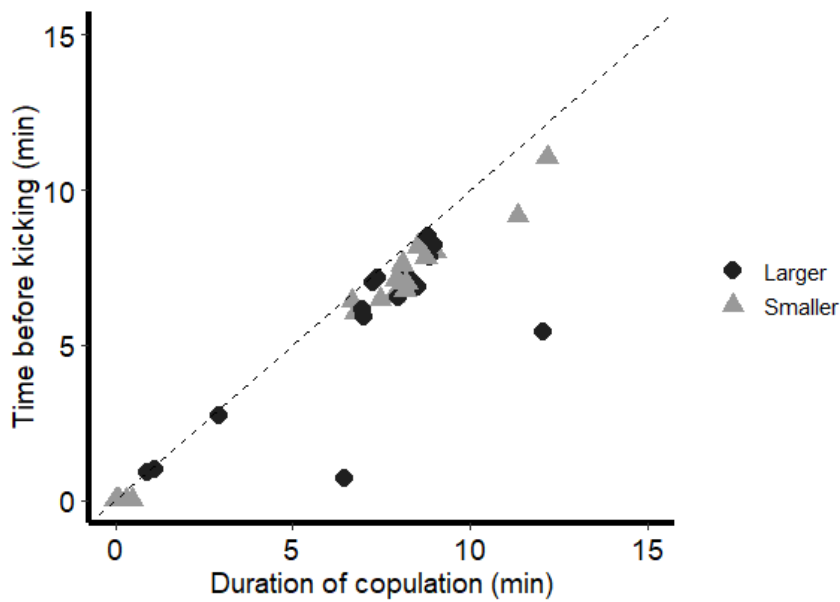

Figure 4: Time before female kicking (minutes) as a function of duration of copulation (minutes). Males had a choice of copulating with two females of different weight. Triangles indicate that the male copulated with the smaller female, whereas diamonds indicate copulations with the larger of the two females. Dashed line represents the identity line (y = x). N = 34.

For R codes used in statistical analyses, see Appendix B

Discussion

Males did not show any preference for mating with larger females over smaller females when copulating for the first time. Neither did male weight affect their female preference. Female weight did not influence the time before copulation, the duration of copulation nor the amount of transferred ejaculate in the first mating. Most copulation attempts happened shortly after the male was exposed to the female, supporting the notion that naive males are eager to copulate and show little selectivity when choosing a mate.

Males were given a choice of mating with a small or a large female that were presented at the same time. However, sequential mating decisions may better reflect the natural setting of *C. maculatus*, rather than simultaneous exposure. Theoreticians have proposed several different tactics that can be used when choosing mates and sequential mate choice is probably more accurate for many taxa (Jennions & Petrie, 1997; but see: Barry & Kokko, 2010). The cricket species *Gryllus bimaculatus* discriminate between mates based on their size in sequential but not in their first mating (P. W. Bateman & Fleming, 2006; P. W. Bateman, Gilson, & Ferguson, 2001). Moreover, both male and female crickets appear to use their previous matings as a yard stick for evaluating other potential mates. Thus, we cannot rule out the possibility that male *C. maculatus* are selective in later matings using the present experimental design. Another possibility to consider is that high quality females can afford to be more selective towards males, and mating attempts towards such females may be associated with a higher probability of rejection. This would complicate the expected pattern of male choice. Whether females discriminate in this manner is unknown, but in the present study, female kicking was not influenced by male weight.

Males did not copulate for a longer time period when mating with a larger female compared to a small one. The majority of copulations lasted between six and nine minutes, indicating that this is the typical copulation duration, regardless of male and female weight. Eady and Brown (2017) similarly found that the duration of copula in *C. maculatus* was unrelated to male and female size. In their study copulations lasted only 5 minutes, while in the study of Brown et al. (2009) mean duration was 9.9 minutes. This demonstrates that there can be significant variation in copulation duration among populations or experimental settings. Copulation duration is found to be a heritable trait in male *C. maculatus* (Brown et al., 2009; but see: Savalli & Fox, 1998a). The trait could thus be subject to sexual conflict, serving as a form of "extended mate guarding" and delay female remating (Parker, 1970). Evidence of this is found in other insect species (Mazzi, Kesäniemi, Hoikkala, & Klappert, 2009). Miyatake and Matsumura (2004)

found that short copulations (one or three minutes) increased remating frequencies of female *C. maculatus* compared to copulations lasting five minutes. This could indicate that it is incomplete sperm transfer that affects remating, although transfer of ejaculates was not measured in this study. Interestingly, the duration of the first copulation has been found to be correlated with a reduced success in sperm competition in *C. maculatus* (Edvardsson & Canal, 2006; Edvardsson & Tregenza, 2005). The influence of copulation duration on fertilisation success is hence more complex, which may explain the lack of correlation between copulation duration and female weight. Furthermore, female kicking may prevent long copulations and restrict the male's ability to selectively regulate duration.

Ejaculate size was not correlated with copulation duration in the present study, which is in line with several other studies on *C. maculatus* (Brown et al., 2009; Edvardsson & Tregenza, 2005; Savalli & Fox, 1998a). However, other studies have found that long copulations facilitate the transfer of larger ejaculates (Edvardsson & Canal, 2006; van Lieshout, McNamara, & Simmons, 2014). The experimental design differs between these studies, there could thus be several explanations for the inconsistencies. One being that ejaculate size and is correlated with duration up until a certain point, but not for longer copulations.

Results indicate that the estimates of ejaculate size are similar to some previous studies (Edvardsson & Canal, 2006; van Lieshout et al., 2014), but somewhat higher than others (Katvala et al., 2008; Rönn, Katvala, & Arnqvist, 2011). The measuring of male relative weight loss, a proxy for ejaculation size, was done by weighing each male before and after copulation. This might be an unreliable method of estimating ejaculate transfer, since weight loss may also be linked to metabolic costs. However, it is likely that this additional weight loss was minimal, as males were stored in small chambers restricting much movement and no more than 1 hour passed between the two measurements. Fox et al. (1995) weighted both males and females twice both before and after mating. A similar method could have yielded more reliable results in the present study, as there was some discrepancy between female weight gain and male loss in the nine trials were this was measured.

Male relative weight loss during copulation was not affected by female weight nor the female to male weight ratio. This indicates that males do not adjust the size of their spermatophore based on female size during first time copulations, which is consistent with the findings of Fox et al. (2006). In contrast, van Lieshout et al. (2014) found that male *C. maculatus* are able to regulate the size of the first ejaculation under certain conditions. Large males tended to transfer larger ejaculates when copulating with smaller, rather than larger females. Interestingly, this

effect was only observed when males were exposed to a sociosexual stimulus, allowing 24 hours of both tactile and olfactory contact with other beetles prior to their first mating (van Lieshout et al., 2014). Furthermore, they found that males reduce their spermatophore when they are exposed to a male biased sociosexual environment prior to mating. This is consistent with "intensity models" of sperm competition, where males reduce their investment as the number of potential competitors increases (Kelly & Jennions, 2011; Parker & Pizzari, 2010). The findings of van Lieshout and colleagues emphasises the importance of cues provided by the sociosexual environment, indicating that this can have an effect on male mating behaviour. In the present study, all beetles were isolated and deprived of visual, tactile and olfactory stimuli prior to mating trials, although some pheromone exchange is likely to have occurred between chambers. This lack of stimuli could possibly serve as information representative of a low population size. In a population with few potential mates, males would not benefit from being selective in their choice of females. Whether different sociosexual environments can have an impact on male mating preference, should thus be further investigated.

The size of the spermatophore decreases successively with each mating, from >5% to 1% of male weight (Fox et al., 1995; Rönn et al., 2008; Savalli & Fox, 1999a), indicating that adjustment of ejaculate transfer is possible. Ejaculate depletion in *C. maculatus* is well documented (Ofuya, 1995), suggesting that spermatophore production gets more limited for each successive mating. As capital breeders, they rely on the resources they acquire during their larval stage, so resources available for adults is clearly restricted. The present study only focused on the first mating, but males may be more selective in later matings when resources become more limiting.

According to the sperm allocation model of Galvani and Johnstone (1998), males should be less withholding of their sperm as the possibility, and uncertainty, of future reproductive opportunities are reduced. This suggests that male *C. maculatus* should be more selective of females in their first matings than in the later stages of their lifespan. Furthermore, they argue that even if sperm allocation is affected by female quality, it is not necessarily optimal to allocate large amounts to high-quality females. This is because high-quality females may be associated with higher sperm competition and increased risk of sperm rejection, and this may offset the benefits of mating with these females (Galvani & Johnstone, 1998). These additional factors increase the complexity of optimal sperm allocation and may be part of the explanation of why the predictions of the present study were not met. In contrast, the model of Reinhold et al. (2002) propose that males should invest more in initial matings, as each male's copulation

17

may be the last one. They also show that males should invest more sperm in high-quality females, and claim that Galvani and Johnstone's (1998) model relies on unrealistic assumptions. Thus, there might not be a simple answer to this issue. Indeed, several studies in several species have revealed that sperm allocation strategies are complex. The optimal strategy is highly depending on the number of competitors, female mating status and phenotypic quality, as well as the amount of information available to males (Kelly & Jennions, 2011; Parker & Pizzari, 2010).

Even if virgin male *C. maculatus* do not selectively adjust the size of their spermatophore, they still might regulate the composition. Marshall and McNeil (1989) found that the levels of hydrocarbons, a potentially important nutrient, found in the spermatophore of a species of moth, remains stable during multiple matings even though spermatophore size is reduced. They further caution against a priori use of spermatophore size as an estimate of male nutrient investment, as size may not reflect the amount of limiting resources. Whether male *C. maculatus* regulate spermatophore composition in response to female traits remains an interesting question for future studies.

Mating behaviour is not solely determined by males but is to a large degree influenced by females. In the present study, most copulations ended shortly after females started kicking, indicating that this behaviour is linked to the termination of copulation. Thus, neither the time before kicking nor the duration of kicking was linked to female or male weight. In contrast, van Lieshout et al. (2014) found that females initiated kicking earlier when copulating with larger males, which transferred larger ejaculates. Larger ejaculates, despite containing more nutrition and increasing female fecundity, may reduce female longevity beyond the point that can be explained by a trade-off with increased fecundity (Eady, Hamilton, & Lyons, 2007). The spermatophore may contain seminal fluid compounds that increase male fitness at the expense of female fitness, which points to a potential sexual conflict in relation to spermatophore size and copulation duration. Followingly, counteracts by females could prevent males from being selective in these mating aspects.

Multiple matings in *C. maculatus* reduces female longevity (den Hollander & Gwynne, 2009) and they regularly experience genital wounding caused by males spines (Crudgington & Siva-Jothy, 2000; Hotzy & Arnqvist, 2009; Rönn & Hotzy, 2012). Furthermore, females unable to kick has been shown to experience increased damage to their genital tract (Crudgington & Siva-Jothy, 2000).

The degree of female control over copulation duration remains somewhat unclear. Some studies demonstrate that female kicking is largely ineffectual, as experimental manipulation have shown that duration of kicking and copulation not necessarily correspond (van Lieshout et al., 2014; Wilson & Tomkins, 2014). While Edvardsson and Canal (2006) found no support for sexual conflict over copulation duration, other studies show that copulations last longer if females are prevented from kicking (Crudgington & Siva-Jothy, 2000; Edvardsson & Tregenza, 2005). These findings are not necessarily contradictory, as males can potentially control copulation duration, irrespective of kicking being required to terminate matings (Wilson & Tomkins, 2014). One possible explanation could be that female kicking assists in the dislodging process, but that this is ineffectual until males decide to end copulation. Taking another approach, Savalli and Fox (1998a) found that copulation duration in *C. maculatus* was only heritable through daughters, but others have also included sons (Brown et al., 2009), further supporting the notion that both sexes may have a role.

Ejaculate and body size may vary between different populations of *C. maculatus* (Fox et al., 2006). Fox and Moya-Laraño (2009) found that females in a population from Burkina Faso preferred to mate with large males when well-fed but not when unfed, while a population from South India did not show this preference. This suggest that mate choice may be both condition- and population-dependent. The population used in the present study has been maintained in a laboratory conditions for several generations and its origin is unknown, and a possible adaption to these conditions could affect the results.

In conclusion, the results of the present study indicate that virgin males of *C. maculatus* do not discriminate in their choice of mates, copulation duration or ejaculate transfer based on female weight. It is still possible that males are more selective during subsequent matings when resources are more limiting, and they have been exposed to a greater number of females. Male choice may also be based on other traits than female size. Female age and her mating status are documented to affect male choice in other insects (Arnaud & Haubruge, 1999; L. W. Simmons et al., 1993; L. W. L. W. Simmons, Llorens, Schinzig, Hosken, & Craig, 1994) .

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Appendix A

Table 2: Test of linear model effects of body weight (mg) on time before kicking (minutes) and kicking duration (minutes). "Relative weight difference" measured as $\frac{female\ weight}{male\ weight}$. N = 34.

RESPONSE VARIABLE	PREDICTOR VARIABLE	EFFECT	SE	$T_{1,\ 32}$	P
Time before kicking	Female weight	0.33	4.1	0.41	0.68
	Male weight	-1.65	1.33	-1.24	0.22
	Relative weight difference	3.2	2.82	1.13	0.27
Kicking duration	Female weight	0.37	0.35	1.08	0.29
	Male weight	-0.3	0.6	-0.5	0.62
	Relative weight difference	1.5	1.24	1.21	0.23

Appendix B

R code:

#Binomial test

```
binom.test(16,34,(1/2),alternative="two.sided")
```

 #Paired t-test

```
with(Data.til.R_test2, t.test(unchosen.f.bweight,chosen.f.bweight, paired = TRUE,
alternative = "two.sided"))
```

#Binary logistic regression – Effect of male weight on preference

```
fit1.glm <- glm(m.choice2~m.bweight, family=binomial,data=Data.til.R_test2)
anova(fit1.glm, test='Chi')
summary(fit1.glm)
```

#Survival analysis – Time before copulation

```
#Choosing error distribution:
fit1.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="logistic",
data=Data.til.R_test2)

fit2.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="extreme",
data=Data.til.R_test2)

fit3.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="gaussian",
data=Data.til.R_test2)

fit4.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="weibull",
data=Data.til.R_test2)

fit5.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="exponential",
data=Data.til.R_test2)

fit6.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="loglogistic",
data=Data.til.R_test2)

fit7.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="lognormal",
data=Data.til.R_test2)

anova(fit1.surv, fit2.surv, fit3.surv, fit4.surv, fit5.surv, fit6.surv, fit7.surv)


#fit7.serv gir lavest unexplained variability-->"lognormal"

fit0.surv <- survreg(Surv(time.b.mating, mating.status)~+1, dist="lognormal",
data=Data.til.R_test2)
```

```
fit1.surv <- survreg(Surv(time.b.mating, mating.status)~m.choice, dist="lognormal",
data=Data.til.R_test2)

anova(fit0.surv, fit1.surv, test="Chi")

summary(fit1.surv)
```

#Survival analysis – Duration of copulation

```
#Choosing error distribution:

fit1.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="logistic",
data=Survival.Duration)

fit2.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="extreme",
data=Survival.Duration)

fit3.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="gaussian",
data=Survival.Duration)

#fit4.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="weibull",
data=Survival.Duration)

#fit5.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="exponential",
data=Survival.Duration)

#fit6.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="loglogistic",
data=Survival.Duration)

#fit7.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="lognormal",
data=Survival.Duration)

anova(fit1.surv, fit2.surv, fit3.surv)


#fit2.serv gir lavest unexplained variability--> "Extreme"

fit02.surv <- survreg(Surv(duration, mating.status)~+1, dist="extreme",
data=Survival.Duration)

fit12.surv <- survreg(Surv(duration, mating.status)~m.choice, dist="extreme",
data=Survival.Duration)

anova(fit02.surv, fit12.surv, test="Chi")

summary(fit12.surv)
```

#Linear models – Duration of copulation

```
fit1.lm<- lm(duration~m.bweight, data=Survival.Duration)

anova(fit1.lm)

summary(fit1.lm)

par(mfrow=c(2,2))
```

28

```
plot(fit1.lm)


fit2.lm<- lm(duration~chosen.f.bweight, data=Survival.Duration)

anova(fit2.lm)

summary(fit2.lm)

par(mfrow=c(2,2))

plot(fit2.lm)


fit3.lm<- lm(duration~weight.difference.chosen.f.m, data=Survival.Duration)

anova(fit3.lm)

summary(fit3.lm)

par(mfrow=c(2,2))

plot(fit3.lm)


#Linear models – Male relative weight loss

fit1.lm<- lm(m.weight.loss.rel~m.bweight, data=Data.til.R_test2)

anova(fit1.lm)

summary(fit1.lm)

par(mfrow=c(2,2))

plot(fit1.lm)


fit2.lm<- lm(m.weight.loss.rel~chosen.f.bweight, data=Data.til.R_test2)

anova(fit2.lm)

summary(fit2.lm)

par(mfrow=c(2,2))

plot(fit2.lm)


fit3.lm<- lm(m.weight.loss.rel~weight.difference.chosen.f.m, data=Data.til.R_test2)

anova(fit3.lm)

summary(fit3.lm)

par(mfrow=c(2,2))

plot(fit3.lm)
```

```
fit4.lm<- lm(m.weight.loss.rel~duration, data=Data.til.R_test2)

anova(fit4.lm)

summary(fit4.lm)

par(mfrow=c(2,2))

plot(fit4.lm)


fit5.lm<- lm(m.weight.loss.rel~m.bweight*duration, data=Data.til.R_test2)

anova(fit5.lm)

summary(fit5.lm)

par(mfrow=c(2,2))

plot(fit5.lm)


#Linear models – Female kicking

    #Female weight – Time before kicking

    fit3.lm<- lm(time.b.kicking~chosen.f.bweight, data=Data.til.R_test2)

    anova(fit3.lm)

    summary(fit3.lm)

    par(mfrow=c(2,2))

    plot(fit3.lm)


    #Male weight – Time before kicking

    fit5.lm<- lm(time.b.kicking~m.bweight, data=Data.til.R_test2)

    anova(fit5.lm)

    summary(fit5.lm)

    par(mfrow=c(2,2))

    plot(fit5.lm)


    #Relative weight difference – Time before kicking

    fit9.lm<- lm(time.b.kicking~weight.difference.chosen.f.m, data=Data.til.R_test2)

    anova(fit9.lm)

    summary(fit9.lm)
```

```
par(mfrow=c(2,2))
plot(fit9.lm)


#Female weight - Duration of kicking
fit3.lm<- lm(duration.of.kicking~chosen.f.bweight, data=Data.til.R_test2)
anova(fit3.lm)
summary(fit3.lm)
par(mfrow=c(2,2))
plot(fit3.lm)


#Male weight - Duration of kicking
fit5.lm<- lm(duration.of.kicking~m.bweight, data=Data.til.R_test2)
anova(fit5.lm)
summary(fit5.lm)
par(mfrow=c(2,2))
plot(fit5.lm)


#Relative weight difference – Duration of kicking
fit9.lm<- lm(duration.of.kicking~weight.difference.chosen.f.m, data=Data.til.R_test2)
anova(fit9.lm)
summary(fit9.lm)
par(mfrow=c(2,2))
plot(fit9.lm)
```