LRH: LUC F. BUSSIERE ET AL.

RRH: POST-COPULATORY SEXUAL CONFLICT IN CRICKETS

SEXUAL CONFLICT AND CRYPTIC FEMALE CHOICE IN THE BLACK FIELD CRICKET, *Teleogryllus commodus*

LUC F. BUSSIÈRE[1,2], JOHN HUNT[1,3], MICHAEL D. JENNIONS[4] AND ROBERT BROOKS[1,5]

1. *School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, NSW 2052, Australia.*
2. *Zoologisches Museum der Universität Zürich, Winterthurerstrasse 190, CH 8057 Zürich, Switzerland. Email: luc.bussiere@access.unizh.ch*
3. *Centre for Ecology and Conservation, The University of Exeter in Cornwall, Tremough Campus, Penryn, TR10 9EZ, United Kingdom. Email: J.Hunt@exeter.ac.uk*
4. *School of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia. Email: michael.jennions@anu.edu.au*
5. *Email: rob.brooks@unsw.edu.au*

Abstract.— The prevalence and evolutionary consequences of cryptic female choice (CFC) remain highly controversial, not least because the processes underlying its expression are often concealed within the female reproductive tract. However, even when female discrimination is relatively easy to observe, as in numerous insect species with externally attached spermatophores, it is often difficult to demonstrate directional CFC for certain male phenotypes over others. Using a biological assay to separate male crickets into attractive or unattractive categories, we demonstrate that females strongly discriminate against unattractive males by removing their spermatophores before insemination can be completed. This results in significantly more sperm being transferred by attractive males than unattractive males. Males respond to CFC by mate-guarding females after copulation, which increases the spermatophore retention of both attractive and unattractive males. Interestingly, unattractive males who suffered earlier interruption of sperm transfer benefited more from mate guarding and guarded females more vigilantly than attractive males. Our results suggest that post-copulatory mate guarding has evolved via sexual conflict over insemination times rather than through genetic benefits of biasing paternity toward vigorous males, as has been previously suggested.

Key words.— Gryllidae, indirect benefits, post-copulatory choice, sexual selection, sperm choice, sperm competition

Sexual selection sometimes continues beyond the choice of mating partners through sperm competition (Parker 1970; Simmons 2001b) and cryptic female choice (hereafter CFC) (Thornhill 1983; Eberhard 1996). The mechanisms and evolutionary consequences of sperm competition are well established (Birkhead and Moller 1998; Simmons 2001b), but the prevalence of CFC remains controversial (Birkhead 1998; Telford and Jennions 1998; Birkhead 2000; Eberhard 2000; Kempenaers et al. 2000; Pitnick and Brown 2000; Birkhead and Pizzari 2002) in spite of its numerous and important evolutionary implications (Eberhard 1996). For example, CFC is central to determining whether post-copulatory processes reinforce or oppose pre-copulatory mate choice (Danielsson 2001) and whether indirect benefits of post-copulatory paternity biasing can offset or complement the direct costs and benefits of mating (Bussière 2002; Cameron et al. 2003; Chapman et al. 2003a; Eberhard and Cordero 2003).

The controversy surrounding CFC stems in part from disagreement over an appropriate definition (Birkhead 2000; Eberhard 2000). Here we adopt a broad definition of CFC that includes both copulatory (e.g., female control of sperm transfer) and post-copulatory (e.g., sperm selection) female processes that may bias paternity towards certain males (Thornhill 1983; Eberhard 1996; Andres and Rivera 2000; Tallamy et al. 2002). More importantly, CFC is controversial because of the numerous challenges that must be overcome to formally demonstrate its existence (Birkhead 1998; Telford and Jennions 1998; Eberhard 2000; Kempenaers et al. 2000; Pitnick and Brown 2000). Many of the underlying processes involved in CFC are concealed within the female's reproductive tract making them difficult to observe and directly manipulate (Eberhard 1996; Edvardsson and Arnqvist 2000). Consequently, empiricists are often left to explain the relative contribution of male and female effects to the total variance

observed in males' share of paternity, making the separation of cause and effect difficult (Birkhead 1998; Telford and Jennions 1998; Eberhard 2000; Pitnick and Brown 2000). Even cleverly designed studies of CFC have noted the difficulties of unambiguously partitioning the effects between the sexes (Edvardsson and Arnqvist 2000; Pizzari and Birkhead 2000; Ward 2000; Bloch Qazi 2003; Engqvist and Sauer 2003; Evans et al. 2003; Pilastro et al. 2004).

Empirical studies face several additional challenges. First, CFC requires at least two male participants who may themselves interact without any overt intervention by the female. Researchers must therefore separate the effects of sperm competition *per se* from those due to female choice (Birkhead 2000; Pitnick and Brown 2000). Second, differences between the males and the context within which females choose may affect the degree or direction of CFC (Ward 2000). For example, if males are encountered and sampled sequentially (Gibson and Langen 1996) females may "trade-up" in sperm use to maximize the genetic quality of their offspring (Halliday 1983; Jennions and Petrie 2000). The response of a female to a particular male may thus depend on the phenotypes of her previous mates. Finally, since CFC may both arise from and generate sexual conflict (Eberhard 1996, 2000), disfavoured males should be strongly selected to oppose female preferences (Chapman et al. 2003b; Eberhard and Cordero 2003; Arnqvist and Rowe 2005). This counter-selection on males may obscure otherwise striking patterns in the cryptic activities of females (Rowe et al. 2003). Given these inherent difficulties it is not surprising that most of the empirical support for CFC is still indirect (Eberhard 1996) (but see Edvardsson and Arnqvist 2000; Tallamy et al. 2002; Pilastro et al. 2004).

Field crickets (Orthoptera: Gryllidae) are ideal subjects for investigations of CFC because females can actively remove the externally attached spermatophore at any stage

following copulation, thus interrupting insemination (Sakaluk 1984; Simmons 1986; Bateman et al. 2001). Spermatophore removal is an effective mechanism for CFC because male paternity typically increases with spermatophore attachment time (Sakaluk 1984; Simmons 1986; Simmons 1987b; Sakaluk and Eggert 1996; Garcia-Gonzalez and Simmons 2005). Establishing CFC, however, requires a demonstration that females discriminate among males by removing the spermatophores of some males sooner than those of others. This has been shown in several courtship feeding insects, where spermatophore retention increases with the size of a courtship food gift (Vahed 1998; Gwynne 2001). However, the extent to which such biased insemination arises via indirect selection for genetic benefits rather than direct selection for food acquisition is not clear (Gwynne 2001; Bussière 2002). In field crickets, where spermatophores are not associated with large nutritional donations (but see Wagner et al. 2001), the evidence for CFC is more equivocal, especially when one considers the probable publication bias against negative results. In most cases the phenotypes favored by females are unknown (Fleischman and Sakaluk 2004) or attractiveness is imperfectly associated with individual features (e.g., size) of the male phenotype (Simmons 1987a). Even within a single species, some studies may successfully demonstrate CFC (Simmons 1986; Bateman et al. 2001) while others fail to do so (Wynn and Vahed 2004). Furthermore, the evidence for indirect benefits that favor the evolution of CFC in crickets is limited and inconsistent (Simmons 1987a, 2001a, 2003; Fleischman and Sakaluk 2004; Head et al. 2005). These inconsistent findings may reflect the diversity of Gryllid mating systems or the inherent difficulties associated with formally demonstrating CFC in non-courtship-feeding crickets.

One such difficulty may arise if male crickets actively and aggressively oppose female spermatophore removal, the timing of which is used to measure CFC. Following spermatophore transfer, males in several field cricket species engage in post-copulatory mate guarding (Alcock 1994). The guarding male directs aggressive behavior towards intruding males to prevent the female from remating (Simmons 1986, 1990; Sakaluk 1991; Simmons 1991; Wynn and Vahed 2004) or to allow the guarding male to secure additional matings (Bateman and MacFadyen 1999). Aggression towards females has sometimes been interpreted as a by-product of this process (Simmons 1986). Alternatively, aggression could be selected for if it prolongs spermatophore attachment time (Loher and Rence 1978; Evans 1988; Hockham and Vahed 1997; Bateman and MacFadyen 1999; Bateman et al. 2001). Several authors have proposed that male behavior during post-copulatory guarding might allow females to assess a male's health and vigor, such that vigilant or vigorous guards, being the most desirable mates, signal their genetic superiority by their ability to harass females (Thornhill and Alcock 1983; Simmons 1986, 1990, 1991; Hockham and Vahed 1997). An alternative explanation, however, is that the intensity of mate guarding is the result of sexual conflict (Parker 1979; Chapman et al. 2003b; Arnqvist and Rowe 2005), so that males who stand to lose the most by CFC are the most vigorous guards. This hypothesis predicts that the most vigilant males are genetically inferior, and that guarding acts in opposition to active female choice.

The native Australian black field cricket, *Teleogryllus commodus* (Walker), is widely distributed across southern Australia (Otte and Alexander 1983) and its basic mating behavior has been studied extensively (Loher and Rence 1978; Evans 1983, 1988). Males commence mate guarding immediately after copulation and display

aggression towards the female if she attempts to remove the spermatophore (Loher and Rence 1978; Evans 1988). Consequently, guarded females retain spermatophores for significantly longer than unguarded females. We do not know, however, whether females remove spermatophores from certain males sooner than others (i.e., whether spermatophore removal constitutes directional CFC *sensu* Birkhead and Pizzari 2002) or whether males favored by CFC mate guard to a greater or lesser extent than rivals.

We conducted a series of pre-copulatory behavioral trials to designate males as either attractive or unattractive. In our first experiment, we varied the attractiveness of both the first and second male to mate with a given female in a two-way factorial design. We determined whether the timing of spermatophore removal represents CFC that biases sperm transfer towards attractive males. We predicted that, if present, this pattern would be stronger when the female's first mate was an attractive male. In the second experiment, we examined whether the effect of post-copulatory mate guarding on spermatophore attachment time differed for attractive and unattractive males. If male guarding has evolved as a response to sexual conflict then the benefits of guarding should be greater for unattractive males, who have relatively more to lose from CFC. Finally, in our third experiment we manipulated spermatophore attachment time to determine the effect of spermatophore removal on the number of sperm transferred to a female.

MATERIALS AND METHODS

Experimental animals

We collected approximately 200 gravid female Australian black field crickets from cattle pastures at Smith's Lake (32°22'S, 152°30'E), NSW, Australia in March 2002 to establish a laboratory breeding stock. We isolated field collected females in

individual plastic containers (5 x 5 x 5 cm), and provided them with commercially-produced cat food (Friskies Go-Cat® Senior), water and a petri-dish containing moist cotton wool for egg laying. We maintained cultures by rearing the offspring of 100 randomly paired adults per generation in six large stock culture containers (80 litres) in a constant temperature room set to 28 ± 1 °C and a 10D: 14L light regime. Before the animals reached the final instar, we separated nymphs into single sex cultures. We kept adults in single sexed populations for 10 days after eclosion to ensure that experimental animals were sexually mature virgins.

Assessing male attractiveness

Mating in *T. commodus* follows a highly stereotypical sequence of events (Loher and Rence 1978). Upon contacting the female with his antennae, the male produces a courtship call during which he moves backwards towards the female while lowering his body to the ground. The female then mounts the male, aligning her body and genital organs to be parallel with his. The male then inserts a sclerotized epiphallus into the female's genital chamber and begins threading the guilding-rod (containing the spermatophore tube) into the aperture of the receptacular duct of the female's reproductive tract. This movement is accompanied by rapid and irregular flicking of the male's caudal cerci. A few seconds later the epiphallus unhooks and the guilding rod is withdrawn from the female leaving only the spermatophore tube behind in the receptacular duct. The male and female genital organs then separate. Mating lasts 3 minutes on average and requires the active co-operation of the female to be successful (Loher and Rence 1978).

As in several other field cricket species (Simmons 1987b; Bateman 1998), latency to mating in male *T. commodus* is a useful indicator of attractiveness

(Shackleton et al. 2005). Previously, "no-choice" trials showed that the time taken for a female to successfully mount a male after the onset of male courtship is a reliable predictor of male mating success in *T. commodus*, both in the short term (93% of mountings lead to spermatophore transfer within 2-hours) and longer term (males with the shortest latency to mounting obtained significantly more matings over a 3-day period) (Shackleton et al. 2005). Moreover, latency to mounting a given male was significantly repeatable (0.50 ± 0.02) (Shackleton et al. 2005).

Consequently, to determine male attractiveness we conducted a two-round tournament that selected males based on the time that elapsed until a female mounted them. We conducted the tournaments under red light to minimize observer disturbance. In the first round, we placed each of 120 sexually naïve males in an individual plastic container (7 x 7 x 5 cm) with a randomly assigned virgin female from our stock culture. When a female successfully mounted a male, but before the transfer of a spermatophore, we separated the pair. We scored a mounting as successful if (*i*) the female remained motionless on top of the male for at least 5 seconds and (*ii*) the male commenced spermatophore transfer, characterized by the rapid flicking of his cerci. Once half of the females had mounted their partner, we also separated the remaining pairs. Round two commenced with a new female being randomly assigned to each male. Of the more attractive males in the prior round, the first thirty males to be remounted became our "attractive" males (the most attractive quartile of the original population), and the thirty remaining males were discarded. Of the males that were not mounted in the first round, the first thirty males to mount in the second round were discarded, and the remaining males designated unattractive. Thus, each tournament yielded 30 attractive and 30 unattractive males. Unlike many previous studies that use single morphological (Miller

and Pitnick 2002; Pilastro et al. 2004) or behavioural (Edvardsson and Arnqvist 2000; Pizzari and Birkhead 2000; Tallamy et al. 2002) traits to assign male attractiveness, our biological assay incorporates all factors contributing to short-range male attractiveness (Boake 1985; Fedorka and Mousseau 2002; Kokko et al. 2003; Head et al. 2005).

Experiment 1: Directional CFC

By definition, CFC is a female's preference that biases paternity towards a subset of the males with whom she has mated, the outcome of which may vary with the relative attractiveness of these males and any interactions between them (Ward 2000). We therefore assigned sexually naïve females two mates and varied the attractiveness of both the first and second mate. We then removed the second male from the female's proximity after copulation to measure how females manipulated spermatophore attachment time in the absence of male interference. To obtain males for the first mating, we ran a tournament to produce 30 attractive (A) and 30 unattractive males (U). On the same night we then mated each male to a randomly assigned experimental virgin female within small mating chambers (7 x 7 x 5 cm). We recorded the interval between the onset of male courtship and mounting to confirm the validity of our attractiveness assay. After copulating, females were physically prevented from removing spermatophores prematurely by confining them to narrow tubes (5cm length, 1cm diameter) for one hour after mating. Each mating pair was allowed to copulate a second time the following night to ensure females had a large store of sperm and were therefore less likely to retain spermatophores due to sperm limitation (Wynn and Vahed 2004). To obtain the second mates, we conducted an additional bioassay tournament (using a new set of sexually naïve males and females) on the third night. On the same night we then mated half of the experimental females to a male from the same attractiveness treatment as the first male,

and the other half to a male from the opposite attractiveness treatment. This produced four groups of experimental female that mated to either: (a) two attractive males (AA), (b) two unattractive males (UU), (c) an attractive then an unattractive male (AU) or (d) an unattractive then an attractive male (UA) (n = 15 in each).

Once again we recorded the interval between the onset of male courtship and mounting. Immediately after mating with the second male, we removed him and measured the time each female took to remove the spermatophore. We measured the pronotum width (as an index of body size) of all experimental animals using an eyepiece graticule in a binocular microscope (Leica MS5) and their body weight (to the nearest 0.5 mg) using an electronic balance (Mettler Toledo AG135).

Experiment 2: Male mate guarding and CFC

To determine how a male's attractiveness affected his ability to influence CFC, we simultaneously manipulated the attractiveness of the second male and his ability to guard the female after mating in a two-way factorial design. Female spermatophore removal behavior is not influenced by the attractiveness of her first mate (see Results below), so we mated each of 60 randomly selected virgin females to a randomly selected stock male twice over consecutive nights, as outlined above. We then ran a tournament to generate 30 attractive (A) and 30 unattractive (U) males that were randomly paired with a single experimental female in an individual rectangular container (12 x 7 x 7 cm). A larger arena was used than in Experiment 1 because our pilot studies showed greater male variance in the ability to guard within these dimensions, and Simmons (Simmons 1991) has suggested that a female's ability to escape is unnaturally low in small containers. Immediately after spermatophore transfer we removed the male from the arena for half the trials, and for the other half we allowed the male to remain and guard

her. This produced four experimental treatments in which a female's second mate was (a) attractive and removed after mating (AR), (b) attractive and permitted to guard after mating (AG), (c) unattractive and removed after mating (UR) and (d) unattractive and permitted to guard after mating (UG) (n = 15 in each). We measured the time taken by each female to remove the spermatophore, the pronotum width and body weight of all experimental animals.

In the guarding treatments (AG and UG), we quantified the intensity of male guarding. During mate guarding, males remain in close proximity to the female and typically contact the female's body with their antennae (Loher and Rence 1978; Evans 1983). We therefore recorded every 10 minutes whether or not the male was in antennal contact with the female. In total, we observed each male for 80 minutes (8 samples) or until the female had removed the spermatophore.

Experiment 3: How male attractiveness and spermatophore attachment time affects sperm transfer

To determine how spermatophore removal affects sperm transfer, we experimentally manipulated the spermatophore attachment time for attractive and unattractive males. We paired each of 30 attractive and 30 unattractive males with a virgin female and allowed them to mate. Immediately after mating, we restrained each female in a narrow plastic tube to prevent her from removing the spermatophore. Females were then randomly assigned to one of six treatments where spermatophore attachment time was 12, 24, 36, 48, 60 and 72 minutes (n =5 per treatment). After the spermatophore had been attached for the required duration we removed it using a pair of fine forceps and immediately froze and stored the female at –20 °C until sperm counts were conducted. We successfully manipulated insemination time for 54 females (in the

remaining trials the spermatophore was accidentally displaced while placing the female in the restraining tube; these animals were discarded from the experiment).

To count the number of sperm transferred to a female as a function of spermatophore attachment time, we dissected females and removed their spermathecae (sperm storage organs). We dispersed the spermathecal contents in 100 µl of distilled water by repeatedly (100 times) drawing 50 µl of the solution into a plastic pipette tip. We then applied 10 µl of this solution to a haemocytometer and counted the number of sperm residing within the central marked grid (a volume of 0.1µl) using a compound microscope (Olympus; 400 x magnification). We successfully removed the spermatheca from 51 females. We conducted two sperm counts per female and scaled the average of these counts to the original volume (100µl) to obtain the total number of sperm transferred to the female. The two sperm counts for each female were highly repeatable (Repeated Measures ANOVA: among males, $F_{50, 101} = 4.94$, $P = 0.0001$; Repeatability = 0.80 ± 0.12).

Statistical analysis

We performed all parametric analyses using SPSS (version 11). For Experiments 1 and 2 we applied a log-transformation to spermatophore attachment times in order to satisfy the assumptions of normality and homogeneity of variances. In the figures we present back-transformed measures of error and central tendency to illustrate differences among treatments, and provide means and standard errors for raw data in the figure legends. Unless otherwise stated, all summary statistics are Mean ± SE and statistical tests are two-tailed.

In Experiment 2, differences in the timing of spermatophore removal meant that the amount of time a male spent guarding his female (and therefore the total number of

measures recorded per male) varied. We therefore express the intensity of male mate guarding as a proportion of the total number of observations made (e.g., for each male, guarding intensity is a fraction in which the numerator is samples in which guarding was observed, and the denominator is the total number of samples before spermatophore removal). This has a binomial rather than normal error distribution, so we analyzed the intensity of male mate guarding behavior using a generalized linear mixed model (GLMM) with a binomial error distribution and *logit link* function (Crawley 2002). We analyzed the data in S-Plus 6.4 using the *Mas*s library of Venables and Ripley (2002) and the *glmmPQL* function. We corrected for over-dispersion in our data by testing the fit of the model using the *F*- statistic (Crawley 2002).

RESULTS

Experiment 1: Directional CFC

In both the first and second mating, attractive males required significantly shorter courtship to obtain a mating than did unattractive males (see Table 1), thereby validating our biological assay of male attractiveness. Females removed the spermatophore of unattractive males significantly sooner than those of attractive males (see Fig. 1; ANOVA: $F_{1,56} = 109.80$, $P < 0.001$). However, the time taken for a female to remove the second male's spermatophore was not affected by the first male's attractiveness ($F_{1,56} = 0.042$, $P = 0.83$), nor by the interaction between the first and second male's attractiveness ($F_{1,56} = 0.696$, $P = 0.41$; see Fig. 1).

Table 1 here

Fig. 1 here

Morphology was a poor predictor of a male's attractiveness, as the differences in pronotum width and weight between attractive and unattractive males were not significant in either the first or second tournament (see Table 1). More importantly, neither the pronotum width of the second male (Regression: $F_{1,59} = 1.20$, $P = 0.28$) nor

his weight ($F_{1,59} = 0.02$, $P = 0.90$) were significant predictors of spermatophore removal time.

Experiment 2: Male mate guarding and CFC

Fig. 2 here

Irrespective of male guarding, females took significantly longer to remove the spermatophores of attractive males (see Fig. 2; ANOVA: $F_{1,56} = 39.76$, $P < 0.0001$). However, females that were guarded by a male took significantly longer to remove the spermatophore than females isolated from males after mating (see Fig. 2; $F_{1,56} = 208.232$, $P < 0.0001$). Interestingly, there was a significant interaction between male attractiveness and mate guarding ($F_{1,56} = 14.88$, $P = 0.0003$). The relative effect of mate guarding on spermatophore attachment time was greater for unattractive males (the difference between guarding and removed-male treatments was greater for unattractive males; see Fig. 2) who guarded more intensely than attractive males (GLM with binomial distribution: $F_{1,28} = 11.01$, $P = 0.0025$; % of spot samples guarding; A = 83.0 ± 3.8%; U = 96.7 ± 1.9%).

Again, neither the body size nor the weight of the second male to mate significantly covaried with male attractiveness (body size: $t_{58} = 0.51$, $P = 0.61$; A = 6.18 ± 0.07 mm, U = 6.13 ± 0.07 mm; weight: $t_{58} = 0.84$, $P = 0.84$; A = 0.59 ± 0.02 g, U = 0.57 ± 0.02g). Moreover, neither the second male's body size (Regression: $F_{1,59} = 0.0003$, $P = 0.99$) nor his weight ($F_{1,59} = 0.25$, $P = 0.62$) were significant predictors of spermatophore attachment time. Finally, a male's guarding intensity was not related to his body size (GLM with binomial distribution: $F_{1,28} = 0.04$, $P = 0.84$) or weight ($F_{1,28} = 0.09$, $P = 0.77$).

Experiment 3: How male attractiveness and spermatophore attachment time affects sperm transfer

Fig. 3 here

Attractive and unattractive males did not differ in their rates of sperm transfer (see Fig. 3; ANOVA: $F_{1,39} = 0.002$, $P = 0.96$). Although the number of sperm transferred to a female at mating increased with spermatophore attachment time ($F_{5, 39} = 10.69$, $P = 0.0001$) this relationship showed diminishing returns (see Fig. 3). The number of sperm transferred increased from 12 to 36 minutes (720 to 2160 s; $P < 0.05$), but was statistically indistinguishable between 36, 48, 60, and 72 minutes (> 2160 s; all Tukey's post-hoc pairwise comparisons). The interaction between male attractiveness and spermatophore attachment time on the number of sperm transferred to the female was not significant ($F_{5, 39} = 0.16$, $P = 0.98$).

We fitted a second order polynomial regression to the combined sperm transfer curve (see Fig. 3) to predict how insemination success changed across our previous experimental treatments. The polynomial regression explained more of the variance (r = 0.736) than linear (r = 0.714) or logarithmic (r = 0.732) regressions. We then used the spermatophore attachment times from Experiment 2 to estimate how much (*i*) CFC in the absence of males reduced the insemination success of unattractive males and (*ii*) male mate guarding increased insemination success for attractive and unattractive males. When the second male to mate was unattractive rather than attractive and prevented from mate-guarding, spermatophore attachment time decreased by an average 1125 s, which resulted in 74% difference in the number of sperm transferred to the females (attractive males: 25518 sperm; unattractive males: 14627 sperm). On average, mate guarding increased the spermatophore attachment time of unattractive males, from 1352 to 4172 s, corresponding to a 121% increase in the number of sperm transferred to the

female. In comparison, mate-guarding by attractive males increased spermatophore attachment time from 2477 to 4679 s, which only corresponds to a 26% increase in the number of sperm transferred to the female. Thus, although mate guarding increased the number of sperm transferred for all males, the benefit was greater for unattractive males.

DISCUSSION

In species with polyandry, a male's mating success is not always equivalent to his reproductive success (Eberhard 1996). Here, we demonstrate that CFC, mediated by the premature removal of the externally attached spermatophore, affects male insemination success in the Australian black field cricket. We further show that when the last male to mate with a female is attractive, she retains his spermatophore for considerably longer, resulting in the transfer of significantly more sperm than if he were unattractive. We have not directly shown that this increased number of sperm transferred biases paternity towards the attractive male. However, in several other cricket species sperm competition is a lottery in which numerical representation in the elastic and spherical spermatheca is a large determinant of a male's relative fertilization success (Simmons 1987b; Parker et al. 1990; Sakaluk and Eggert 1996; Simmons 2001b; Garcia-Gonzalez and Simmons 2005). Therefore, it is likely that spermatophore removal by females has direct fitness consequences for males.

Sexual conflict over spermatophore attachment time

Even if there are no direct costs to retaining a spermatophore until it empties, CFC via spermatophore removal arises as a consequence of sexual conflict between the reproductive interests of males and females, and it also escalates this conflict (Eberhard 1996; Partridge and Hurst 1998; Arnqvist and Rowe 2005). A male will benefit most if

every female with whom he mates uses only his sperm to fertilize eggs for her entire lifespan. However, any female that exercises CFC will necessarily fail to do so for at least some of her mates (Eberhard 1996).

We predicted that if male harassment were a signal of male quality, it should generally covary positively with premating preferences and postmating preferences exerted in the absence of males. Instead, we found that males disfavoured by precopulatory choice were the most vigilant guards and benefited more by guarding than attractive males. We argue that this strongly suggests that male harassment is maintained through sexual conflict over insemination (but see Arnqvist and Rowe 2005). We cannot reject the hypothesis that the ability to harass females indicates condition or quality to some extent but, if this is true, it is unclear why females favour different classes of males during premating choice and when isolated from males as compared to when males are present after copulation. This would also beg the question as to why females allow attractive male spermatophores to remain attached for longer than those of unattractive males even when attractive males are less vigilant guards (Fig 2).

Our results demonstrate that males actively restrict the efficiency of CFC. Male aggression towards females during mate-guarding prolongs the spermatophore attachment times of both attractive and unattractive males. However, unattractive males guard more intensely, perhaps because mate guarding has a relatively larger impact on sperm storage for unattractive males. In another species, Simmons (1990) has previously demonstrated that heavily parasitized male crickets guard more intensely. However, he suggested that this was consistent with a role for mate guarding in sexual advertisement, since low-quality males had to invest more for the same level of insemination. In

contrast, we propose that mate guarding evolved via sexual conflict, and that the males most likely to provide indirect benefits are those who guard least.

One intriguing possibility is that spermatophore removal has both indirect and direct fitness consequences for females. Females may benefit indirectly if CFC biases paternity toward males of high genetic quality and benefit directly from removing the spermatophore if there are dose-dependent costs to seminal transfer. The level of sexual conflict over spermatophore attachment time in crickets that arises from ejaculate products that manipulate female physiology is still unknown. In *Drosophila melanogaster*, the sex peptide associated with sperm is known to regulate female oviposition (Liu and Kubli 2003), and is also exploited by males at a net cost to female fitness (Wigby and Chapman 2005). In what might be an analogous system (Wagner and Harper 2003), male cricket (including *T. commodus*) spermatophores contain prostaglandin synthetase, an enzyme that converts arachidomic acid in the female's body into prostaglandin (Loher 1981; Tobe and Loher 1983; Murtaugh and Denlinger 1987). Male *T. commodus* also transfer large amounts of arachidomic acid in their ejaculate, which further elevates female haemolymph prostaglandin levels (Ai et al. 1986; Stanley-Samuelsson et al. 1987), and thus stimulates increased rates of egg-laying (Stanley-Samuelsson and Peloquin 1986). Female *T. commodus* have evolved adaptations that may reduce the rate at which male-derived prostaglandins enter the haemolymph (Sugawara 1987) and excrete excess prostaglandins (Stanley-Samuelsson and Loher 1985). Females may benefit from spermatophore removal by reducing the transfer of these chemicals, and thereby lowering any costs of males chemically manipulating their reproductive effort. If spermatophore retention is costly, then it is especially interesting

that females retain the spermatophores of attractive males for longer than those of unattractive males. Although some models of mate choice evolution suggest that females may make mating decisions that incur direct costs to obtain genetic benefits (Weatherhead and Robertson 1979; Cordero and Eberhard 2003; Kokko et al. 2003) this idea has been criticized by others who argue that indirect benefits are trivial compared to any direct costs of choosiness (Kirkpatrick 1985; Cameron et al. 2003; Arnqvist and Rowe 2005). Evaluating these intriguing hypotheses will require more complete measures of the direct and indirect fitness consequences of spermatophore removal to assess their relative importance in the evolution of post-copulatory interactions in *T. commodus*.

Episodes of sexual selection in field crickets

We predicted that female's CFC decisions would be influenced by the attractiveness of the previous male either directly or through an interaction with the attractiveness of the second male. The ability of females to "trade-up" with regard to mate quality (Halliday 1983; Jennions and Petrie 2000) has been documented in a range of species that exhibit sequential mate choice (Bakker and Milinski 1991; Brooks and Caithness 1995; Gabor and Halliday 1997; Pitcher et al. 2003), including the field cricket, Gryllus bimaculatus (Bateman et al. 2001). In *T. commodus*, we found no evidence that CFC depended on the relative attractiveness of previous mates. This suggests that females base their decision on how long to retain a spermatophore primarily on the current mate's attractiveness. We note, however, that other factors must influence retention time. In Experiment 1, unguarded females retained an attractive male's spermatophore for much longer than unguarded females in Experiment 2.

Variation in the absolute time of spermatophore retention, and the difference in retention between guarded and non-guarded females is also apparent in earlier reports of male influence on spermatophore attachment (mean ± SE; 1950 ± 320 s versus 6339 ± 134 s, (Loher and Rence 1978), 438 ± 74 s versus 4278 ± 266 s, (Evans 1988)). Importantly, however, in both our experiments females retained the spermatophores of attractive males for a significantly longer time.

Sexual selection often occurs in discrete episodes (Arnold and Wade 1984a, 1984b) and there is no *a priori* reason to expect that selection will be in the same direction on males in each consecutive selective episode (Moore and Moore 1999; Bonduriansky and Rowe 2003). Here we show that, in the absence of mate guarding, CFC reinforces pre-copulatory mate choice decisions in *T. commodus*. The selection imposed on males by male mate guarding, however, opposes selection via pre-copulatory mate choice decisions because males that were unattractive in pre-copulatory choice benefited more by guarding females. Disentangling the effects of the three processes of pre-copulatory choice, spermatophore removal and post-copulatory harassment on net sexual selection remains an important research challenge.

ACKNOWLEDGMENTS

We thank P. De Luca, J. Evans, D. Gwynne, T. Ivy, A. Timenes Laugen, L. Simmons and P. Ward for valuable comments on the experiment, and N. Spyrou for technical assistance. We are also grateful to the associate editor and two anonymous reviewers who provided very helpful suggestions for improving the manuscript. This research was supported by a Natural Sciences and Engineering Research Council (Canada) fellowship to L.F.B. and an Australian Research Council grant to J.H., M.D.J.

and R.B. J.H. was funded by a Natural Environment Research Council (United Kingdom) Research Fellowship during the writing of this manuscript.

LITERATURE CITED

Ai, N., S. Komatsu, I. Kubo, and W. Loher. 1986. Manipulation of prostaglandin-mediated oviposition after mating in *Teleogryllus commodus*. Intl. J. Inv. Repr. Dev. 10:33-42.

Alcock, J. 1994. Postinsemination associations between males and females in insects: the mate-guarding hypothesis. Ann. Rev. Entomol. 39:1-21.

Andres, J. A., and A. C. Rivera. 2000. Copulation duration and fertilization success in a damselfly: an example of cryptic female choice? Anim. Behav. 59:695-703.

Arnold, S. J., and M. J. Wade. 1984a. On the measurement of natural and sexual selection: applications. Evolution 38:720-734.

———. 1984b. On the measurement of natural and sexual selection: theory. Evolution 38:709-719.

Arnqvist, G., and L. Rowe. 2005. Sexual Conflict. Princeton University Press, Princeton.

Bakker, T. C. M., and M. Milinski. 1991. Sequential female choice and the previous male effect in sticklebacks. Behav. Ecol. Sociobiol. 29:205-210.

Bateman, P. W. 1998. Assortative mating by both sexes in the cricket *Platygryllus primiformis* (Orthoptera: Gryllidae: Gryllinae). Trans. Amer. Ent. Soc. 124:63-68.

Bateman, P. W., L. N. Gilson, and J. W. H. Ferguson. 2001. Male size and sequential mate preference in the cricket *Gryllus bimaculatus*. Anim. Behav. 61:631-637.

Bateman, P. W., and D. N. MacFadyen. 1999. Mate guarding in the cricket *Gryllodes sigillatus*: Influence of multiple potential partners. Ethology 105:949-957.

Birkhead, T. R. 1998. Cryptic female choice: Criteria for establishing female sperm choice. Evolution 52:1212-1218.

———. 2000. Defining and demonstrating postcopulatory female choice -- again. Evolution 54:1057 - 1060.

Birkhead, T. R., and A. P. Moller. 1998. Sperm Competition and Sexual Selection. Academic Press, San Diego.

Birkhead, T. R., and T. Pizzari. 2002. Postcopulatory sexual selection. Nat. Rev. Genet. 3:262-273.

Bloch Qazi, M. 2003. A potential mechanism for cryptic choice in a flour beetle. J. evol. Biol. 16:170-176.

Boake, C. R. B. 1985. Genetic consequences of mate choice: a quantitative genetic method for testing sexual selection theory. Science 227:1061-1063.

Bonduriansky, R., and L. Rowe. 2003. Interactions among mechanisms of sexual selection on male body size and head shape in a sexually dimorphic fly. Evolution 57:2046-2053.

Brooks, R., and N. Caithness. 1995. Female choice in a feral guppy population: are there multiple cues? Anim. Behav. 50:301 - 307.

Bussière, L. F. 2002. A model of the interaction between "good genes" and direct benefits in courtship feeding animals: when do males of high genetic quality invest less? Phil. Trans. R. Soc. Lond. B 357:309-317.

Cameron, E., T. Day, and L. Rowe. 2003. Sexual conflict and indirect benefits. J. evol. Biol. 16:1055-1060.

Chapman, T., G. Arnqvist, J. Bangham, and L. Rowe. 2003a. Response to Eberhard and Cordero, and Córdoba-Aguilar and Contreras-Garduño: Sexual conflict and female choice. Trends Ecol. Evol. 18:440-441.

———. 2003b. Sexual conflict. Trends Ecol. Evol. 18:41-47.

Cordero, C., and W. G. Eberhard. 2003. Female choice of sexually antagonistic male adaptations: a critical review of some current research. J. evol. Biol. 16:1-6.

Crawley, M. 2002. Statistical Computing: An Introduction to Data Analysis Using S-Plus. John Wiley & Sons, Chichester.

Danielsson, I. 2001. Antagonistic pre- and post-copulatory sexual selection on male body size in a water strider (*Gerris lacustris*). Proc. R. Soc. Lond. B 268:77-81.

Eberhard, W. G. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton.

———. 2000. Criteria for demonstrating postcopulatory female choice. Evolution 54:1047 - 1050.

Eberhard, W. G., and C. Cordero. 2003. Sexual conflict and female choice. Trends Ecol. Evol. 18:438-439.

Edvardsson, M., and G. Arnqvist. 2000. Copulatory courtship and cryptic female choice in red flour beetles, *Tribolium castaneum*. Proc. R. Soc. Lond. B 267:559-563.

Engqvist, L., and K. Sauer. 2003. Determinants of sperm transfer in the scorpionfly *Panorpa cognata*: male variation, female condition and copulation duration. J. evol. Biol. 16:1196-1204.

Evans, A. R. 1983. A study of the behaviour of the Australian field cricket *Teleogryllus commodus* (Walker) (Orthoptera: Gryllidae) in the field and in habitat simulations. Z. Tierpsychol. 62:269-290.

———. 1988. Mating systems and reproductive strategies in three Australian gryllid crickets: *Bobilla victoriae* Otte, *Balamara gidya* Otte and *Teleogryllus commodus* Walker (Orthoptera: Gryllidae: Nemobiinae; Trigonidiinae; Gryllinae). Ethology 78:21-52.

Evans, J. P., L. Zane, S. Francescato, and A. Pilastro. 2003. Directional postcopulatory sexual selection revealed by artificial insemination. Nature 421:360-363.

Fedorka, K. M., and T. A. Mousseau. 2002. Material and genetic benefits of female multiple mating and polyandry. Anim. Behav. 64:361-367.

Fleischman, R. R., and S. K. Sakaluk. 2004. No direct or indirect benefits to cryptic female choice in house crickets (*Acheta domesticus*). Behav. Ecol. 15:793-798.

Gabor, C. R., and T. R. Halliday. 1997. Sequential mate choice by multiply mating smooth newts: females become more choosy. Behav. Ecol. 8:162-166.

Garcia-Gonzalez, F., and L. W. Simmons. 2005. Sperm viability matters in insect sperm competition. Curr. Biol. 15:271-275.

Gibson, R., and T. Langen. 1996. How do animals choose their mates? Trends Ecol. Evol. 11:468-470.

Gwynne, D. T. 2001. Katydids and Bush-crickets: Reproductive Behavior and Evolution of the Tettigoniidae. Cornell University Press, Ithaca, NY.

Halliday, T. 1983. Do frogs and toads choose their mates? Nature 306:226-227.

Head, M. L., J. Hunt, M. D. Jennions, and R. Brooks. 2005. The indirect benefits of mating with attractive males outweigh the direct costs. PLoS Biology 3:289-294.

Hockham, L. R., and K. Vahed. 1997. The function of mate guarding in a field cricket (Orthoptera, Gryllidae, *Teleogryllus natalensis* Otte and Cade). J. Insect Behav. 10:247-256.

Jennions, M. D., and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. Biol. Rev. 75:21-64.

Kempenaers, B., K. Foerster, S. Questiau, B. C. Robertson, and E. L. M. Vermeirssen. 2000. Distinguishing between female sperm choice versus male sperm competition: a comment on Birkhead. Evolution 54:1050 - 1052.

Kirkpatrick, M. 1985. Evolution of female choice and male parental investment in polygynous species: the demise of the "sexy son". Am. Nat. 125:788-810.

Kokko, H., R. Brooks, M. D. Jennions, and J. Morley. 2003. The evolution of mate choice and mating biases. Proc. R. Soc. Lond. B 270:653-664.

Liu, H. F., and E. Kubli. 2003. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 100:9929-9933.

Loher, W. 1981. The effect of mating on female sexual behavior of *Teleogryllus commodus* Walker. Behav. Ecol. Sociobiol. 9:219-225.

Loher, W., and B. Rence. 1978. The mating behavior of *Teleogryllus commodus* (Walker) and its central and peripheral control. Z. Tierpsychol. 46:225-259.

Miller, G. T., and S. Pitnick. 2002. Sperm-female coevolution in *Drosophila*. Science 298:1230-1233.

Moore, A. J., and P. J. Moore. 1999. Balancing sexual selection through opposing mate choice and male competition. Proc. R. Soc. Lond. B 266:711-716.

Murtaugh, M., and D. Denlinger. 1987. Regulation of long-term oviposition in the house cricket, *Achaeta domesticus*: roles of prostaglandin and factors associated with sperm. Arch. Ins. Biochem. Physiol. 6:59-72.

Otte, D., and R. D. Alexander. 1983. The Australian Crickets (Orthoptera: Gryllidae). The Academy of Natural Sciences of Philadelphia, Philadelphia, PA.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45:525-567.

———. 1979. Sexual selection and sexual conflict. In: Sexual Selection and Reproductive Competition in Insects (Eds. M. S. Blum and N. A. Blum), Academic Press, New York.:123-166.

Parker, G. A., L. W. Simmons, and H. Kirk. 1990. Analysing sperm competition data: simple models for predicting mechanisms. Behav. Ecol. Sociobiol. 27:55-65.

Partridge, L., and L. D. Hurst. 1998. Sex and conflict. Science 281:2003-2008.

Pilastro, A., M. Simonato, A. Bisazza, and J. P. Evans. 2004. Cryptic female preference for colorful males in guppies. Evolution 58:665-669.

Pitcher, T. E., B. D. Neff, F. H. Rodd, and L. Rowe. 2003. Multiple mating and sequential mate choice in guppies: females trade up. Proc. R. Soc. Lond. B 270:1623-1629.

Pitnick, S., and W. D. Brown. 2000. Criteria for demonstrating female sperm choice. Evolution 54:1052 - 1056.

Pizzari, T., and T. R. Birkhead. 2000. Female feral fowl eject sperm of subdominant males. Nature 405:787-789.

Rowe, L., E. Cameron, and T. Day. 2003. Detecting sexually antagonistic coevolution with population crosses. Proc. R. Soc. Lond. B 270:2009-2016.

Sakaluk, S. K. 1984. Male crickets feed females to ensure complete sperm transfer. Science 223:609-610.

———. 1991. Postcopulatory mate guarding in decorated crickets. Anim. Behav. 41:207-216.

Sakaluk, S. K., and A. K. Eggert. 1996. Female control of sperm transfer and intraspecific variation in sperm precedence: Antecedents to the evolution of a courtship food gift. Evolution 50:694-703.

Shackleton, M. A., M. D. Jennions, and J. Hunt. 2005. Fighting success and attractiveness as predictors of male mating success in the black field cricket, *Teleogryllus commodus*: the effectiveness of no-choice tests. Behav. Ecol. Sociobiol. 58:1-8.

Simmons, L. W. 1986. Female choice in the field cricket *Gryllus bimaculatus* (De Geer). Anim. Behav. 34:1463-1470.

———. 1987a. Female choice contributes to offspring fitness in the field cricket, *Gryllus bimaculatus* (De Geer). Behav. Ecol. Sociobiol. 21:313-321.

———. 1987b. Sperm competition as a mechanism of female choice in the field cricket, *Gryllus bimaculatus*. Behav. Ecol. Sociobiol. 21:197-202.

———. 1990. Post-copulatory guarding, female choice and the levels of gregarine infections in the field cricket, *Gryllus bimaculatus*. Behav. Ecol. Sociobiol. 26:403-407.

———. 1991. On the post-copulatory guarding behaviour of male field crickets. Anim. Behav. 42:504-505.

———. 2001a. The evolution of polyandry: an examination of the genetic incompatibility and good-sperm hypotheses. J. evol. Biol. 14:585-594.

———. 2001b. Sperm Competition and its Evolutionary Consequences in the Insects. Princeton University Press, Princeton, NJ.

———. 2003. The evolution of polyandry: patterns of genotypic variation in female mating frequency, male fertilization success and a test of the sexy-sperm hypothesis. J. evol. Biol. 16:624-634.

Stanley-Samuelsson, D., R. Jurenka, G. Blomqvist, and W. Loher. 1987. Sexual transfer of prostaglandin precursor in the field cricket, *Teleogryllus commodus*. Physiol. Entomol. 12:347-354.

Stanley-Samuelsson, D., and W. Loher. 1985. The disappearance of injected prostaglandins from the circulation of adult female Australian field crickets, *Teleogryllus commodus*. Arch. Ins. Biochem. Physiol. 2:367-374.

Stanley-Samuelsson, D., and J. Peloquin. 1986. Egg-laying in response to prostaglandin injections in the Australian field cricket, *Teleogryllus commodus*. Physiol. Entomol. 12:347-354.

Sugawara, T. 1987. Cuticular lining in the genital chamber of the cricket - an obstacle to prostaglandin diffusing? Intl. J. Inv. Repr. Dev. 12:213-216.

Tallamy, D. W., B. E. Powell, and J. A. McClafferty. 2002. Male traits under cryptic female choice in the spotted cucumber beetle (Coleoptera: Chrysomelidae). Behav. Ecol. 13:511-518.

Telford, S. R., and M. D. Jennions. 1998. Establishing cryptic female choice in animals. Trends Ecol. Evol. 13:216-218.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122:765-788.

Thornhill, R., and J. Alcock. 1983. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge, MA.

Tobe, S., and W. Loher. 1983. Properties of the prostaglandin synthetase complex in the cricket *Teleogryllus commodus*. Ins. Biochem. 13:137-141.

Vahed, K. 1998. The function of nuptial feeding in insects: review of empirical studies. Biol. Rev. 73:43-78.

Venables, W., and B. Ripley. 2002. Modern Applied Statistics with S. Springer-Verlag, New York.

Wagner, W. E., and C. J. Harper. 2003. Female life span and fertility are increased by the ejaculates of preferred males. Evolution 57:2054-2066.

Wagner, W. E., R. J. Kelley, K. R. Tucker, and C. J. Harper. 2001. Females receive a life-span benefit from male ejaculates in a field cricket. Evolution 55:994-1001.

Ward, P. I. 2000. Cryptic female choice in the yellow dung fly *Scathophaga stercoraria* (L.). Evolution 54:1680-1686.

Weatherhead, P. J., and R. J. Robertson. 1979. Offspring quality and the polygyny threshold: "the sexy son hypothesis". Am. Nat. 113:201-208.

Wigby, S., and T. Chapman. 2005. Sex peptide causes mating costs in female *Drosophila melanogaster*. Curr. Biol. 15:316-321.

Wynn, H., and K. Vahed. 2004. Male *Gryllus bimaculatus* guard females to delay them from mating with rival males and to obtain repeated copulations. J. Insect Behav. 17:53-66.

Corresponding Editor: R. Harrison

TABLE 1. The mean (±SE) latency to mating and morphological characteristics of attractive and unattractive males (determined by biological assay) that were mated to females in either the first or second male role.

Trait	1st Mating		2nd Mating	
	Attractive	*Unattractive*	*Attractive*	*Unattractive*
Latency to mounting (s)	101 ± 30	220 ± 38*	242 ± 45	1082 ± 193*
Pronotum width (mm)	6.21 ± 0.66	6.09 ± 0.08	5.90 ± 0.08	5.67 ± 0.07
Weight (g)	0.65 ± 0.02	0.62 ± 0.02	0.54 ± 0.02	0.50 ± 0.02

* $P < 0.05$; for each mating the attributes of attractive and unattractive males are compared using an unpaired t-test with df = 58.

FIGURE LEGENDS

FIGURE 1. The spermatophore attachment time (back-transformed mean ± SE) of the second male as a function of his own attractiveness and the attractiveness of the female's previous mate. AA: 1st and 2nd male attractive; AU: 1st male attractive, 2nd male unattractive; UA: 1st male unattractive, 2nd male attractive; UU: 1st and 2nd male unattractive. See text for more details. Means ± SE for raw data: AA: 4257 ± 252 s; AU: 1292 ± 193 s; UA: 4095 ± 343 s; UU: 1430 ± 207 s.

FIGURE 2. Spermatophore attachment time (back-transformed mean ± SE) as a function of the second male's attractiveness and the presence or absence of post-copulatory mate guarding. AG: male attractive and permitted to mate guard; UG male unattractive and permitted to mate guard; AR: male attractive and removed after spermatophore transfer; UR: male unattractive and removed after spermatophore transfer. See text for more details. Means ± SE for raw data: AG: 4678 ± 121 s; UG: 4172 ± 280 s; AR: 2477 ± 114 s; UR: 1352 ± 83 s.

FIGURE 3. Sperm transfer of attractive and unattractive males as a function of spermatophore attachment time. Closed symbols = attractive males, open symbols = unattractive males. As sperm transfer did not depend on male attractiveness, we fit a second order polynomial regression to the combined data to estimate the impact that CFC and male mate guarding had on the number of sperm transferred to a female ($F_{2,51} = 28.74$, $P = 0.0001$, $r^2 = 0.57$, $y = -0.002x^2 + 17.339x - 5159.437$).

Figure 1

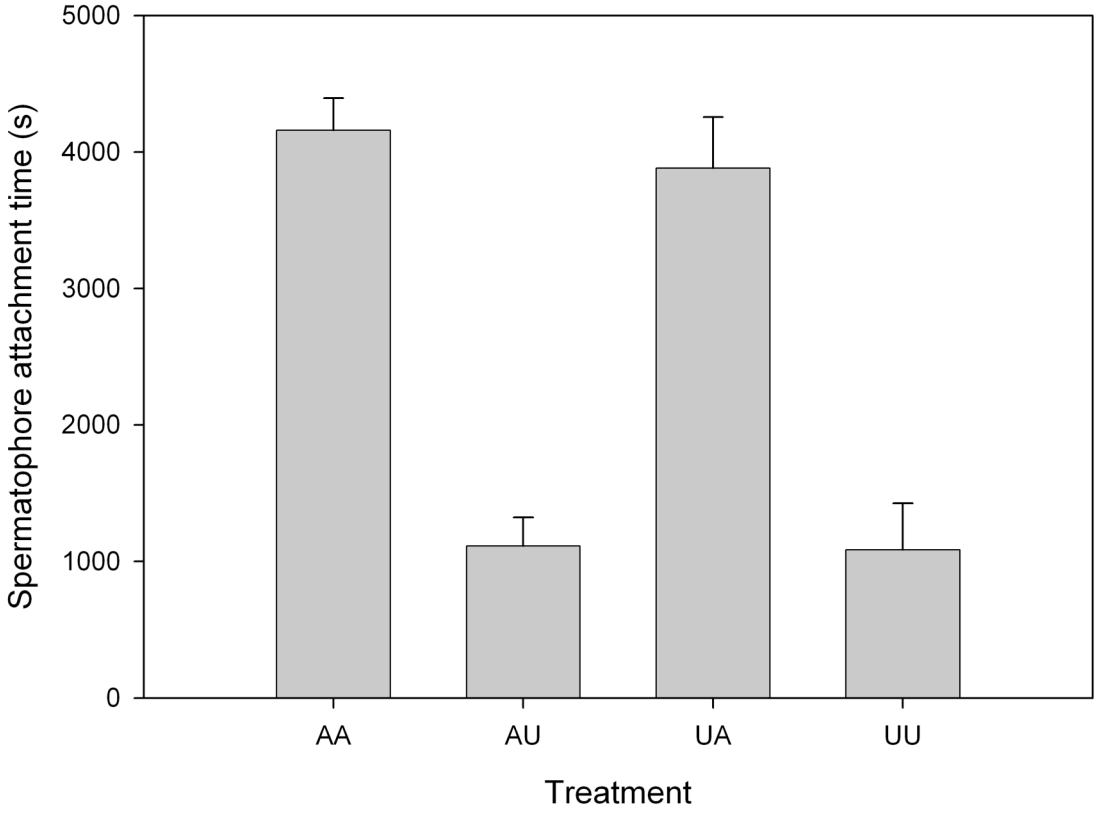

5000
4000
3000
2000
1000
0
Spermatophore attachment time (s)
AA
AU
UA
UU
Treatment

Figure 2

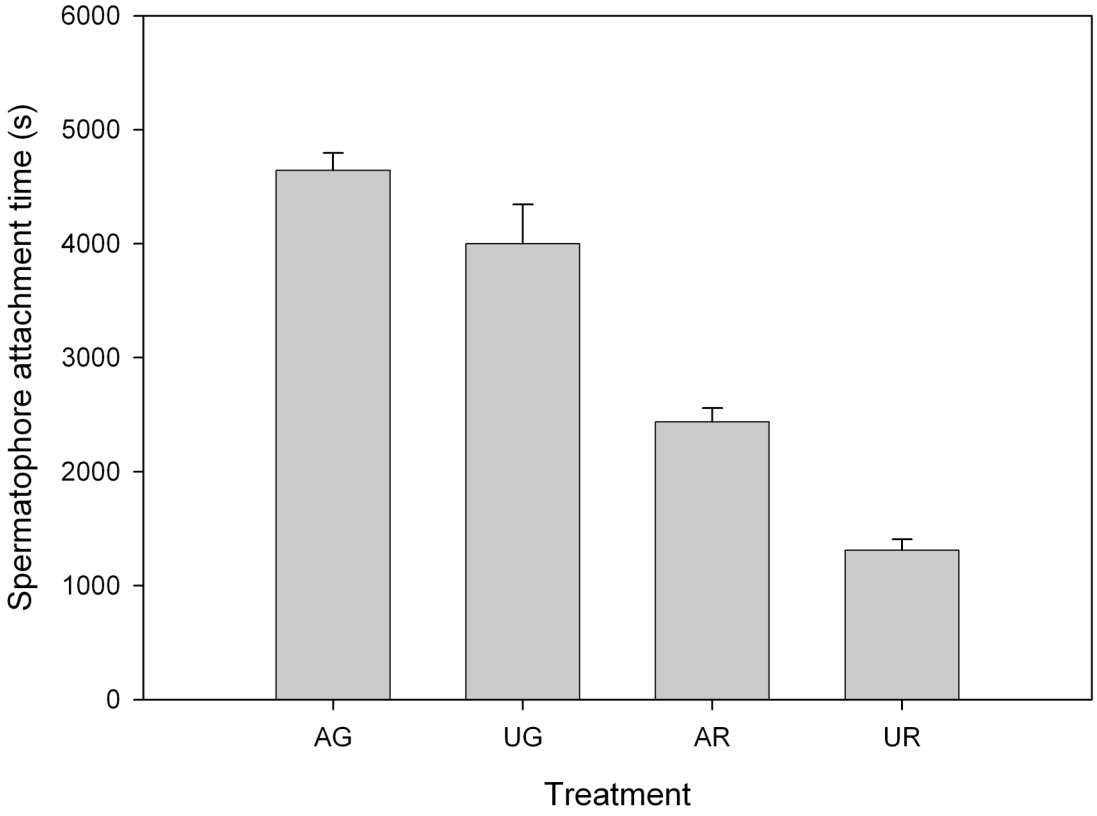
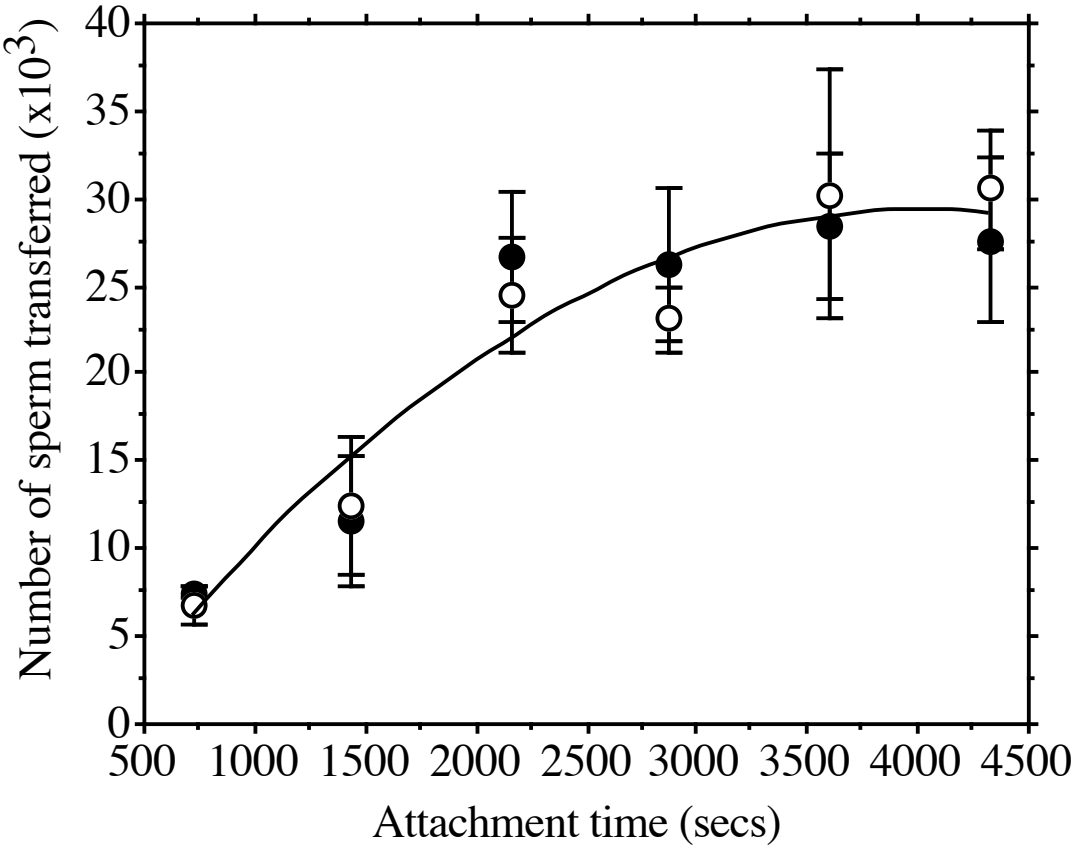

6000
5000
4000
3000
2000
1000
0
Spermatophore attachment time (s)
AG
UG
AR
UR
Treatment

Figure 3.


Number of sperm transferred (x10^3)
40
35
30
25
20
15
10
5
0
500
1000
1500
2000
2500
3000
3500
4000
4500
Attachment time (secs)