MOLECULAR ECOLOGY

Molecular Ecology (2010) **19**, 610–619 doi: 10.1111/j.1365-294X.2009.04496.x

Competitive PCR reveals the complexity of postcopulatory sexual selection in *Teleogryllus commodus*

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Abstract

The outcome of mate choice depends on complex interactions between males and females both before and after copulation. Although the competition between males for access to mates and premating choice by females are relatively well understood, the nature of interactions between cryptic female choice and male sperm competition within the female reproductive tract is less clear. Understanding the complexity of postcopulatory sexual selection requires an understanding of how anatomy, physiology and behaviour mediate sperm transfer and storage within multiply mated females. Here we use a newly developed molecular technique to directly quantify mixed sperm stores in multiple mating females of the black field cricket, *Teleogryllus commodus*. In this species, female postcopulatory choice is easily observed and manipulated as females delay the removal of the spermatophore in favour of preferred males. Using twice-mated females, we find that the proportion of sperm in the spermatheca attributed to the second male to mate with a female (S_2) increases linearly with the time of spermatophore attachment. Moreover, we show that the insemination success of a male increases with its attractiveness and decreases with the size of the female. The effect of male attractiveness in this context suggests a previously unknown episode of mate choice in this species that reinforces the sexual selection imposed by premating choice and conflicts with the outcome of postmating male harassment. Our results provide some of the clearest evidence yet for how sperm transfer and displacement in multiply mated females can lead directly to cryptic female choice, and that three distinct periods of sexual selection operate in black field crickets.

Keywords: cryptic choice, DNA quantification, field cricket, insemination success, mate choice, sperm competition

Received 16 June 2009; revision received 16 November 2009; accepted 21 November 2009

Introduction

Understanding postcopulatory sexual selection remains a difficult empirical challenge as fertilization success depends not only on precopulatory mating decisions, but also on sperm competition (Parker 1970), cryptic female choice (Thornhill 1983; Eberhard 1996) and antagonistic interactions between males and females (Parker 1979; Arnqvist & Rowe 2005). Empirical studies have traditionally inferred the mechanisms underlying postcopulatory sexual selection by combining behavioural manipulations with the analysis of paternity (Boorman & Parker 1976). By mating females twice and then quantifying the proportion of offspring sired by the second male (P_2), fertilization success can be related to a number of male and female characteristics including sperm morphology (García-González & Simmons 2007),

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the quality and quantity of sperm transferred (Wedell & Cook 1998; García-González & Simmons 2005; Jones & Elgar 2008), the morphology of the female genitalia and sperm storage structures (Walker 1980; Fedina & Lewis 2004) and the quality or attractiveness of the male (Pilastro *et al.* 2002; Hosken *et al.* 2003). By quantifying variation in the share of paternity, however, these studies have focused on the ultimate outcome of male and female interactions, rather than the processes that occur during or after mating (Simmons 2001).

Directly quantifying the transfer and storage of sperm can illuminate how behaviours that contribute to paternity, such as copulation duration, harassment or the number of matings, can affect the utilization of sperm by females. In a wide variety of species, particularly insects, sperm transfer rates have been estimated by observing or manipulating copulation durations and then quantifying the number of sperm in storage for a once-mated female (Birkhead & Møller 1998; Simmons 2001). Increasingly, however, techniques such as artificial insemination (Evans *et al.* 2003) or differentially labelled sperm (Civetta 1999; Simmons *et al.* 1999; Schärer *et al.* 2007) have been developed that allow the physiological mechanisms mediating sperm transfer and storage within multiply mated females to be examined directly. In a recent paper, Bussière *et al.* (in press) described a method for quantifying male contributions to mixed sperm stores using a form of competitive PCR based on amplifying microsatellite markers (cf. quantification of target DNA; Zentilin & Giacca 2007). This technique uses the ratio of allelic signals in amplified samples of stored sperm in order to quantify the relative contributions of males with known genotypes to doubly mated females. A benefit of this approach is that the natural physiology of males and females remains unaltered, thereby avoiding the need to assess or control for the potential effects that sperm-labelling techniques such as radioisotopes or DNA staining could have on sperm behaviour or mating performance. Bussière *et al.* (in press) used this approach to demonstrate an influence of mating interval on biases in sperm storage across multiple spermathecae in yellow dung flies.

Here we present the results of an experiment using the same method to examine the processes affecting male insemination success in the black field cricket, *Teleogryllus commodus*. In this species, males transfer an external spermatophore during mating and vigorously harass females to prevent the premature removal of the spermatophore (Loher & Rence 1978; Evans 1988), with the unattractive males harassing the most intensely (Bussière *et al.* 2006). Females then actively remove this spermatophore before all sperm is transferred, biasing spermatophore attachment times in favour of attractive males (Bussière *et al.* 2006). This sexual conflict between males and females over spermatophore attachment duration has important consequences for which combinations of traits are favoured by sexual selection, as male harassment both significantly weakens the intensity and changes the form of selection acting on male courtship calls (Hall *et al.* 2008). Importantly, however, the evolutionary consequences of sexual conflict over spermatophore attachment and postcopulatory sexual selection via spermatophore removal depend on how variation in the duration of attachment relates to fitness. Attempts to address this important issue have found inconsistent results. A number of studies have found that increasing the duration of spermatophore attachment results in greater sperm transfer and share of paternity (Sakaluk 1984; Simmons 1986, 1987; Sakaluk & Eggert 1996). In another species of field cricket, for example, paternity is almost entirely predicted by the number of sperm stored from each male (*Gryllus bimaculatus*, Bretman *et al.* 2009). In contrast, Simmons *et al.* (2003) have demonstrated that neither sperm numbers nor sperm length influences paternity in the field cricket *Teleogryllus oceanicus*, in part because of the confounding influence of sperm viability (García-González & Simmons 2005).

The difficulty in interpreting these contrasting findings is often attributed to the lack of information regarding how sperm is stored and transferred in multiply mated females. Likewise, as paternity will depend on both male and female driven processes, it is equally important to resolve how insemination varies with male and female characteristics such as body size, condition or attractiveness, rather than assuming that copula duration alone, for example, will predict paternity. In this study, therefore, we were interested in the relationship between spermatophore attachment and sperm transfer when females had mated previously. Using a series of classical double mating trials (Boorman & Parker 1976; Parker *et al.* 1990), we manipulated the duration of spermatophore attachment for the second male to mate with a given female. We then quantified the proportion of sperm stored in the spermatheca that belongs to the second male (S_2) using the method developed by Bussière *et al.* (in press). Combining this method with standard multiple regressions, we show that the relationship between spermatophore attachment time and insemination success (S_2) follows a linear trend and explored the influence of male and female body size and male attractiveness on sperm transfer in multiply mated females. This allows us to address two important questions for which there are few existing data: how does sperm transfer and storage occur when females have previously mated, and how does sperm transfer and storage relate to male attractiveness?

Materials and methods

Animals for this experiment originated from a culture of *Teleogryllus commodus* collected in 2004 from Smith's Lake (32°22′S, 152°30′E), New South Wales, Australia. The culture was maintained in a controlled temperature chamber (28 °C) using a 14:10-h light:dark regime. During the experiment, we checked for newly enclosed adults daily, weighed any new adults and measured their pronotum width. From these data, we estimated the overall body size as a single principal component (over 90% of variation explained) extracted from the principal components analysis of weight and pronotum width. We individually housed the adults in small plastic jars, provided with cat food pellets, vials of water stoppered with cotton wool and egg carton for shelter. We replaced the food and water weekly.

Microsatellite screening of adults

Before we conducted any behavioural trials, we first genotyped all individuals to be used in the experiment. From 77 males and 92 females, we extracted DNA from the middle tibia using a DNeasy® Tissue Kit (QIAGEN AG) and then used the QIAGEN® Multiplex PCR Kit to amplify the following six microsatellite loci: Totri55a, Totri57, Totri59, Totri78, Totri88a and Totri9a (Beveridge & Simmons 2005). PCR amplification was performed using a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) with the following cycling conditions: 95 °C for 15 min, then 27 cycles at 94 °C for 30 s, 55 °C for 3 min and 72 °C for 1 min, and finally 55 °C for 30 min. Total PCR reaction volume was 6 μL: 1 μL DNA template, 3 μL QIAGEN Multiplex PCR Master Mix, 1.4 μL distilled water and 0.6 μL microsatellite primer mix (100 μM). We then visualized the PCR products on an ABI-3730 Sequencer (Applied Biosystems) and sized fragments using GeneMapper (version 3.7, Applied Biosystems).

A summary of the microsatellite screening is shown in Table 1. Using Genepop (web version, Raymond & Rousset 1995), we tested each locus for Hardy–Weinberg equilibrium. The characteristics of the microsatellites from our Smiths Lake population (NSW, Australia) differ in some instances (Totri59, Totri78 and Totri88a) from the microsatellites screened by Beveridge & Simmons (2005) based on the Walpole population (WA, Australia) in both the number of alleles at a loci and the size range of alleles found are different. In total four loci showed a significant heterozygote deficit: Totri88a is believed to be X-linked (Beveridge & Simmons 2005); Totri59 was found to have only two alleles and was not used in any subsequent analyses. For the remaining two loci, Totri55a and Totri9a, we avoided using data concerning the allelic concentrations and relied on the other informative loci unless both males were heterozygous at the locus in question (and therefore null alleles could not possibly affect the data).

Table 1 Characterization of the six microsatellite loci (Beveridge & Simmons 2005) in *Teleogryllus commodus*

Locus	*n*	N_A	Size range	H_o	H_e
Totri55a	169 (3)	11	81–242	0.64	0.80
Totri57	169 (1)	11	203–230	0.78	0.85
Totri59	169 (38)	2	69–91	0.03	0.08
Totri78	169	8	125–164	0.82	0.79
Totri88a	169	9	138–183	0.38	0.70
Totri9a	169	6	138–157	0.41	0.56

The number of individuals genotyped (*n*), including the number of individuals for which no product was detected (in brackets), the number of alleles (N_A), the allele size range in base pairs and the observed (H_o) and expected (H_e) heterozygosities are shown for each locus.

Behavioural trials

Using a series of behavioural trials, we experimentally manipulated the duration of spermatophore attachment in doubly mated females. Each experimental unit consisted of two males and two females, grouped such that the male contributions to spermathecal contents could be distinguished from one another and the female using two or more microsatellite loci. We conducted the trials over two consecutive nights beginning 2 h after dark as this is when the crickets are most active and using crickets that were between 10 and 14 days old posteclosion to ensure that they were sexually mature. We ensured that all males had produced spermatophores before the trials in order to control for any potential modification of spermatophore content based on female characteristics. On the first night, we mated each male to a female within the same group and then confined the female to a small plastic tube (5 × 1 cm) for 60 min to prevent spermatophore removal. This ensures that the females are less likely to be sperm limited in subsequent mating trials (Wynn & Vahed 2004). On the following night, we then mated each male to the alternate female within the group and again confined the female to a small plastic tube. This time, however, we removed the spermatophore of the focal male after 6, 12, 18, 24, 30, 36, 48 or 60 min (360–3600 s), depending on the treatment to which the group had been randomly assigned. In this way, each female was mated first to a nonfocal (60 min attachment time) male and then to a focal (manipulated attachment time) male. Finally, for each mating trial, we also estimated a male's attractive-

ness as the inverse of the time taken for the female to mount the male, as mating latency is a commonly used metric of male attractiveness in many insect species (Shackleton *et al.* 2005; Taylor *et al.* 2007; McGuigan *et al.* 2008; Hall *et al.* 2009). A total of 31 groups (62 males and 62 females) were used in the experiment.

Competitive PCR analysis of spermatheca content

Following the focal (i.e. second) mating, females were individually housed with food and water for 24 h before being placed in microcentrifuge tubes and frozen. To ensure that no sperm was used during this period, we prevented females from egg-laying (during which fertilization occurs, Huber *et al.* 1989) by not providing a suitable oviposition material. We later dissected the spermatheca and separated the ejaculates from female tissue by dehydrating the spermatheca in 70% ethanol following Tripet *et al.* (2001). After removing the sperm pellet from the spermatheca, we extracted the DNA using a QIAamp® DNA Micro Kit (QIAGEN AG) and amplified the microsatellite loci using the same protocol as outlined earlier. In total, we conducted three replicate amplifications for each spermatheca using a subsample of the DNA extracted from the sperm pellet. In each of the amplified subsamples of DNA, we then used GeneMapper to measure the relative signal strengths of the alleles unique to each of the two males. For each allele, relative signal strength was assessed as the area under an allelic peak divided by the total area of all the informative peaks in the run (those belonging to one of the males and not shared by the other male or the female in question).

Before the relative signal strengths can be used to provide information on the ratio of DNA concentrations belonging to each of the two males, the estimates must be corrected to account for the specific properties of each allele in the PCR and genotyping run. Following the process outlined in detail in Bussière *et al.* (in press), we first generated regressions of observed signal strength on factors known to influence amplification by randomly pairing the DNA from 20 adult crickets in a series of varying concentrations (1:1, 2:3, 3:7, 1:4, 1:9 and 1:19). We included the following allele and run characters in our regressions: the initial relative allelic concentrations (i.e. the predicted ratio if all alleles amplify equally well); the number of alleles being amplified in the reaction; the number of shorter alleles (relative to the focal allele) amplified in the reaction; the relative length of the focal allele (focal allele length – mean allele length); and, whether or not the focal allele was homozygous in the focal individual. We then rearranged the equations to correct observed values of allele signal strength for the PCR runs of spermatheca content where the starting DNA concentrations are unknown (Table 2). Finally, after correcting for the properties of the PCR and fragment analysis runs, we adjusted estimates of the corrected DNA ratios based on the number of informative allele copies for each male.

We then used the corrected signal strengths to estimate the fraction of sperm in the spermatheca that belongs to the second male mated to the female (the male's S_2 value). For each focal male, we calculated the repeatability of its S_2 value both within a locus but across replicate PCR runs (to assess the repeatability of ratios that should begin with identical reagent conditions) and then across the different microsatellite loci (to determine how much variation is due to differences in the properties of alleles as opposed to their ratios). As per Becker (1992), we calculated the repeatability using the variance components derived from ANOVA with unequal sample sizes. In total, we were able to collect data for 56 different mating pairs from the original 62, as one male died before completing its focal mating, we failed to remove the spermatheca undamaged in two cases, no sperm were found in the spermatheca in

Table 2 The unstandardized parameter estimates for the linear regression of allele properties on observed signal strength for the microsatellite loci used during the competitive PCR analysis

Locus	*N* = peaks (PCR runs)	Initial DNA concentration	No. shorter alleles	Relative allele length	Focal allele heterozygosity	No. of alleles in run
Totri55a	770 (304)	0.929	−0.058	0.001	0.104	−0.021
Totri57	1189 (381)	0.971	0.011	−0.001	0.014	−0.007
Totri78	1404 (429)	0.895	−0.008	−0.004	0.052	−0.008
Totri88a	946 (355)	0.960	0.028	−0.007	0.028	−0.017
Totri9a	575 (255)	0.988	<0.001	−0.008	0.038	−0.017

The five equations for correcting observed proportion signal strength were based on these original parameters, but the resulting equations were rearranged to isolate the initial DNA concentration. For example, for Totri55a: initial [DNA] = ([observed proportion signal + 0.058 × number of shorter alleles −0.001 × relative allele length −0.104 × heterozygosity + 0.021 × number of alleles in run]/0.929).

one case, and DNA extraction or PCR failed in the remaining two cases.

Characterizing how insemination success relates to male and female characteristics

We used a series of multiple regression models known as response surface analysis (Draper & John 1988; Chenoweth & Blows 2005) to characterize how insemination success (S_2 values) was related to male and female characteristics, including the manipulated duration of spermatophore attachment. Unlike other model building approaches that seek to find the best minimal model (e.g. stepwise regression and backwards deletion), the sequential model-building approach that we use is primarily concerned with the form (linear or nonlinear) of the relationships between our fitness measure (S_2 values) and the other traits of interest. In general, we estimated a model containing only the intercept, a model containing linear terms (β) and, finally, the full response surface as estimated by a second-order polynomial regression that includes linear (β), cross-product interaction (γ_{ij}) and quadratic (γ_{ii}) terms. To evaluate if our traits of interest predicted male insemination success in a linear fashion, we used the Wald chi-squared test to assess if the addition of the linear terms improved the fit of the model containing only an intercept. Evidence for nonlinear trends would then be assessed by the addition of the nonlinear terms (cross-product interaction and quadratic) to the linear only model using the same test statistic. Once the final model is determined, we then inspect the individual regression coefficients to assess which traits were responsible for the significance of the Wald chi-squared tests. All statistical analyses were performed in R (version 2.8.0, R development core team, http://www.R-project.org) using mixed model analyses as implemented with the lmer function of the lme4 package (Bates *et al.* 2008), where the behavioural trial group of a given male was included as a random factor to account for interdependence amongst group members.

Using this approach, we first assessed if the relationship between manipulated attachment times and S_2 followed a linear or nonlinear trend. A positive linear trend would suggest that there are important fitness gains for males with increased spermatophore attachment times, whereas a nonlinear trend (convex or concave) would indicate that the fitness consequences of spermatophore attachment may be more complex. We then characterized the nature of the relationships between S_2 and focal male attachment time, focal male attractiveness, female body size, focal male body size and nonfocal male body size. Before analysis, we transformed attractiveness using natural logarithms as the variable was positively skewed. We also standardized all predictor traits to a mean of 0 and standard deviation of 1 (Lande & Arnold 1983), as this allows for the relative strengths of the relationships between S_2 and our various predictor variables to be more easily compared. In all cases, we used the mean S_2 value for each male (averaged across all loci and subsample runs) in untransformed form as the distribution of these proportion values was not bounded by 1 or 0 and the residuals extracted from all models were normally distributed (Shapiro–Wilk test, $P > 0.05$). A subsequent analysis using an arcsine square root transformation of S_2 yielded the same results; however, we present the analyses based on the untransformed data as this allows for parameter estimates and figures to relate directly to changes in the proportion of sperm within the spermatheca.

Results

Using the competitive PCR analysis, we found that estimates of the fraction of sperm in the spermatheca that belongs to the focal male (S_2) were highly repeatable for each individual across subsample PCR runs (repeatability = 0.96) and across microsatellite loci (repeatability = 0.82). Based on the average S_2 values for each male ($n = 56$), our results indicate that insemination success increased with the duration of spermatophore attachment (Fig. 1). This relationship between attachment time and S_2 followed a significant linear trend ($\chi^2 = 10.822$, d.f. = 1, $P = 0.001$) rather than a nonlinear trend, as the addition of the quadratic term did not improve the fit of the overall model ($\chi^2 = 0.090$, d.f. = 1, $P = 0.764$). For example, the proportion of sperm in

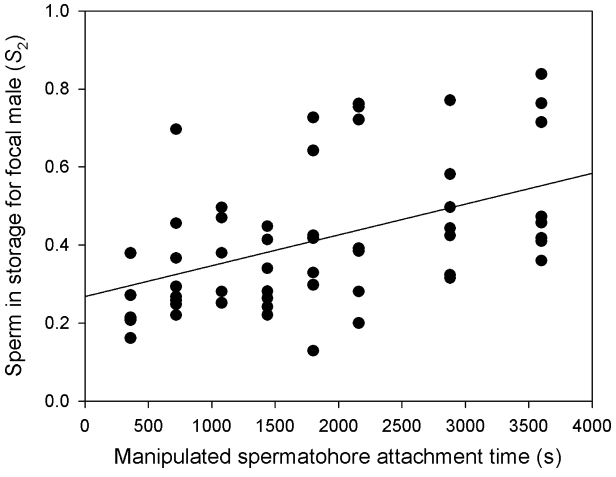

Fig. 1 The fraction of sperm in the spermatheca that belongs to the focal male (S_2) as a function of the manipulated spermatophore attachment time of the focal male in twice-mated females.

storage attributed to the focal male increased from 25% after 6 min (360 s, 0.247 ± 0.037) to over 50% after 60 min (3600 s, 0.554 ± 0.066).

We also examined the relationship between insemination success (S_2), the duration of spermatophore attachment, and male and female phenotypes using multiple regression. In addition to spermatophore attachment time (β = 0.087, P = 0.002), we found that insemination success was significantly influenced by the attractiveness of the focal male (β = 0.066, P = 0.004) and the overall size of the female (β = −0.053, P = 0.020). However, neither focal male body size (β = 0.013, P = 0.546) nor non-focal male body size (β = 0.018, P = 0.394) significantly influenced the insemination success of the focal male. All nonlinear interactions were removed from the regression model, as the addition of the cross-product interaction (χ^2 = 7.671, d.f. = 10, P = 0.661) and quadratic terms (χ^2 = 4.279, d.f. = 5, P = 0.510) did not significantly improve the fit of the model containing only the linear terms (linear model significance: χ^2 = 23.033, d.f. = 5, P < 0.001). The nature of the relationships between male insemination success and spermatophore attachment time, male attractiveness and female body size as described by the regression analysis are shown in Fig. 2. Specifically, there was a positive relationship between S_2 values and both the duration of spermatophore attachment and focal male attractiveness and a negative relationship between S_2 values and overall female size.

Discussion

By combining newly developed competitive PCR-based methods (Bussière *et al.* in press) with the manipulation of postcopulatory spermatophore removal, we have explored one important process underlying postcopulatory sexual selection in multiply mated females. Our findings demonstrate that there is a significant relationship between spermatophore attachment time and insemination success in twice-mated females of *Teleogryllus commodus*. In particular, we found that the proportion of sperm in the spermatheca that belongs to the focal male (S_2) was significantly influenced by the duration of spermatophore attachment, the overall size of the female and the attractiveness of the male. Together, these results address two unresolved evolutionary issues regarding postcopulatory sexual selection: the nature of sperm transfer and storage in multiply mated females; and how insemination success relates to male attractiveness.

The nature of sperm transfer and storage

Experimental studies have established that changing the length of copulation (Lorch *et al.* 1993; Engqvist *et al.*

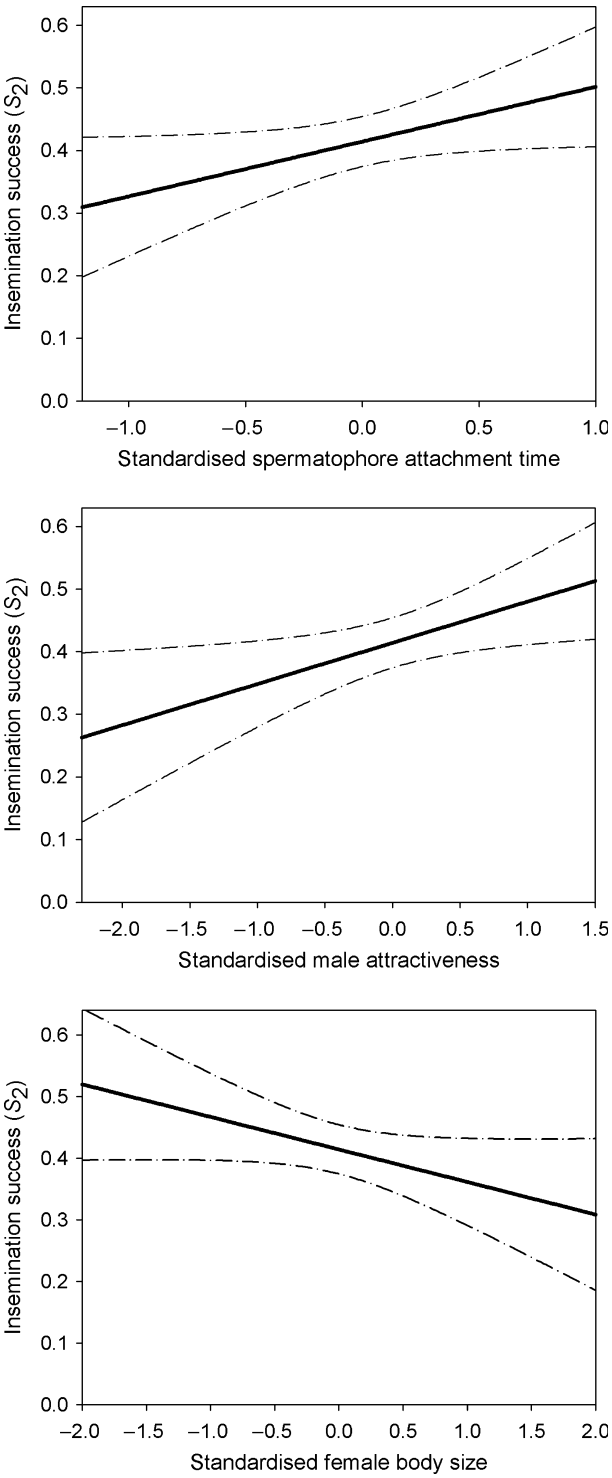

Fig. 2 Visualization of the relationships between focal male insemination success (S_2) and spermatophore attachment time, male attractiveness and female body size. The dark black lines represent the standardized regression coefficients (β) from the multiple regression that included spermatophore attachment time, male attractiveness, female size, focal male size and non-focal male size. The dashed grey lines describe the 95% confidence intervals around the regression estimates.

2007; Pilastro *et al.* 2007) or the duration of spermatophore attachment (Sakaluk 1984; Simmons 1986; Simmons & Achmann 2000; Simmons *et al.* 2003; Bussière *et al.* 2006) will influence the number of sperm transferred for a given male when mated to a virgin female. This relationship between the number of sperm transferred and time is used by both theoretical and empirical studies in combination with paternity data to infer the extent and form of sperm displacement and the optimal length of time for copulation (Parker *et al.* 1990; Parker & Simmons 1991, 2000; Sakaluk & Eggert 1996; Neff & Wahl 2004). In some insect species, however, the specialized sperm storage organs are of fixed or partially constrained volume (Loher & Rence 1978; Hosken & Ward 2000; Simmons 2001). Accordingly, the number of sperm transferred in a single mating may not adequately predict the changes in the mix of sperm that will occur if a female has previously mated.

In *T. commodus*, for example, Bussière *et al.* (2006) have previously shown that the total number of sperm contained within the spermatheca of once-mated females does not significantly increase after 36 min (2160 s). This finding on its own would suggest that there is very little fitness benefit to spermatophore attachment times after this time, and raises the important question of why males and females conflict over the duration spermatophore attachment, particularly when the typical removal times for females from this population exceeds this time (2886 ± 181.4 s; Hall *et al.* 2008). By manipulating spermatophore attachment times and directly quantifying mixed sperm stores, however, our results show that, in previously mated females, the proportion of sperm in storage for the second male increases linearly with the attachment time of its spermatophore. In combination with the knowledge that the expansion of the spermatheca in *T. commodus* is relatively constrained (Loher & Rence 1978), our findings together with those of Bussière *et al.* (2006) suggest that although there is a finite capacity for females to store sperm that will be reached at approximately 36 min (2160 s, 25750 ± 2408 sperm), a male's contribution to the mixture of sperm within the spermatheca can continue to change after this time through sperm displacement. Given that sperm are predicted to mix randomly within the spermatheca (Simmons 1987; Sakaluk & Eggert 1996; García-González & Simmons 2005) and a male's share of paternity is almost completely predicted by his representation of sperm in storage (Bretman *et al.* 2009), our findings indicate that the fitness benefits to males of increased attachment times will continue until 60 min and possibly beyond.

In addition to spermatophore attachment duration, we found that the insemination success of a given male also depends on the size of the female. The effect of female size suggests sperm displacement is occurring because for a given attachment time and level of male attractiveness, there was a higher proportion of sperm from the second male found in the spermatheca of smaller females. Likewise, in the yellow dung fly, smaller females experience greater levels of sperm displacement due to the reduced sperm storage volumes (Parker *et al.* 1999). Although we cannot rule out that other female size effects such as longer spermathecal ducts may also contribute to the observed negative relationship with S_2, if sperm displacement is occurring in *T. commodus* then the most likely mechanism of displacement is one of indirect displacement as the spermatophore tube does not directly enter the spermatheca, but carries sperm into the convoluted receptacular (i.e. spermathecal) duct (Loher & Rence 1978). From our results, we would predict that males should try to prolong spermatophore attachment with larger females not only due to the increased fecundity of such females, but also due to the lower levels of sperm displacement (e.g. Parker *et al.* 1999). Although this hypothesis is untested in *T. commodus*, in another gryllid cricket, *Gryllodes sigillatus*, males guard larger females for longer in order to delay premature spermatophore removal (Bateman *et al.* 2001).

Insemination success, male attractiveness and cryptic female choice

Although the importance of cryptic female choice remains a controversial topic (Birkhead 1998, 2000; Birkhead & Pizzari 2002), the potential mechanisms of cryptic female choice are well described (Eberhard 1996). Females, for example, can selectively favour paternity towards certain males by biasing the transfer and storage of sperm (Hellriegel & Bernasconi 2000; Bussière *et al.* 2006; Bretman *et al.* 2009), discarding sperm (Pizzari & Birkhead 2000; Snook & Hosken 2004), selectively using sperm (Ward 2000) or differentially allocating resources towards zygotes (Gil *et al.* 1999; Cunningham & Russell 2000). Demonstrating the existence of most forms of cryptic female choice, however, remains a difficult empirical challenge as many of the underlying processes are hidden within the female reproductive tract. Furthermore, it is unclear whether cryptic female choice usually acts to reinforce previous decisions made earlier during the mating process (Pizzari *et al.* 2002; Evans *et al.* 2003), or selects males based on characteristics that may be difficult or impractical to assess prior to mating, for example, selecting for genetic compatibility (Bishop *et al.* 1996).

In field crickets, females are known to exert postcopulatory choice by delaying the removal of the spermatophore of attractive (*T. commodus*, Bussière *et al.* 2006) or

large (*Gryllus bimaculatus*, Simmons 1986) males. Our findings show that attractive males my also experience increased insemination success for a given attachment time, as male attractiveness was positively related to the representation of sperm in the spermatheca. One explanation for this pattern would be that attractive males, in general, transfer more sperm. In a previous study, however, Bussière *et al.* (2006) demonstrated that when mated to virgin females there was no significant difference in the sperm transfer rates between attractive and unattractive males. Similarly, given the previously reported link between male attractiveness and male size in another cricket species (e.g. Simmons 1986), it is also possible that attractive males transfer larger spermatophores as a consequence of their increased size. In *T. commodus*, however, based on the data from this study, it appears that male size is not phenotypically correlated with either male attractiveness ($r = 0.096$, $P = 0.484$) nor male insemination success ($r = 0.027$, $P = 0.842$). Moreover, even after controlling for variation in spermatophore attachment times, female size and male attractiveness as part of the multiple regressions, neither the size of the first or second male to mate with a female had any influence on the insemination success of the focal male. Together, these findings strongly indicate that there is no male size effect on sperm transfer in this species (cf. yellow dung flies; Parker & Simmons 2000; Ward 1998).

Instead, we suggest that when multiple mating occurs females are either assisting or inhibiting sperm transfer as a form of cryptic female choice that favours attractive males over unattractive males, or that there is a property of the reproductive apparatus or sperm from attractive males that increases their competitive ability in such situations. Empirical evidence from other insect species suggests that cryptic female choice is most likely to be involved in the increased insemination success of attractive males. In the yellow dung fly, for example, females are known to aid sperm transfer between the bursa and spermathecae via muscular movements (Hellriegel & Bernasconi 2000; Hosken & Ward 2000). Similarly in the bushcricket *Requena verticalis*, Simmons & Achmann (2000) found evidence for the role of female musculature in transporting sperm, as females anaesthetized with carbon dioxide had lower numbers of sperm in their spermatheca. Future studies examining how the morphological and chemical characteristics of sperm covary with male attractiveness may be able to dissect the relative roles of male sperm characteristics and cryptic female choice in generating the observed increase in insemination success for attractive males.

Regardless of the mechanism behind the elevated insemination success of attractive males (cryptic female choice or sperm characteristics), there is now evidence that in *T. commodus* female choice is consistent over several consecutive episodes of the mating sequence. Attractive males, those that are favoured by precopulatory choice in terms of the shortest latency to mate (Shackleton *et al.* 2005), are also favoured via postcopulatory choice by having their spermatophores attached for longer (Bussière *et al.* 2006) and by postcopulatory sexual selection (cryptic female choice or sperm competition) through greater insemination success for a given attachment time. This reinforcement of pre- and postcopulatory mating decisions suggests that the strength of sexual selection may be stronger than predicted based on estimates from individual stages of mate choice. If female choice for attractive males is underestimated, then so too might be the intensity of sexual conflict over spermatophore attachment times. Unattractive males will have even more to gain by harassing females to prevent spermatophore removal, as not only are their spermatophores removed sooner (Bussière *et al.* 2006), but also the transfer of their sperm within the female reproductive tract is reduced. Our findings highlight the potential for techniques that quantify mixed sperm stores (Civetta 1999; Simmons *et al.* 1999; Schärer *et al.* 2007; Bussière *et al.* in press) for disentangling the roles that cryptic female choice and sperm competition may have in determining fertilization success. In combination with behavioural manipulations and paternity analyses, which remain lacking for *T. commodus*, these techniques will allow a greater understanding of the mechanisms underlying postcopulatory sexual selection in general.

Acknowledgements

We thank A. Pemberton for advice and assistance, and the staff of the Molecular Biology Institute of the University of Zurich for granting access to their sequencer. Likewise, D. Gwynne, R. Snook, J. Tomkins and O. Martin contributed with comments on the manuscript. The research was funded by grants from UNSW, the University of Zurich and the Australian Research Council.

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