Sperm competition, alternative mating tactics and context-dependent fertilization success in the burying beetle, *Nicrophorus vespilloides*

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Fertilization success in sperm competition is often determined by laboratory estimates of the proportion of offspring sired by the first (P_1) or second (P_2) male that mates. However, inferences from such data about how sexual selection acts on male traits in nature may be misleading if fertilization success depends on the biological context in which it is measured. We used the sterile male technique to examine the paternity of the same male in two mating contexts in the burying beetle, *Nicrophorus vespilloides*, a species where males have alternative mating strategies based on the presence or absence of resources. We found no congruence in the paternity achieved by a given male when mating under different social conditions. P_2 estimates were extremely variable under both conditions. Body size was unrelated to success in sperm competition away from a carcass but, most probably through pre-copulatory male–male competition, influenced fertilization success on a carcass. The contribution of sperm competition is therefore dependent on the conditions under which it is measured. We discuss our findings in relation to sperm competition theory and highlight the need to consider biological context in order to link copulation and fertilization success for competing males.

Keywords: context-dependent fertilization success; male–male competition; P_2; sperm competition

1. INTRODUCTION

Sperm competition occurs whenever the sperm of two or more males are present within the reproductive tract of a single female and compete for the fertilization of a given set of ova (Parker 1970*a*). It is now widely recognized that sperm competition is a pervasive force in evolution, favouring adaptations in males for the engagement in and/or avoidance of sperm competition (reviewed by Parker 1970*a*; Smith 1984; Birkhead & Møller 1992, 1998; Simmons 2001). Adaptations to sperm competition are taxonomically widespread (Birkhead & Møller 1998; Simmons 2001) and have been shown to influence the evolution of male anatomy (e.g. Waage 1979; Arnqvist & Danielsson 1999; House & Simmons 2003), reproductive physiology and development (e.g. Wedell & Cook 1999; Froman *et al.* 2002; Harris & Moore 2005), as well as pre- and post-copulatory behaviour (e.g. Smith 1979; Edvardsson & Arnqvist 2000; Preston *et al.* 2003).

A great deal of our understanding of the operation and evolutionary consequences of sperm competition has come from laboratory studies on invertebrates, particularly insects (Simmons 2001). In such studies, paternity is often determined by conventional double-mating trials and expressed as the proportion of offspring sired by the second male to mate with the female, commonly referred as P_2 (Boorman & Parker 1976). All patterns of P_2 have been found: either the first or second male fertilizes the majority of eggs ($P_1 > P_2$ or $P_2 > P_1$, i.e. sperm precedence) or both males fertilize an equal number of eggs ($P_1 = P_2 = 0.5$, i.e. sperm mixing). However, associating P_2 estimates with specific evolutionary processes can be misguided because

the same outcome of sperm competition can be achieved through very different proximate mechanisms (see table 2.4 in Simmons 2001). Even within species, the outcome can be extremely variable, with some families highly successful and others poor competitors, even if the population mean measured by P_2 indicates sperm mixing (Corley *et al.* 2006). Moreover, relatively little is known about how P_2 estimates measured in a conventional double mating relate to last-male paternity estimates derived when females mate with more than two different males (Simmons & Siva-Jothy 1998; Simmons 2001; but see Wilkes 1966; Parker 1970*b*; Zeh & Zeh 1994; Drnecich 2003). Even less is known about patterns of sperm utilization in natural populations (Simmons 2001; but see Cobbs 1977; Turner & Andersson 1984; Dickinson 1988; LaMunyon & Eisner 1993; LaMunyon 1994). Given the ubiquity of female multiple mating in insects (Simmons 2001), understanding the strength and form of selection imposed by sperm competition depends on the extent to which the laboratory studies reflect natural conditions. Therefore, in this study, we examine how social factors reflecting the conditions under which mating occurs might influence P_2 estimates within the same individuals in a species where matings can occur under different conditions. We test the prediction that despite variation in P_2 among males, individuals will be consistent in their ability to engage in sperm competition. Such consistency is a necessary condition for using P_2 to infer selection and evolutionary outcomes.

In the burying beetle, *Nicrophorus vespilloides*, all males have plastic mating strategies (Eggert 1992). In one tactic, males actively search for oviposition sites, small vertebrate carcasses, to which females are also attracted. If no female is present on the carcass when it is located, the male

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superficially buries the carcass and emits a sex pheromone to attract females (Eggert & Müller 1989*a*,*b*; Eggert 1992). Other males may also be attracted and relatively larger males are more successful in male–male competition for the carcass (Bartlett 1988; Bartlett & Ashworth 1988; Otronen 1988). On the carcass, the larger resident male repeatedly copulates with the female and consequently sires the majority of offspring developing on the carcass (Bartlett 1988; Müller & Eggert 1989). However, there is a great variation in the success of male–male competition in ensuring paternity. Eggert (1992) found that males that lost in competition on carcasses sire between 0 and 35% of the subsequent offspring that survived to adult.

All males adopt an alternative tactic if they are unable to locate a carcass or have lost in male–male competition. These males emit a pheromone and attract females who mate, even though reproduction cannot occur until a female finds a carcass (Eggert & Müller 1989*a*,*b*; Eggert 1992). Females apparently cannot distinguish between males that are emitting a pheromone on and off a carcass and those without a carcass are equally successful at attracting females (Eggert & Müller 1989*a*). Off a carcass, if they mate, males and females typically mate only once (Eggert & Müller 1989*a*). However, the net result is that a female nearly always arrives at a carcass with stored sperm (Müller & Eggert 1989). This is important because females can locate carcasses with no male present on their own and are able to bury and reproduce on this resource without male assistance. Eggert (1992) found that in a population of *N. vespilloides* in Germany, 39% of female reproduction is with no male present, with fertilization resulting from sperm obtained by mating with multiple males off a carcass. Thus, pheromone signalling without resources can result in reproduction for males if the female finds a carcass that has no male and if they win in sperm competition. It pays males to adopt both tactics because despite the potentially higher fitness payoffs associated with attracting a female when in the possession of a carcass, carcasses are highly unpredictable in space and time (Scott 1998). Thus, all males readily employ either tactic (Müller & Eggert 1987; Eggert 1992).

Here, we examine the fertilization success of a focal *N. vespilloides* male when the same pair of males mated in two different biological contexts: away from a carcass where competition is limited to sperm competition and on a carcass where there is both sperm competition and male–male competition for access to females. Given the previous work of Müller & Eggert (1989) and Eggert (1992) in this species, which shows that males on carcasses ensure paternity by repeatedly mating, we were specifically interested in the extent that an individual male's fertilization success was repeatable across the two different mating contexts. Sperm derived from matings off a carcass compete directly, while on a carcass sperm from previous matings appear to be subverted by repeated matings of the male that is successful in male combat—but just how effective is male combat for all males? Are there males that specialize in sperm competition and others that rely on male–male combat? Or are males superior or inferior in both contexts? Finally, we discuss our findings in relation to sperm competition theory and the importance of the biological context in which copulation occurs for the outcome fertilization success for competing males.

2. MATERIAL AND METHODS

(a) *Experimental animals*

Over 100 female and 100 male *N. vespilloides* were trapped from a natural population located in a deciduous forest at Sunbank Wood, Manchester, UK, to form our stock population. These individuals were attracted to commercial Japanese beetle (funnel) traps baited with rotting meat. A small quantity of meat was used to ensure that no reproduction occurred in the traps. Both males and females were attracted towards most traps. Each trap ($n = 34$) was left in place for two weeks, with beetles removed every 4 days.

In the laboratory, each female was placed in an individual breeding chamber ($17 \text{ cm} \times 12 \text{ cm} \times 6 \text{ cm}$ plastic container) filled with 2 cm of moist soil, provided with approximately 20 g of defrosted mouse carcass (Livefoods Direct Ltd, UK) and allowed to breed. All females were previously mated and produced larvae. All larvae were collected when they dispersed from the carcass and housed in individual rearing chambers ($8 \text{ cm} \times 2 \text{ cm} \times 13 \text{ cm}$ plastic container) filled with 2 cm of moist soil. At eclosion, each virgin offspring was fed two decapitated mealworm (*Tenebrio*) larvae, twice a week. These adults were then randomly mated at sexual maturity to form an F_2 population with no inbreeding. At 16 days post-eclosion, sexually mature virgin F_2 offspring served as our experimental animals. All experiments and rearing of offspring were conducted in a constant temperature room at $20 \pm 1°C$ with a 16 L : 8 D light regime.

(b) *Experimental design*

We examined the fertilization success of a pair of males when mating in two different contexts using the sterile male technique (Boorman & Parker 1976). Males were irradiated by exposure to 10 krad of gamma radiation using a ^{60}Co source. Beetles were anaesthetized using nitrogen gas prior to irradiation. Irradiated sperm are competent to fertilize an ovum, but the zygote fails to hatch due to lethal mutations (Boorman & Parker 1976). Consequently, eggs that are fertilized by a sterile male are reliably identified when a normal (N) and irradiated (R) male copulate with the same female (see Simmons (2001) for a review of the technique and its utility). In *N. vespilloides*, irradiation at this dosage does not influence male mating behaviour or longevity for more than two weeks after exposure (Bartlett 1988; House 2006, unpublished data).

In total, we established 96 pairs of male *N. vespilloides* at random and each pair was randomly allocated a virgin female to mate in two mating contexts. In each experimental pair, one male was irradiated and the other remained fertile. To avoid pseudoreplication, the fertilization success of a single focal male in each pair was assessed in two different mating contexts. In the first mating context, males and females mated without a carcass. Each male copulated once with the female, a typical mating frequency when no carcass present (Bartlett 1988; Eggert & Müller 1989*a*; Eggert 1992). This first mating context is therefore directly analogous to a conventional double mating in a typical P_2 laboratory study (Simmons 2001). In all 96 pairs, the focal male was always the second male to mate (i.e. mating order was standardized). In half of the pairs, the focal male was irradiated (NR, $n = 48$) and in the remaining half it was fertile (RN, $n = 48$). All

matings were observed under low white light in transparent Petri dishes (5 cm diameter × 1 cm depth) lined with a sheet of moistened filter paper, with a 1 h interval between subsequent matings. Twice-mated females and both males were returned to their individual rearing containers and provided with two decapitated mealworms. The female was released into an individual breeding chamber containing 2 cm of moist soil and a defrosted mouse carcass (mean ± s.e. carcass weight = 20.86 ± 0.46 g) 24 h after the last mating. These females then began to prepare the carcass and lay eggs.

In the second mating context, we examined the fertilization success of the same pair of males 24 h later when they competed for matings on a mouse carcass. Both males and a virgin female were released into a breeding chamber containing 2 cm of moist soil and a defrosted mouse carcass (21.43 ± 0.5 g). The males were removed from the breeding chamber 24 h after their introduction and placed in individual 1.5 ml Eppendorf vials and frozen prior to size measurements. Females were again allowed to continue to prepare the carcass and lay eggs.

Further we established 78 males paired at random to control for natural levels of infertility and residual fertility when females mate with fertile and irradiated males, respectively. In half of the pairs, both males were fertile (NN, $n = 39$) and in the remaining half, both males were irradiated (RR, $n = 39$). Each male pair was mated in both contexts following the protocol outlined above for experimental pairs. Unfortunately, we could not reciprocate the order that the focal male mated in each context (i.e. pairs always competed in the absence of a resource first) as preliminary experiments showed that males occasionally injure or kill one another when competing on a carcass (see also Müller & Eggert 1989). The fact that the mean and variance in paternity of the focal male did not differ across mating contexts (figure 1) suggests that our inability to reciprocate the mating order is unlikely to bias our results.

After 60 h following introduction into the breeding chamber, before eggs began to hatch (Smiseth *et al.* 2006), each female and her carcass were temporarily removed from the breeding chamber and placed into an empty container (17 cm × 12 cm × 6 cm). Eggs were removed from the soil using fine forceps and placed on moist cotton pads in batches of 10 or less per Petridish. The soil, carcass and female were then returned to the breeding chamber and the process was repeated at 120 and 180 h. To assign paternity, the number of eggs that hatched in each Petri dish was recorded twice a day until all eggs had either hatched (i.e. fertilized by a normal male) or decomposed (i.e. fertilized by a sterile male, controlling for natural levels of infertility; see below).

Digital images of all experimental males were captured with a digital camera (ColorView II, Soft Imaging System) mounted on a binocular microscope (Leica MZ5). The maximum length of the pronotum was measured on these images using IMAGE J (freely available at http://rsb.info.nih. gov/ij/). Repeated measures of male pronotum length taken from a subsample of 40 beetles showed that this size measurement was highly repeatable ($R_E = 0.99$, $F_{1,39} = 100.21$, $p = 0.0001$). We quantified the size difference between males in each pair by subtracting the pronotum length of the focal male from that of his competitor.
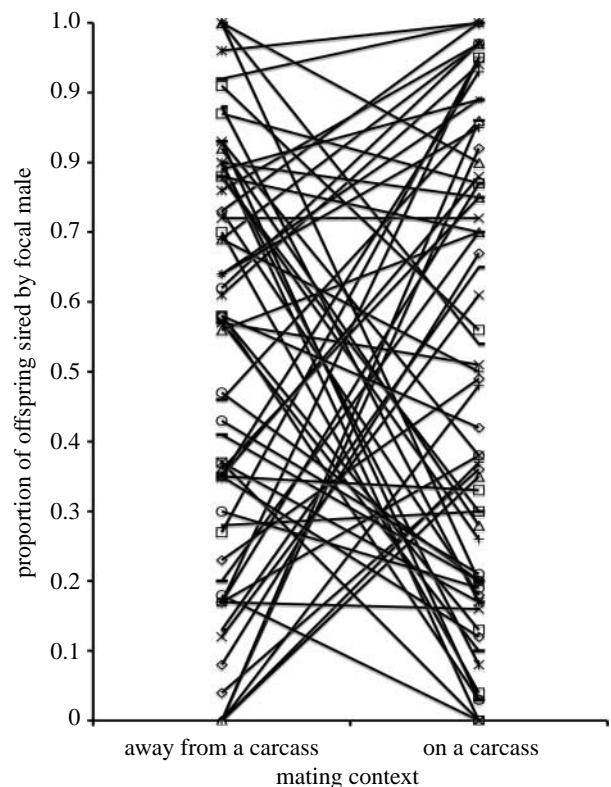
Figure 1. Reaction norms of focal males from each of the 65 different pairs of males in competition, illustrating the lack of congruence between fertilization success in the presence and absence of the resource required for reproduction. Each line represents a single male and only one randomly chosen male per pair was analysed. There was a very low and non-significant correlation between a given male's fertilization successes ($r = 0.028$, $p = 0.828$), showing that fertilization success away from the resource required for reproduction was a poor predictor of success on the resource.

(c) Statistical analysis

Estimates of fertilization success were calculated using the formula given by Cook *et al.* (1997). The proportion of eggs sired by the normal male in the pair (P_N) was calculated as

$$P_N = \frac{x - z}{p - z},$$

where x is the proportion of eggs that hatch; p is the proportion hatching after a NN mating; and z is the proportion hatching after a RR mating. We calculated p and z separately for each mating context (without resource: $p = 0.58 \pm 0.05$, $z = 0.03 \pm 0.01$; with resource: $p = 0.79 \pm 0.03$, $z = 0.04 \pm 0.01$). In cases where the focal male was irradiated (P_R), his paternity was estimated as $P_R = 1 - P_N$. Paternity estimates greater than 1 or less than 0 can occur when values of x are higher than p or lower than z, respectively (Cook *et al.* 1997). We therefore corrected our paternity estimates using the formula given by Cook *et al.* (1997) so that the data lie within the range 0–1. These paternity estimates were arcsine square-root transformed prior to statistical analyses and they conformed to a normal distribution in both the first ($K = 0.099$, $n = 65$, $p = 0.19$) and the second ($K = 0.079$, $n = 65$, $p = 0.20$) mating context. However, untransformed proportions are presented for ease of interpretation.

Although our design started with 96 experimental male pairs, some females failed to produce sufficient eggs.

We therefore restrict our analyses to those male pairs ($n = 65$) where females mating in both contexts produced 10 or more offspring to estimate paternity. We analysed the focal male's fertilization success in the two contexts using a repeated-measures ANCOVA with irradiation sequence (RN or NR) as the main effect, the difference in body size between the two males in a pair as a covariate and the paternity of the focal male in the two mating contexts as the repeated measure. We also ran the analysis with the body size of each male as a covariate to ensure that results relating to difference in body size did not reflect absolute size of the males involved.

We calculated the repeatability (R) of the focal males fertilization success in the different mating contexts using variance components derived from ANOVA (Becker 1984),

$$R = \frac{\sigma_W^2}{(\sigma_W^2 + \sigma_E^2)},$$

where σ_W^2 is the variance among males (estimated from $[MS_W - MS_E]/k$; where MS_W is the mean squares between individuals; MS_E is the error mean squares from the ANOVA; and k is the number of repeat measures per male pair (i.e. $k = 2$)). σ_E^2 is the variance among measures (equal to MS_E). The standard error of R was calculated as described by Becker (1984),

$$s.e. = \sqrt{\frac{2(1-R)^2[1 + R(k-1)^2]}{k(k-1)(n-1)}},$$

where n is the total number of male pairs examined (equal to 65) and k is as above. All analyses were performed in JMP (v. 5.0.1a) and all data are presented as mean $\pm$ 1 s.e.

3. RESULTS

The fertilization capacity of irradiated sperm is often lower than that of normal sperm (Simmons 2001). However, we found that irradiation sequence did not significantly influence the fertilization success of the focal male in a pair, either through its main effect or via interactions with the covariate or the repeat measures (table 1). We therefore simplified our model by removing this main effect, as recommended by Crawley (2005). Removal of this main effect did not significantly increase the error sums of squares within (partial F-test: $F_{3,60} = 0.482$, $p = 0.696$) or between subjects ($F_{3,60} = 0.136$, $p = 0.938$).

Analysis of the minimal adequate model revealed that the fertilization success did not significantly differ across mating contexts with either absolute sizes ($F_{1,60} = 0.574$, $p = 0.452$) or relative size ($F_{1,62} = 0.220$, $p = 0.640$) in the model. The range of mating success in each context varied from 0 to 1 (figure 1). However, there was little congruence between a male's fertilization successes in each mating context (figure 1), with much greater variation in paternity within than between male pairs. Accordingly, a male's fertilization success was not repeatable across the different mating contexts ($R = 0.04 \pm 0.12$).

The relative size of the focal male had a significant effect on his fertilization success, but this was dependent on the particular context that the male mated in (mating context $\times$ relative size difference: $F_{1,62} = 6.787$, $p = 0.012$). Neither absolute size of the focal male (mating context $\times$ absolute size of focal male: $F_{1,60} = 0.674$, $p = 0.415$) nor of

Table 1. Repeated-measures ANCOVA model examining the relationship between the fertilization successes of a single focal male from 65 pairs mating in two mating contexts, with and without resources required for reproduction. While we present the maximal model here, irradiation sequence and its lower-order interactions were removed from this final model by a backward deletion process of model simplification (see text for more details).

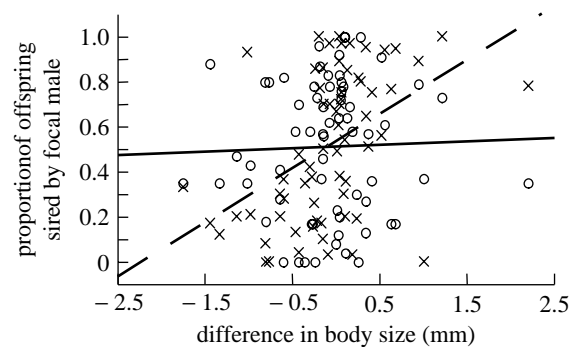| source	SS	$F_{1,60}$	p-value
between subjects			
irradiation sequence (A)	0.118	1.271	0.264
size difference (B)	0.567	6.107	0.016
$A \times B$	0.001	0.006	0.940
within subjects			
mating context (C)	0.016	0.177	0.676
$A \times C$	0.028	0.316	0.576
$B \times C$	0.681	7.635	0.008
$A \times B \times C$	0.091	1.017	0.317

Figure 2. The relationship between the fertilization successes of the focal male mating when the resource required for reproduction was absent (open circles, solid line), or on the resource required for reproduction (crosses, dashed line), and the difference in body size between the focal male and his competitor. The difference in body size was calculated as the size of the focal male minus the size of his competitor. Thus, positive values for this axis indicate that the focal male was larger than his competitor. There is a strongly significant relationship between larger body size and greater fertilization success in males when they are in competition on a carcass, but no relationship between fertilization success and body size when there are no resources present.

the competitor male (mating context $\times$ absolute size of competitor: $F_{1,60} = 0.262$, $p = 0.611$) had a significant effect on fertilization success. When interacting on a carcass, the focal male gained a higher fertilization when he was larger than his opponent (figure 2). There was no size advantage when mating away from a resource ($F_{1,62} = 0.070$, $p = 0.793$) (figure 2). Paternity when mating off a carcass (0.51 ± 0.04) was not significantly different from that expected under random sperm mixing (t-test against a mean of 0.5, $t_{64} = 0.328$, $p = 0.744$).

4. DISCUSSION

We examined the fertilization success of a focal male burying beetle, *N. vespilloides*, when using a conditional mating tactic, i.e. when competing against the same male in two different biological contexts. When the resource

required for reproduction was absent in a conventional double mating, the focal male sired half of the offspring on average ($P_1 = P_2 = 0.5$) and his fertilization success was unrelated to his relative size. In contrast, when in direct competition on a carcass, the success of the same male was largely determined by his relative size. In this mating context, the larger male in the pair sired majority of offspring. This suggests that when there is no carcass immediately available, competition for fertilization is limited to sperm competition but that other factors predominate when the resource required for reproduction is present. While the most parsimonious explanation is that larger males are better able to monopolize the carcass through pre-copulatory male–male competition and secure repeated copulations with the female (Bartlett 1988; Müller & Eggert 1989; Eggert 1992), other alternative explanations cannot be discounted. For example, a similar pattern could arise if females bias paternity towards relatively larger males on a carcass but not off a carcass or if relatively smaller males sire less progeny on a carcass because they strategically reduce their ejaculate investment by virtue of observing larger males increase their expenditure (Enqvist & Reinhold 2006). Regardless of the precise mechanism, we found little congruence between the fertilization successes of a male when competing in the different mating contexts. Thus, our study clearly demonstrates that male fertilization success in *N. vespilloides* depends on the social context in which mating takes place, with different mechanisms influencing fertilization success.

Considerable research in sperm competition has supported the sperm allocation models of Parker (1990*a*,*b*) that considered the tactics adopted by males when they find themselves competing in either a favoured or disfavoured role. If males are limited to a single role, as is commonly the case when fixed alternative mating tactics exist, it should pay disfavoured males to increase their expenditure on the ejaculate to compensate for their role (Parker 1990*b*). Indeed, a large number of animal species have been shown to adjust their ejaculate expenditure when mating in the disfavoured role (e.g. Stockley *et al.* 1994; Gage *et al.* 1995, 2004; Simmons *et al.* 1999; Neff *et al.* 2003; Pizzari *et al.* 2003; Rudolfsen *et al.* 2006; Simmons & Emlen 2006; but see Simmons *et al.* 2000; Byrne 2004). Why, then, do males not adopt consistent tactics when there is plasticity in their mating behaviour? For example, given the effect of size on male fertilization success in *N. vespilloides*, why do males not use a strategy based on size? Our results confirm that smaller males still achieve some reproductive success when mating on a carcass (Bartlett 1988; Eggert 1992) and sperm competition may always play a role. One possible explanation for lack of an association between size and sperm allocation is that males may not be able to predict the frequency with which they play different roles. As pointed out by Müller & Eggert (1989) and Eggert (1992), despite the potentially higher fitness benefits associated with mating on a carcass, the adoption of this tactic requires that a male must both locate a carcass and be able to monopolize it for breeding by either excluding an already present resident male or preventing a takeover by rival males. Since both of these factors are likely to be highly variable over ecological time, mating roles are more likely to be random so that

large and small males are equally likely to occupy a favoured or disfavoured role when mating. In such instances, it no longer pays the disfavoured male to increase his ejaculate investment (Parker 1990*a*). Rather, both favoured and disfavoured males should invest equally in their ejaculate, despite any knowledge they may have of their current role (Parker 1990*a*).

Male mating behaviour is often opportunistic and plastic (Dominey 1984; Shuster & Wade 2003). Under such conditions, it is essential that P_2 values are obtained for males in all of the roles they might play. If P_2 values for a given male are inconsistent across multiple mating conditions, then we cannot generalize the role of sperm competition in selection and evolution. Selection requires a predictable association between variation in traits and variation in fitness. In addition, even when average P_2 values fit expectations, as Corley *et al.* (2006) and the results we present here show, there can be tremendous variation among individuals in their specific P_2 value. Thus, we need to quantify the nature and source of the variation in P_2 for individuals. Finally, we have to know the frequency with which specific males find themselves in the different mating roles. Then, in general, P_2 studies under such conditions will not be very informative or valuable. Given the ubiquity of plastic mating behaviour and variable social conditions for mating, P_2 studies may have limited applications for most species.

Thus, our study supports the suggestion that researchers should interpret P_2 studies with caution (Simmons & Siva-Jothy 1998; Simmons 2001; Corley *et al.* 2006). Although there are instances where conventional P_2 estimates are remarkably similar to paternity estimates that are derived under different laboratory mating regimes (e.g. Parker 1970*b*; Eady & Tubman 1996) and field conditions (e.g. Cobbs 1977; Turner & Andersson 1984), many exceptions do exist (e.g. Wilkes 1966; LaMunyon & Eisner 1993; LaMunyon 1994; Zeh & Zeh 1994). It is important to recognize that such measures may not always be informative outside the experimental conditions in which they are measured. Although P_2 estimates derived from one experimental setting can provide insight into the mechanisms underlying sperm utilization and the potential adaptations that sperm competition can generate (Simmons 2001), they will provide little insight into the current selection operating on male traits unless male fertilization remains constant across different mating contexts and the variation among males is minimal. Studies examining the degree of congruence between paternity estimates in different biological contexts for the same male provide an important first step in understanding how sperm competition might shape the evolution of male traits.

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