Male phenotype predicts insemination success in guppies

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Theory predicts that mate choice can lead to an increase in female fecundity if the secondary sexual traits used by females to assess male quality covary with the number of sperm transferred during copulation. Where females mate multiply, such a relationship between male attractiveness and ejaculate size may, additionally (or alternatively), serve to augment the effect of indirect selection by biasing paternity in favour of preferred males. In either case, a positive correlation between male attractiveness and the size of ejaculates delivered at copulation is predicted. To date, some of the most convincing (indirect) evidence for this prediction comes from the guppy, a species of fish exhibiting a resource-free mating system in which attractive males tend to have larger sperm reserves. We show that, during solicited copulations, male guppies with preferred phenotypes actually transfer more sperm to females than their less-ornamented counterparts, irrespective of the size of their initial sperm stores. Our results also reveal that, during coercive copulations, the relationship between ejaculate size and the male's phenotype breaks down. This latter result, in conjunction with our finding that mating speed—a factor under the female's control—is a significant predictor of ejaculate size, leads us to speculate that females may exert at least partial control over the number of sperm inseminated during cooperative matings.

Keywords: sexual selection; direct benefits; cryptic female choice; sperm competition; male ornaments

1. INTRODUCTION

Mate choice can benefit females indirectly through the enhanced survival or reproductive success of their offspring (Fisher 1930; Williams 1966), or directly via the provision of resources that increase their own reproductive success (Andersson 1994). In species exhibiting resource-free (i.e. 'sperm-only') mating systems, authors generally favour indirect models of sexual selection to explain the evolution of female choice, even though subtle direct benefits can play an important role in the mating system. For example, Sheldon (1994) argued that the evolution of female choice in resource-free mating systems could just as easily be explained by direct fertility benefits, if the traits used by females to assess male quality covary with the size or quality of the male's ejaculate. This so-called 'phenotype-linked fertility hypothesis' (Sheldon 1994) predicts that males with the most developed sexually selected characters provide larger (or better quality) ejaculates to the female.

In species where females mate with multiple partners during a single reproductive cycle (i.e. polyandrous mating systems), receiving more sperm from preferred males may augment the effectiveness of precopulatory female mate choice, because of the advantages conferred on attractive males during sperm competition (Parker 1998). Consequently, in addition to (or indeed instead of) securing direct fertility benefits through mate choice, females may benefit indirectly by mating with 'sperm-rich' males. Thus, the possibility that females obtain more sperm from preferred males has important, and hitherto unexplored, implications for sperm competition in polyandrous mating systems (Møller 1998).

To date, support for the idea that attractive males deliver more sperm to females has been limited to studies demonstrating that sperm production characteristics (e.g. testis size or the number of sperm per stripped ejaculate) are correlated with particular secondary sexual traits in males (Matthews *et al.* 1997; Engen & Folstad 1999; Merila & Sheldon 1999; Pilastro & Bisazza 1999; Gomendio *et al.* 2000; Pitcher & Evans 2001). However, as noted by the authors of some of these studies, possessing large sperm stores does not necessarily imply that attractive males produce large ejaculates. Indeed, the opposite can be true if, for example, males with preferred phenotypes partition their ejaculates more sparingly among several females (Warner *et al.* 1995), or are more likely to deplete their sperm stores following frequent copulations (Gray 1997; Westneat *et al.* 1998). Thus, to test whether females obtain more sperm from phenotypically attractive males it is necessary to demonstrate that the size of ejaculates delivered at copulation correlates with aspects of the male's phenotype.

Here we use the guppy (*Poecilia reticulata*), a species of live-bearing fish with internal fertilization, to provide an explicit test of the hypothesis that phenotypically attractive males deliver larger ejaculates at copulation. Guppies are small tropical freshwater fish, exhibiting a promiscuous mating system in which female choice plays an important role (Houde 1997). Females typically copulate with two or more males during each monthly receptive phase (Kelly *et al.* 1999; Evans & Magurran 2000), and base their choice of partner on several phenotypic traits including

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coloration, body size and courtship display intensity (Houde 1997). Outside periods of female sexual receptivity, males have the ability to use forced copulations in order to circumvent female choice (Pilastro & Bisazza 1999). Although male phenotypic traits such as ornamentation, body size and courtship vigour appear to be condition-dependent indicators of male genetic quality (Reynolds & Gross 1992; Nicoletto 1995), they have also been shown to predict the size of the male's sperm reserves (Matthews *et al.* 1997; Pitcher & Evans 2001). As a consequence, it has been argued that female guppies have the potential to assess male reproductive condition, and may benefit either directly through increased fecundity, or indirectly through postcopulatory sexual selection, by choosing to mate with phenotypically attractive males (Jennions & Petrie 2000).

We investigated the possibility that female guppies obtain more sperm from males with attractive phenotypes, using recently devised techniques to estimate the size of ejaculates delivered during natural copulations (Pilastro & Bisazza 1999). Our basic protocol was as follows. We examined the relationship between ejaculate size and male phenotype following cooperative, and forced, copulations. In both cases, we determined the number of sperm delivered during a single copulation and related this to (i) mode of copulation (i.e. solicited or forced); and (ii) the expression of male phenotypic traits including body size, body coloration and mating behaviour, all of which are known to influence female mating preferences in guppies (Houde 1997).

2. MATERIAL AND METHODS

(a) *Matings*

The guppies used in this experiment were descendants of fishes collected from a feral population near Manaus (Brazil) and maintained unselected in the laboratory (Pilastro & Bisazza 1999). In our first experiment, we measured the number of sperm delivered during solicited copulations, which were obtained using sexually receptive virgin females (Houde 1997). In each trial, a virgin female was introduced into an experimental tank (150 l tank with water maintained at 25 ± 0.5 °C) containing natural weed and an airstone. After acclimation for 30 min, a test male was placed with the female and behavioural observations commenced as soon as he performed his first mating attempt (see below). In the second experiment, we obtained only forced copulations by individually pairing test males with a non-virgin, mid-cycle (i.e. sexually unreceptive) female (Houde 1997). In both experiments, we observed each pair of fishes until they successfully copulated. Prior to the 'forced copulation' trials, non-virgin females were isolated from males for 10 days to ensure that sperm from previous matings were not present in their gonoducts (Pilastro & Bisazza 1999; Matthews & Magurran 2000). Similarly, in both experiments all test males were isolated from females for 48 h prior to the trials to ensure that they entered the mating trials with replenished sperm reserves (Kuckuck & Greven 1997). The males participating in both experiments were randomly assigned to their treatment group and used only once.

During the behavioural observations in both experiments, we recorded the total number of mating attempts performed by males, and noted the time taken for each male to successfully inseminate the female. Courtship by male guppies is charac-

terized by an elaborate 'sigmoid' display used to attract prospective mates. In contrast, sneaky mating is a cryptic strategy in which males attempt to forcibly inseminate females (Houde 1997). In both cases, copulations were only considered successful if they were followed by a series of post-copulatory jerks by males. Such jerking by male guppies invariably signals successful sperm transfer (Liley 1966). In the trials involving sexually receptive virgin females, we ensured that we considered only copulations solicited by the female ($n = 31$). Solicited copulations were always preceded by male courtship and included the characteristic female 'glide' towards males (Liley 1966). Conversely, in trials involving non-receptive females, we observed only forced copulations without female cooperation or prior male display ($n = 19$). As a measure of male sexual behaviour, we recorded the number of mating attempts (either through courtship or sneaky mating) during the 5 min period prior to copulation.

Following the mating trials, test males were isolated for 3 days in order to fully replenish their sperm stores (see Kuckuck & Greven 1997) before being manually stripped of sperm. This made it possible to estimate sperm store at rest using the methods outlined below (see next section). To evaluate our assertion that the experimental males in both groups had fully replenished their sperm supplies after the 3 day rest period, we compared the size of sperm stores at rest among these males with sperm counts obtained from an additional group of rested males ($n = 22$) that did not participate in the mating trials, but were otherwise treated in exactly the same way as experimental ones.

(b) *Ejaculate collection*

We anaesthetized each female within 10 min of copulation and retrieved sperm from her gonoduct (Pilastro *et al.* 1997; Pilastro & Bisazza 1999). A glass micropipette was used to flush the female's gonoduct five times with 10 μl of physiological solution (0.9% NaCl). The resultant solution was diluted into distilled water, and sperm were coloured with rose bengal dye and subsequently fixed by adding formalin (Pilastro & Bisazza 1999). The solution was then passed through a Millipore filter (pore size 0.22 μm) under vacuum and placed on a glass slide, where it was cleared with immersion oil (Shapiro *et al.* 1994). Sperm counts, which were done 'blind' of the experimental group, were performed under ×400 magnification on an area of the filter measuring 0.122 mm × 0.122 mm (Pilastro & Bisazza 1999). The count was repeated for each of 10 separate portions of the filter and the mean value of these readings was multiplied by the total filter area (1133.5 mm²) to provide us with a measure of the total number of sperm per ejaculate. The above procedures were repeated for both experiments. Three days after the mating trials, sperm were artificially stripped (following Matthews *et al.* 1997) from the experimental and control males to provide a measure of sperm store, at rest, for each male. In each case, the number of sperm per stripped ejaculate was estimated using the methods outlined above.

(c) *Male phenotype*

Following the extraction of sperm, all males were photographed with a digital camera, and an image analysis software package (NIH image) was used to measure standard length (SL) and analyse body coloration. Specifically, we considered three main components (orange, blue and black) of these colour patterns (Endler & Houde 1995), and controlled for variation in body size among males by expressing each colour component as

Table 1. Predictors of ejaculate size following solicited copulations in guppies.
(β, partial regression coefficient.)

variables included in final model[a]	β	s.e.	r	t	p
constant	-3.493	1.163	—	-3.004	0.006
proportion of orange[b]	6.474	1.403	0.613	4.613	<0.001
male SL (mm)	0.132	0.046	0.411	2.869	0.008
proportion of black[b]	4.284	1.514	0.403	2.829	0.009
mating speed (min)[c]	-0.201	0.097	-0.276	-2.066	0.049
variables excluded from final model[a]					
number of courtship displays	-0.148	—	-0.204	-1.044	0.306
sperm store at rest ($\times 10^6$)[c]	0.102	—	0.150	0.761	0.454
number of forced copulation attempts	0.075	—	0.108	0.544	0.591
proportion of blue[b]	0.041	—	0.054	0.270	0.789
female SL (mm)	-0.004	—	-0.006	-0.030	0.976

[a] Stepwise multiple regression, p to enter, 0.05; p to remove, 0.10. Dependent variable: sperm retrieved from the female ($\times 10^6$) following log transformation. None of the independent variables in the forced copulation trials was included in the final model (all p-values greater than 0.10).
[b] Following arcsine transformation.
[c] Following log transformation.

the percentage cover of total body area (Pitcher & Evans 2001). Male phenotypic traits (coloration, body size and mating behaviour) and sperm store at rest were fitted into a stepwise multiple regression model, with ejaculate size as the dependent variable (for further details see table 1). All data were tested for normality and proportional data were arcsine transformed prior to statistical tests. When normality was not attained following transformation, appropriate non-parametric tests were used.

3. RESULTS

(a) *Courtship behaviour*

Ejaculate size ranged from 0 to 6.12×10^6 in courtship copulations (mean $\pm$ s.d. $= (2.14 \pm 1.72) \times 10^6$, and 0 to 1.13×10^6 in forced copulations $(0.21 \pm 0.32) \times 10^6$; $U = 66$, $p < 0.0001$). In the solicited copulation trials, males performed significantly more courtship displays and fewer sneaky attempts (courtship displays per min, 1.31 ± 0.52; sneaky attempts per min, 0.62 ± 0.63) than those in the forced copulation group (courtship, 0.25 ± 0.34; sneaky, 1.50 ± 0.62; $t_{48} = 4.81$, $p < 0.001$, $t_{48} = 7.90$, $p < 0.001$, respectively). However, total sexual activity (courtship displays + sneaky attempts) did not significantly differ between the two groups ($t_{48} = 1.01$, $p = 0.32$).

(b) *Male phenotype and ejaculate size in solicited copulations*

The results from the stepwise regression analysis revealed that in solicited copulations the number of sperm inseminated was significantly and positively related to the relative area of orange and black spots, and to body size, and negatively to mating speed (table 1). Overall, these four predictors accounted for 58% of the variance in ejaculate size ($F_{4,26} = 8.96$, $R^2 = 0.58$, $p < 0.001$); none of the remaining variables (relative area of blue, male mating behaviour and female body size) was included in the final model (table 1). Simple pairwise correlations confirmed that ejaculate size was significantly correlated with relative
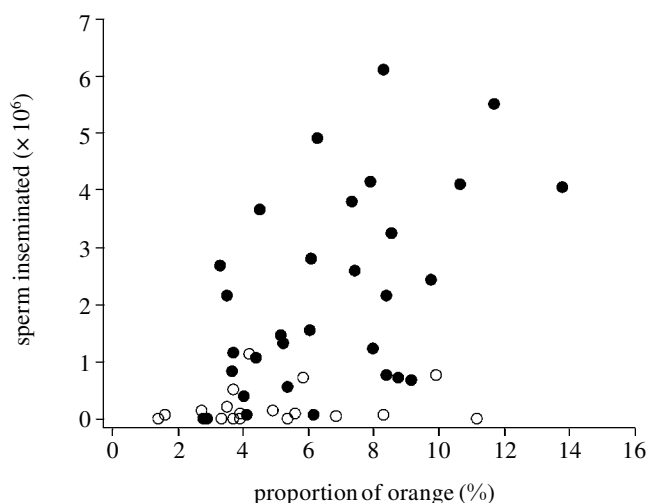

Figure 1. The relationship between ejaculate size and the proportion of orange pigment in males. Correlations are shown following solicited (black circles) and forced (open circles) copulations. Ejaculate size was positively correlated with the extent of orange coloration following solicited copulations only.

orange area (Pearson's correlation coefficient, $r = 0.49$, $n = 31$, $p = 0.036$; figure 1) and mating speed (Pearson's correlation coefficient, $r = -0.38$, $n = 31$, $p = 0.036$; figure 2), but not with male SL ($r = 0.04$, $n = 31$, $p = 0.083$) or black pigmentation ($r = 0.26$, $n = 31$, $p = 0.16$). The relationship between the number of sperm inseminated and relative orange area remained significant, after statistically controlling for differences in sperm store at rest among males (partial correlation in which sperm store at rest was held statistically constant, $r_{\text{partial}} = 0.51$, $p = 0.004$; Sokal & Rohlf 1995). Mating speed remained significantly, and negatively, correlated with ejaculate size after statistically controlling for body size, sperm store at rest and relative orange area ($r_{\text{partial}} = -0.40$, d.f. $= 26$, $p = 0.033$).
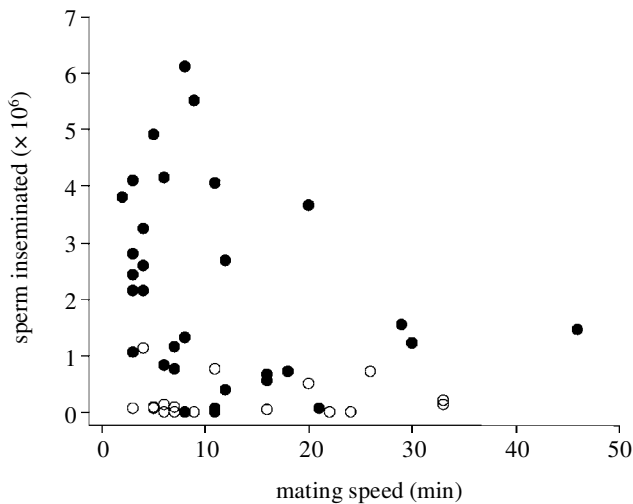
Figure 2. The relationship between ejaculate size and the time elapsed before copulation. Correlations are shown following solicited (black circles) and forced (open circles) copulations. Ejaculate size was negatively associated with mating speed, expressed as the time elapsed from the introduction of the male into the experimental tank and copulation. The association between mating speed and ejaculate size was not evident in the forced copulation group.

(c) *Male phenotype and ejaculate size in forced copulations*

In the second experiment, in which ejaculates were retrieved from females following forced copulations, none of the independent variables was significantly associated with ejaculate size (no variables were selected by the model and all pairwise correlations $p > 0.10$).

(d) *Sperm stores and secondary sexual characteristics*

Our analysis confirmed that sperm store at rest did not significantly differ among the experimental and control groups ($F_{2,69} = 0.982$, $p = 0.38$). In subsequent pairwise correlations we detected no significant association between sperm store at rest and any of the phenotypic traits measured, either when the groups were analysed separately (all p-values greater than 0.10) or when data from the three (solicited, forced and control) groups were pooled ($n = 72$, all p-values greater than 0.10).

4. DISCUSSION

We provide support for the prediction that the expression of preferred traits in males is proportional to the quality or size of ejaculates delivered at copulation (Sheldon 1994). Our results revealed that during solicited copulations, female guppies obtain more sperm from phenotypically attractive males, irrespective of the size of the males' initial sperm stores. Previous studies have shown that the phenotypic traits chosen for our study as predictors of ejaculate size are important mate choice cues in most populations of guppy (reviewed by Houde 1997). In particular, studies have repeatedly shown that the relative area of orange influences female choice in this species (Houde 1997). The accumulated evidence from much of this work has supported the idea that female guppies benefit indirectly by favouring certain male phenotypes, for

example by producing viable offspring or attractive sons (e.g. Reynolds & Gross 1992; Godin & Dugatkin 1996; Brooks 2000; Brooks & Endler 2001). While our results do not contradict the conclusions from these studies, they do uncover the potential for direct (fertility) benefits to simultaneously play a role in resource-free mating systems (Sheldon 1994). An important direction for further work is to determine whether female fecundity in guppies is influenced by ejaculate size.

Although our study uncovered a positive association between ejaculate size and male phenotype (in cooperative matings), sperm store at rest did not correlate with any of the phenotypic traits measured in the study, or significantly predict the size of ejaculates at copulation. These findings contrast with two previous studies reporting positive associations between the number of sperm per stripped ejaculate and both courtship rate (Matthews *et al.* 1997) and certain colour pattern components (Pitcher & Evans 2001). However, Pitcher & Evans (2001) also showed that there were significant interpopulation differences with respect to which components of the male's phenotype were associated with sperm load. Indeed, the lack of correlation between the size of sperm stores and phenotype in the present study is consistent with previous work on this population (Pilastro & Bisazza 1999). Thus, our findings suggest that even when sperm production traits (e.g. testis size or the number of stripped sperm) are not associated with male phenotype (e.g. Birkhead & Fletcher 1995), females may potentially benefit by obtaining more sperm from preferred males.

Female guppies typically mate with more than one male during their brief sexually receptive phase (Houde 1997). Our finding that attractive males deliver more sperm during solicited copulations may have important implications for sexual selection through sperm competition. Large ejaculate size, whether mediated by males or females (see below), may enhance the effectiveness of precopulatory mate choice, and further bias paternity towards preferred males (Møller 1998). Thus, although our results suggest that female mate choice may facilitate increased insemination success by preferred males, the consequences for female reproductive success might be related to the 'quality' of offspring produced, rather than the quantity of offspring as predicted by the phenotype-linked fertility hypothesis (Sheldon 1994). An important advance towards establishing the type of benefit obtained by females is (i) to determine whether female fecundity (brood size) is a function of the number of sperm transferred at copulation; and (ii) to examine the relative paternity share of preferred and non-preferred males following multiple mating.

An intriguing question is why colourful males should inseminate more sperm than their less ornamented counterparts. One possibility is that certain males are intrinsically better 'inseminators' than others, for instance, because they are more agile or vigorous during copulation. Although this idea is consistent with previous work demonstrating a relationship between male swimming ability and body coloration (Nicoletto 1991), it does not explain why colourful males were no more efficient than drab ones during sneaky copulations. An alternative possibility is that females may themselves exert control over the number of sperm inseminated during (solicited) copulations,

for example by manipulating the duration of copulation, penetration depth, or by selectively ejecting part of the ejaculate (Pizzari & Birkhead 2000; Elgar *et al.* 2000). Our study provides indirect support for this latter possibility. We found that following solicited copulations, ejaculate size was negatively associated with mating speed—a factor that was clearly under the control of the female—and remained so after statistically controlling for body size and body coloration. Importantly, the association between mating speed and ejaculate size was not evident in the forced copulation group in which precopulatory female control over copulation was undermined. The possibility that females exert control over ejaculate transfer during cooperative copulations is also consistent with the results from a previous study of sperm precedence patterns in guppies (Evans & Magurran 2001). In that study, mating speed was a significant predictor of paternity, with shorter insemination intervals between two successive males resulting in second-male sperm precedence. Our present finding that shorter mating times resulted in the insemination of more sperm provides a plausible mechanism to account for the patterns of paternity documented in that study (Evans & Magurran 2001). It has already been postulated that females may exert control over the timing of fertilization in guppies (Evans & Magurran 2000). Clearly, control over ejaculate transfer is a further mechanism by which they may influence the outcome of post-copulatory sexual selection. Such 'cryptic' forms of female choice (Eberhard 1996) remain intriguing possibilities that await further investigation.

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