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Patterns of sperm precedence and predictors of paternity in the Trinidadian guppy

Jonathan P. Evans* **and Anne E. Magurran**

Environmental and Evolutionary Biology, University of St Andrews, St Andrews, Fife KY16 9TS, UK

Despite its widespread occurrence in animals, sperm competition has been studied in a limited range of taxa. Among the most neglected groups in this respect are internally fertilizing fish in which virtually nothing is known about the dynamics of sperm competition. In this study, we examined the outcome of sperm competition when virgin female guppies mated with two males. Behavioural cues were used to ensure that each male mated once (with female cooperation) and that sperm were successfully inseminated at copulation. Two polymorphic microsatellite loci were used to estimate the proportion of offspring sired by the second male (P_2) and the results revealed a bimodal distribution with either first or (more often) second male priority. The observed P_2 distribution differed from that expected under the 'fair raffle' model of sperm competition. Random sperm mixing is therefore unlikely to account for the observed variance in P_2 in this study. A further aim of our study was to identify predictors of male reproductive success. Using logistic linear modelling, we found that the best predictors of paternity were time to remating and the difference in courtship display rate between first and second males. Males that mated quickly and performed relatively high numbers of sigmoid displays obtained greater parentage than their slower and less vigorous counterparts. Since females are attracted to high-displaying males, our results suggest that female choice may facilitate sperm competition and/or sperm choice in guppies.

Keywords: *Poecilia reticulata*; poeciliid; guppies; sperm competition; P_2

1. INTRODUCTION

It is now recognized that sperm competition (Parker 1970) is ubiquitous in the animal kingdom, occurring in taxa as diverse as birds, insects, amphibians, mammals and fish (Smith 1984; Birkhead & Møller 1998). Indeed, with the aid of paternity markers even species previously thought to be strictly monogamous have been shown to mate promiscuously (e.g. Birkhead & Møller 1992). Theory predicts that a male's fertilization success will increase proportionately with the number of his sperm relative to that of other males (Parker 1990; Parker *et al*. 1990). In support of this prediction, comparative studies across a wide range of taxa have shown that male ejaculate expenditure is higher in species experiencing high levels of sperm competition (Harcourt *et al*. 1981; Stockley *et al*. 1997). A number of studies have also shown that individual males have the ability to adjust their ejaculate size according to the magnitude of the sperm competition they face (Gage 1991, 1995; Fuller 1998).

One of the primary objectives of researchers working on sperm competition (in internally fertilizing species) is establishing patterns of paternity when two males mate consecutively with a female (e.g. Birkhead & Møller 1998). Usually, paternity patterns are described in terms of second (or last) male sperm precedence (P_2), that is the proportion of offspring sired by the second male to mate (Boorman & Parker 1976). The majority of studies on sperm precedence have been confined to insects (Gwynne 1984; Simmons & Siva-Jothy 1998) and birds (Birkhead & Møller 1992) and very little is known about the patterns of paternity in other groups of internal fertilizers. This is particularly true of fish in which the outcome of sperm competition in internal fertilizers has never been studied in detail (Petersen & Warner 1998). In the poeciliids, for example, sperm competition is known to be intense (Hildemann & Wagner 1954; Constantz 1984; Zane *et al*. 1999) but is nonetheless poorly understood. Among these, the guppy has been the subject of intense study, particularly relating to the evolution of secondary sexual characters through female choice (Houde 1997). Guppies have a promiscuous mating system in which female choice plays an important role (Kodric-Brown 1985; Houde 1987). As virgins and during their brief receptive phase (which is approximately monthly), female guppies solicit copulations from several males (Houde 1997; Evans & Magurran 2000) and subsequently produce mixed-paternity broods (Hildemann & Wagner 1954; Kelly *et al*. 1999). Female guppies store sperm for several months within the folds of their ovaries (Constantz 1984) where they are nourished by extracellular ovarian sugars (Gardiner 1978).

In order to attract females, male guppies use a highly conspicuous courtship posture that is known as a sigmoid display (Baerends *et al*. 1955; Liley 1966). This display serves to show off the male's coloration and may signal viability (e.g. Nicoletto 1993) or fertility (Matthews *et al*. 1997). In addition, males employ the sneaky mating tactic, which is termed gonopodial thrust, in order to circumvent female choice. Gonopodial thrusts are forced copulations that are not preceded by a courtship display and in which the male attempts to thrust his gonopodium (the intromittent organ) into the female's genital pore (Baerends *et al*. 1955; Liley 1966). While both strategies are used interchangeably (and continually) by individual males, the degree to which either tactic is employed can depend on several factors including social environment (Rodd & Sokolowski 1995; Evans & Magurran 1999), predation pressure (Endler 1987; Magurran & Seghers 1990; Dill *et al*. 1999) and female receptivity (Liley 1966).

*Author for correspondence (jpe1@st-and.ac.uk).

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Some of the earliest work on sperm competition was performed using poeciliids as a model system. Indeed, long before the term sperm competition was coined, Winge (1937) noted that multiply mated female guppies gave birth to successive broods over several months. Later work examined the pattern of sperm precedence when post-partum females subsequently mated with a second male. This work uncovered a strong last male advantage and suggested that fresh sperm were at a competitive advantage over previously stored sperm (Hildemann & Wagner 1954; Matthews 1998). However, no study has examined the outcome of sperm competition when two ejaculates are inseminated within the same brood cycle. Furthermore, although the number of stripped sperm correlates with several male traits including courtship intensity (Matthews *et al*. 1997) and body size (Pilastro & Bisazza 1999), it is not known whether these predict male reproductive success accurately. The aims of the present study were therefore to examine paternity patterns when receptive virgin females were sequentially paired with two randomly chosen males and to relate these patterns to several male traits with the aim of uncovering possible predictors of male reproductive success.

2. METHODS

(a) *The study population and its maintenance*

The guppies used in this experiment were descendants of wild-caught fish from the lower Tacarigua River in Trinidad, a high-predation site in which guppies coexist with the pike cichlid *Crenicichla alta* (Magurran & Seghers 1994). In common with male guppies inhabiting other high-predation rivers, those in the lower Tacarigua River are characterized by low levels of carotenoid coloration (see Endler 1980). The high fecundity of fish from such high-predation populations (e.g. Reznick & Endler 1982) makes them well suited for parentage studies such as the one described here.

Virgin females were reared for this experiment in single-sex groups as soon as they became morphologically distinguishable from males (approximately five to six weeks) (see Houde 1997). The male guppies used in this study were maintained in mixed-sex aquaria (*ca*. 1:1 sex ratio) until used in the experiment. On the morning of the mating trials all fish were fed to satiation with commercially prepared flake food (TetraMin).

(b) *Mating trials*

Mating trials took place between 09.00 and 11.00 h in an aquarium (60 cm × 30 cm × 38 cm) containing gravel, a small clump of Java moss (*Vesicularia dubyana*) and an air filter. The tank temperature was maintained at 25 ± 0.5 °C and light was provided by an 18 W fluorescent bulb on a 12 L:12 D cycle. Virgin female guppies aged six months and approximately matched for size were used for the mating trials. In each trial, a female was sequentially paired with two randomly chosen males taken from stock aquaria. Copulation success was judged according to Liley's (1966) detailed description of guppy mating behaviour. Copulations were considered successful only if they were followed by post-copulatory jerks by the male. Such jerking movements by male guppies are highly conspicuous and invariably signal sperm transfer (Liley 1966). Post-copulatory jerks usually last several minutes and are followed by a refractory period that can last up to 1 h (Houde 1997). Copulations not followed by post-copulatory jerks do not result in successful sperm transfer (Liley 1966) and males immediately resume courtship after such attempts (authors' personal observation).

The protocol for the mating trials was simple: each female was allowed to settle overnight in the mating arena and on the following morning a male (male 1) was carefully placed into the tank. As soon as the pair successfully copulated, male 1 was removed and placed in a small holding tank (30 cm × 20 cm × 22 cm) for five days in order to replenish his sperm reserves prior to measuring his behaviour and sperm number (see below). At this point, the time taken for male 1 to copulate successfully with the female was noted. Exactly 1 h after male 1 had copulated, a second male (male 2) was placed in the tank and was observed until he successfully inseminated the female. The time taken for the second male to mate was recorded. After copulation, male 2 was removed and placed with male 1 in the holding tank for five days. Each male's colour pattern was then sketched to aid subsequent recognition of the fish. If male 2 did not copulate successfully with the female within 30 min he was replaced by a third male. If this third male was successful, the time taken for him to mate (from introduction to copulation) was recorded. In practice, only three of the successful mating trials (where females were double mated) involved a third male. If the third male was unsuccessful, the trial was terminated and the female and (three) males were returned to a stock tank. Females and males from failed trials played no further part in subsequent mating trials. The 1 h gap between matings was necessary in order to allow the female to settle and resume normal swimming and foraging activities. In all cases matings were initiated by the female and did not result from forced copulations by males.

A total of 55 mating trials were performed yielding 30 successful double matings. The female was only inseminated once by each male in each of these successful trials and the interval between matings never exceeded 2 h. Following double matings, females were isolated in plastic bottles (4 l volume), each containing a small clump of Java moss and an airstone, until the production of their first brood. Twenty-one of the 30 double-mated females gave birth to broods, of which 19 comprised three or more offspring (mean brood size = 7.9 ± 0.72 s.e.). Only broods containing three or more offspring were used for the paternity analysis. In practice, only one brood comprised three individuals; all other broods contained at least four offspring.

(c) *Estimating P_2*

Second-male sperm precedence (P_2) was estimated using two polymorphic microsatellite DNA markers specifically designed for poeciliids. The first, which was isolated and characterized by J. S. Taylor and F. Breden (unpublished data) (GenBank accession number AF164205), had a core $(TAA)_n$ repeat with primer sequences 5′-GTG ACC GAA CGA AAG GAT A-3′ and 5′-CCC CAA AGG AAC ACT GTA-3′. The second locus (Pooc-G49) was isolated by Parker *et al.* (1998) (accession number AF026459) and had a $(GT)_6$, GC, $(GT)_4$, GC, $(GT)_7$ repeat with primer sequences 5′-CAT AGA TTC TGC AGG CAG TG-3′ and 5′-CTC AGT GAC TAT AAG GCC AAC-3′.

After giving birth, females were anaesthetized in a water bath containing 0.4 g l^{-1} benzocaine (ethyl *p*-amino benzoate). When fully subdued, each female's standard length was recorded and a small clipping (*ca*. 25 µg) was taken from her caudal fin. Fin-clipped females were revived in conditioned fresh water (Stress Coat®, Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA) and placed in stock aquaria. Juveniles

were too small to survive the fin-clipping procedure and were therefore humanely killed in a water bath containing a lethal dose of benzocaine. Caudal fin tissue was taken from the two putative sires after observing their behaviour and stripping their sperm (see §§ (d) and (e) below).

Genomic DNA was extracted from the tissue samples taken from each family (two putative sires, mother and offspring) using a rapid, one-tube extraction method (Estoup *et al*. 1996). Polymerase chain reaction (PCR) amplifications were performed on a PTC-100 thermal cycler (MJ Research, Inc., Incline Village, NV, USA). Reaction volumes of 10 µl for the first $(TAA)_n$ locus consisted of 1.6 µl of DNA template, 0.07 µl of each dNTP (each dNTP = 10 mM), 2.0 mM $MgCl_2$, 5 pmol of each primer pair and 0.4 units of Taq polymerase (Promega, Southampton, UK). Thermal cycles consisted of 1 min at 95 °C followed by 27 cycles at 52 °C for 30 s, 72 °C for 30 s and 95 °C for 10 s. A terminal extension step of 5 min at 72 °C completed the programme. The PCR conditions for Pooc-G49 were identical except for an annealing temperature of 63.2 °C and an $MgCl_2$ concentration of 1.5 mM. PCR products were resolved on 6% polyacrylamide gels (Sambrook *et al*. 1989) and paternity was assigned according to allele sharing between putative sires, mother and offspring.

A total of 146 offspring were genotyped, of which 134 (91.8%) could be unambiguously assigned to one of the putative sires. The results of the paternity analysis were 'blind' checked by an impartial observer not involved in the study but experienced in gel-scoring techniques. Offspring that could not be unambiguously assigned to one of the two males ($n = 12$) using both microsatellite loci were not included in the subsequent paternity analysis. Four of the 12 offspring for which paternity could not be assigned constituted an entire brood and therefore a total of 18 families were used in the final analysis.

(d) *Male mating behaviour*

Five days after the mating trials, pairs of males from each trial were moved into an observation tank (45 cm × 30 cm × 30 cm) containing two unfamiliar, non-virgin females already acclimatized to the tank (*ca*. 24 h). The observation tank contained gravel, moss and an air filter. Male courtship behaviour (sigmoid display and gonopodial thrust rate) towards the two females was recorded over a 10 min period as soon as the males settled into the tank and commenced courtship. Sigmoid displays were recorded when the focal male moved in front (or to one side) of the female, arched his body in a pronounced S-shape and quivered (Baerends *et al*. 1955; Liley 1966). Gonopodial thrusts were recorded when the focal male attempted to make (or succeeded in making) physical contact with a female's genital region without her cooperation.

(e) *Sperm counts*

Following behavioural observations, male pairs were humanely killed in a water bath containing a lethal dose of benzocaine. After removing excess water from each male, standard length (±0.1 mm) was determined and sperm were extracted. In order to strip sperm, each male was placed on an inverted Petri dish under low-power magnification. The gonopodium was swung forward and gentle pressure was applied to the abdomen just anterior to the base of the gonopodium (where the testes are located). This procedure released sperm in the form of spermatozeugmata (sperm bundles) and was repeated in order to ensure that all sperm bundles were extracted. Following removal, sperm bundles were drawn up a Gilson™ pipette and added to 100 µl of 0.9% saline solution. The samples were repeatedly drawn up and expelled from the pipette in order to break down the sperm bundles and distribute sperm cells evenly. Sperm count was estimated by counting sperm cells on an 'improved Neubauer chamber' haemocytometer under ×400 magnification. The distribution of sperm cells across the haemocytometer was checked visually for evenness before counts commenced. The number of sperm was determined by multiplying the mean sperm count (from five counts) by the sample's dilution factor and initial volume. Sperm counts were expressed as the total number of spermatozoa per stripped ejaculate.

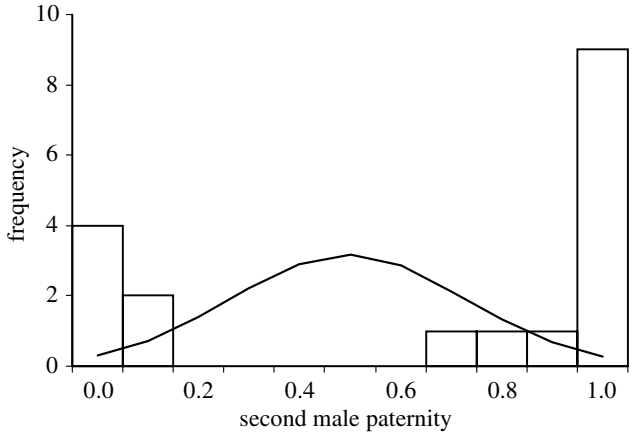

Figure 1. Expected P_2 distribution (line) if sperm mix randomly compared with observed data (bars). The observed distribution is bimodal and shows either first or last male sperm precedence. For further details see the text.

(f) *Analysis*

Second male paternity (P_2) was estimated by determining the proportion of offspring within each brood sired by the second male to mate. Observed values of P_2 were compared with theoretical expected values of sperm precedence based on a model assuming random sperm mixing (Parker *et al*. 1990). Assuming a 'fair raffle' in which individual sperm from both males have an equal chance of fertilizing ova and are not used preferentially by the female, the probability of second male paternity will be $P_2 = S_2/(S_1 + S_2)$, where S_1 and S_2 represent the number of sperm ejaculated by the first and second males, respectively. Recent work has suggested that the number of stripped sperm is a good predictor of ejaculate size in guppies (Pilastro & Bisazza 1999) and, therefore, values of S_1 and S_2 were estimated by comparing the mean (stripped) sperm counts from first and second males. This generated a range of expected P_2-values for each of the 18 families tested. The mean and standard deviation of these data were used to generate a normal curve (see line in figure 1) that was compared with the observed P_2 distribution (bars in figure 1) using a Kolmogorov–Smirnov goodness-of-fit test (see Cook *et al*. 1997).

Generalized linear regression was used to determine whether the independent variables measured for each male (sperm number, sigmoid rate, thrust rate, body size and time to remating) significantly affected P_2. Because P_2 data are proportional, they were analysed using logistic modelling with a logit link function (Genstat 5 Committee 1993). Four of the independent variables represented differences in the explanatory variables between first and second males (i.e. male 2 trait minus male 1 trait). The fifth independent variable to be fitted into the model was time to remating (which is the time from

Table 1. *Results from the generalized logistic linear modelling of P_2 against five independent variables*

(The significance of terms is inferred from changes in the deviance of the model due to each variable, which is distributed as χ^2. Non-significant (n.s.) probabilities occur where $p > 0.05$.)

source of deviance	d.f.	deviance (% of total)	probability
sperm number	1	0.126 (0.04%)	n.s.
thrusts	1	0.791 (0.05%)	n.s.
sigmoid rate	1	21.442 (14.68%)	< 0.001
body size	1	1.032 (0.7%)	n.s.
time to remating	1	70.595 (48.33%)	< 0.001
residual	10	52.059 (36.2%)	—

introduction to copulation for second—or third—males). Male 2 success and failure (success was the number of offspring sired by male 2 within each brood and failure the number sired by male 1) were entered as the binary response variable.

3. RESULTS

(a) *Pattern of P_2*

The proportion of offspring sired by the second male ranged from 0 to 100% (mean ± s.d. = 0.64 ± 0.45) and clearly showed a bimodal distribution (figure 1). The number of stripped sperm did not differ significantly among first and second males ($t_{34} = 0.53$ and $p = 0.60$) and these data provided the basis for the expected P_2 distribution under the fair raffle model of sperm competition. The expected and observed distribution of P_2 differed significantly (Kolmogorov–Smirnov goodness-of-fit test, $D_{0.5} = 0.56$, $n = 18$ and $p < 0.01$) (figure 1), suggesting non-random mixing of sperm. There was either first or (more often) last male priority and in no instance was paternity shared equally by both males.

(b) *Predictors of fertilization success*

Table 1 shows the results from the logistic linear regression and reveals that time to remating and relative differences in sigmoid rate among competing males accounted for more than 63% of the deviance in the model. Relative male size showed a sequence effect when entered into the model and was non-significant if relative difference in sigmoid rate among male pairs was entered first (table 1). This arises because the sigmoid rate and body size were correlated in this data set; the time to remating and relative differences in the sigmoid rate were always significant irrespective of input order. Figure 2 shows the fitted curves for the sigmoid rate and remating interval and reveals that males who performed relatively more sigmoids obtained greater parentage (figure 2*a*) with P_2 declining rapidly when the time to remating exceeded 15 min (figure 2*b*).

Because the distribution of P_2 was bimodal in this data set, individual males could be classified as successful (sired more than 50% of the brood) or unsuccessful (sired less than 50% of the brood) (e.g. Wedell & Cook 1998). On average, successful males mated 7.1 (±1.4 s.e.) min after being introduced to the female. However, unsuccessful males took an average of 14.6 (±3.5) min to copulate (two-tailed *t*-test comparing mean time to mating for successful and unsuccessful males, $t_{32} = 1.98$ and $p = 0.062$). Interestingly, successful males had similar sperm counts (stripped ejaculate size = 3.65×10^6 ± 0.45 s.e.) to unsuccessful males ($3.36 \times 10^6 \pm 0.58$) ($t_{34} = 0.4$ and $p = 0.69$). Furthermore, successful males were no larger than their unsuccessful counterparts ($t_{34} = 0.36$ and $p = 0.72$), nor did they have higher rates of gonopodial thrusting ($t_{32} = 0.28$ and $p = 0.78$).

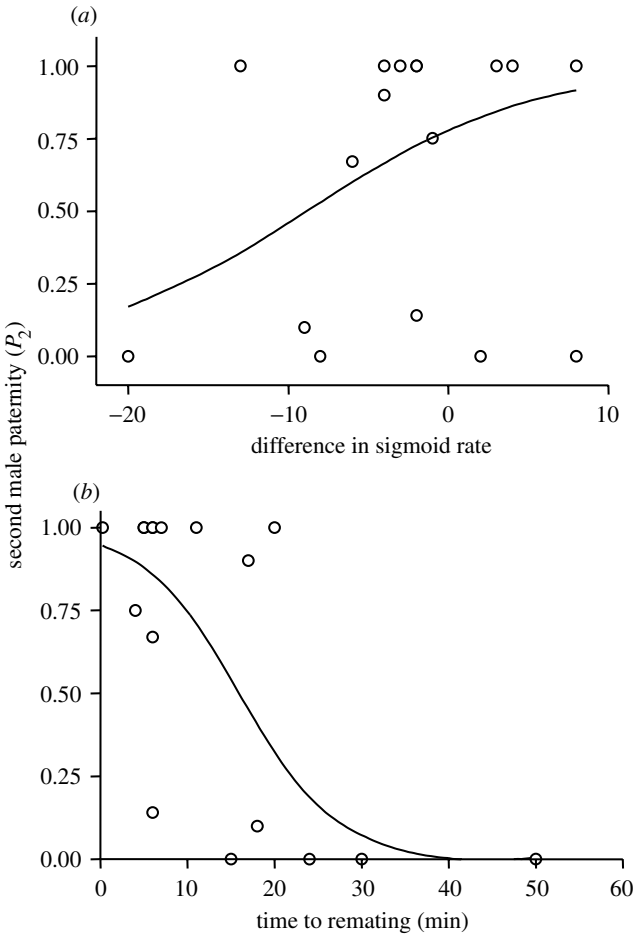

Figure 2. Proportion of offspring sired by the second male (P_2) as a function of (*a*) differences in the display rate among first and second males and (*b*) time to remating. Curves are the fitted lines from the generalized linear model.

4. DISCUSSION

In summary, this study of sperm precedence in guppies revealed a bimodal distribution of paternity that was slightly skewed towards second males. Our subsequent analysis showed that the outcome of sperm competition did not occur by random sperm mixing following a fair raffle (Parker *et al.* 1990). Interestingly, sperm number was not a good predictor of fertilization success. Instead, the logistic regression analysis revealed two predictors that together explained a significant proportion of the variance in P_2. The speed with which females mated with second males significantly affected the outcome of paternity, with P_2 declining as the time to remating increased. We also found that males with relatively high sigmoid display rates sired proportionately more offspring than less vigorous males.

The skew towards second males in the distribution of paternity may have been a consequence of the experimental design in which females were able to choose whether or not to copulate with either of the two males. In the case of first males, all females invariably copulated with them in a matter of minutes. Indeed, virgin females often mate indiscriminately when first encountering a male (Houde 1997). However, females were more reluctant to mate with subsequent males (as aptly demonstrated by our difficulty in achieving a high number of successful double matings) and the skewed pattern of paternity towards second males may have been a consequence of females choosing to remate with superior males. This form of 'trading up' (e.g. Møller 1992) is common in birds and may account for the widespread occurrence of last male sperm precedence reported in several avian studies (Birkhead & Møller 1992). It would be interesting to examine the outcome of paternity in cases where females are denied the choice of partner (e.g. using artificial insemination—see below) in order to determine whether a skewed pattern still persists in absence of female choice.

Our results revealed a negative association between time to remating and P_2. Possible explanations for negative relationships between remating interval and P_2 have been discussed for insects, where similar results have been obtained (see the review by Simmons & Siva-Jothy 1998). For example, Retnakaran (1974; cited in Simmons & Siva-Jothy 1998) and Allen *et al.* (1994) suggested that, when a female's sperm storage organ(s) are filled by the first male's sperm, P_2 would be zero because there would be no room for sperm from subsequent males. However, shortening the remating interval beyond a critical point could result in a reversal of this pattern (i.e. high P_2) if the first male's sperm had not reached the storage organ (or site of fertilization) and the ejaculate from the second male was better positioned to do so first. Clearly this is one of several possible mechanisms that might account for the observed pattern of P_2 in our study. A first and crucial step towards elucidating such mechanisms is to understand the complex processes of sperm transfer, storage and fertilization. These are clearly challenges for further study.

Although the mechanisms underlying the distribution of paternity in this study remain to be investigated, the fact that time to remating (our strongest predictor) was under female and not male control highlights the role that females play in influencing the paternity of their broods. Male courtship intensity and the time to remating may be linked because males with high display rates are likely to attract females more quickly (Nicoletto 1993). We know from an earlier study that sigmoid display rate correlates positively with sperm production in male guppies from the lower Tacarigua River population (Matthews *et al.* 1997). In that study, Matthews *et al.* (1997) proposed that females use pre-copulatory mate choice cues in order to assess male fertility and argued that males with high sigmoid rates would be successful during sperm competition. The results presented in the current study support this prediction but suggest that sperm number is not necessarily the most important component of sperm competition success in guppies. We found that the number of stripped sperm did not differ between successful and unsuccessful males and that the observed pattern of sperm precedence was not consistent with random sperm mixing. However, our estimate of ejaculate size was based on the use of stripped ejaculates and not natural ejaculates delivered at copulation. Thus, we cannot rule out the possibility that males were exerting control over their ejaculate size during the mating trials, for example in response to olfactory cues from the female. While recent work has suggested that the size of stripped ejaculates reliably predicts natural ejaculate size in guppies (Pilastro & Bisazza 1999), future work should determine whether males can facultatively adjust their ejaculate size and to what extent.

It has been argued that strong bimodal distributions of P_2, such as the one presented in this study, are suggestive of female choice of ejaculates (e.g. Cook *et al.* 1997). Given the current, intense interest in post-copulatory female choice (Eberhard 1996, 2000; Birkhead 1998, 2000; Pitnick & Brown 2000), guppies are an ideal species in which to investigate this topic further. For example, artificial insemination would be a useful approach in determining whether 'high-quality' males achieve disproportionately high reproductive success when the insemination event is uncoupled from pre-copulatory mate choice cues (e.g. body ornamentation and courtship display). Such an experiment would enable researchers to assess the relative importance of male and female roles in sperm competition whilst controlling for possible confounding effects such as mating order, sperm number and mating interval (see Birkhead 2000). Artificial insemination would also control for the potentially confounding effect of poor (or 'sloppy') sperm mixing, which may also affect subsequent distributions of paternity (Harvey & Parker 2000). We strongly advocate the development and implementation of such an approach in future studies of sperm choice and sperm competition in guppies.

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REFERENCES

Allen, G. R., Kazmer, D. J. & Luck, R. F. 1994 Post-copulatory male behaviour, sperm precedence and multiple mating in a solitary parasitoid wasp. *Anim. Behav.* **48**, 635–644.

Baerends, G. P., Brouwer, R. & Waterbolk, H. T. 1955 Ethological studies on *Lebistes reticulatus* (Peters), I. An analysis of the male courtship pattern. *Behaviour* **8**, 249–334.

Birkhead, T. R. 1998 Cryptic female choice: criteria for establishing female sperm choice. *Evolution* **52**, 1212–1218.

Birkhead, T. R. 2000 Defining and demonstrating postcopulatory female choice—again. *Evolution* **54**, 1057–1060.

Birkhead, T. R. & Møller, A. P. 1992 *Sperm competition in birds*. London: Academic Press.

Birkhead, T. R. & Møller, A. P. (eds) 1998 *Sperm competition and sexual selection*. San Diego, CA: Academic Press.

Boorman, E. & Parker, G. A. 1976 Sperm (ejaculate) competition in *Drosophila melanogaster* and the reproductive value of females to males in relation to female age and mating status. *Ecol. Entomol.* **1**, 145–155.

Constantz, G. D. 1984 Sperm competition in poeciliid fishes. In *Sperm competition and the evolution of animal mating systems* (ed. R. L. Smith), pp. 465–485. Orlando, FL: Academic Press.

Cook, P. A., Harvey, I. F. & Parker, G. A. 1997 Predicting variation in sperm precedence. *Phil. Trans. R. Soc. Lond.* B **352**, 771–780.

Dill, L. M., Hedrick, A. V. & Fraser, A. 1999 Male mating strategies under predation risk: do females call the shots? *Behav. Ecol.* **10**, 452–461.

Eberhard, W. G. 1996 *Female control: sexual selection by cryptic female choice.* Princeton University Press.

Eberhard, W. G. 2000 Criteria for demonstrating postcopulatory female choice. *Evolution* **54**, 1047–1050.

Endler, J. A. 1980 Natural selection on color patterns in *Poecilia reticulata*. *Evolution* **34**, 76–91.

Endler, J. A. 1987 Predation, light intensity and courtship behaviour in *Poecilia reticulata* (Pisces: Poeciliidae). *Anim. Behav.* **35**, 1376–1385.

Estoup, A., Largiader, C. R., Perrot, E. & Chourrout, D. 1996 Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Mol. Mar. Biol. Biotechnol.* **5**, 295–298.

Evans, J. P. & Magurran, A. E. 1999 Male mating behaviour and sperm production characteristics under varying sperm competition risk in guppies. *Anim. Behav.* **58**, 1001–1006.

Evans, J. P. & Magurran, A. E. 2000 Multiple benefits of multiple mating in guppies. *Proc. Natl Acad. Sci. USA* **97**, 10 074–10 076.

Fuller, R. C. 1998 Sperm competition affects male behaviour and sperm output in the rainbow darter. *Proc. R. Soc. Lond.* B **265**, 2365–2371.

Gage, M. J. G. 1991 Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. *Anim. Behav.* **42**, 1036–1037.

Gage, M. J. G. 1995 Continuous variation in reproductive strategy as an adaptive response to population density in the moth *Plodia interpunctella*. *Proc. R. Soc. Lond.* B **261**, 25–30.

Gardiner, D. M. 1978 Utilization of extracellular glucose by spermatozoa of two viviparous fishes. *Comp. Biochem. Physiol.* A **59**, 165–168.

Genstat 5 Committee 1993 *Genstat 5 release 3 reference manual.* Oxford University Press.

Gwynne, D. T. 1984 Male mating effort, confidence of paternity, and insect sperm competition. In *Sperm competition and evolution of animal mating systems* (ed. R. L. Smith), pp. 117–149. Orlando, FL: Academic Press.

Harcourt, A. H., Harvey, P. H., Larson, S. G. & Short, R. V. 1981 Testis weight, body weight and breeding system in primates. *Nature* **293**, 55–57.

Harvey, I. F. & Parker, G. A. 2000 'Sloppy' sperm mixing and intraspecific variation in sperm precedence (P_2) patterns. *Proc. R. Soc. Lond.* B **267**, 2537–2542.

Hildemann, W. H. & Wagner, E. D. 1954 Intraspecific sperm competition in *Lebistes reticulatus*. *Am. Nat.* **88**, 87–91.

Houde, A. E. 1987 Mate choice based on naturally occurring color-pattern variation in a guppy population. *Evolution* **41**, 1–10.

Houde, A. E. 1997 *Sex, color, and mate choice in guppies.* Princeton University Press.

Kelly, C. D., Godin, J.-G. J. & Wright, J. 1999 Geographic variation in multiple paternity within natural populations of the guppy (*Poecilia reticulata*). *Proc. R. Soc. Lond.* B **266**, 2403–2408.

Kodric-Brown, A. 1985 Female preference and sexual selection for male coloration in the guppy. *Behav. Ecol. Sociobiol.* **17**, 199–205.

Liley, N. R. 1966 Ethological isolating mechanisms in four sympatric species of poeciliid fishes. *Behaviour* **13** (Suppl. 13), 1–197.

Magurran, A. E. & Seghers, B. H. 1990 Risk sensitive courtship in the guppy (*Poecilia reticulata*). *Behaviour* **112**, 194–201.

Magurran, A. E. & Seghers, B. H. 1994 Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proc. R. Soc. Lond.* B **255**, 31–36.

Matthews, I. M. 1998 Mating behaviour and reproductive biology of the guppy, *Poecilia reticulata.* PhD thesis, University of St Andrews, UK.

Matthews, I. M., Evans, J. P. & Magurran, A. E. 1997 Male display rate reveals ejaculate characteristics in the Trinidadian guppy *Poecilia reticulata*. *Proc. R. Soc. Lond.* B **264**, 695–700.

Møller, A. P. 1992 Frequency of female copulation with multiple mates and sexual selection. *Am. Nat.* **139**, 1089–1101.

Nicoletto, P. F. 1993 Female sexual response to condition-dependent ornaments in the guppy, *Poecilia reticulata*. *Anim. Behav.* **46**, 441–450.

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

Parker, G. A. 1990 Sperm competition games: raffles and roles. *Proc. R. Soc. Lond.* B **242**, 120–126.

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

Parker, K. M., Hughes, K., Kim, T. J. & Hedrick, P. W. 1998 Isolation and characterization of microsatellite loci from the Gila topminnow (*Poeciliopsis o. occidentalis*) and their utility in guppies (*Poecilia reticulata*). *Mol. Ecol.* **7**, 357–363.

Petersen, C. W. & Warner, R. R. 1998 Sperm competition in fishes. In *Sperm competition and sexual selection* (ed. T. R. Birkhead & A. P. Møller), pp. 435–463. San Diego, CA: Academic Press.

Pilastro, A. & Bisazza, A. 1999 Insemination efficiency of two alternative male mating tactics in the guppy (*Poecilia reticulata*). *Proc. R. Soc. Lond.* B **266**, 1887–1891.

Pitnick, S. & Brown, W. D. 2000 Criteria for demonstrating female sperm choice. *Evolution* **54**, 1052–1056.

Retnakaran, A. 1974 The mechanism of sperm precedence in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae) *Can. Entomol.* **106**, 1189–1194.

Reznick, D. N. & Endler, J. A. 1982 The impact of predation on life history evolution in Trinidadian guppies (*Poccilia reticulata*). *Evolution* **36**, 160–177.

Rodd, F. H. & Sokolowski, M. B. 1995 Complex origins of variation in the sexual behaviour of male Trinidadian guppies, *Poecilia reticulata*: interactions between social environment, heredity, body size and age. *Anim. Behav.* **49**, 1139–1159.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular cloning. A laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Simmons, L. W. & Siva-Jothy, M. T. 1998 Sperm competition in insects: mechanisms and the potential for selection. In *Sperm competition and sexual selection* (ed. T. R. Birkhead & A. P. Møller), pp. 341–434. San Diego, CA: Academic Press.

Smith, R. L. (ed.) 1984 *Sperm competition and the evolution of animal mating systems.* Orlando, FL: Academic Press.

Stockley, P., Gage, M. J. G., Parker, G. A. & Møller, A. P. 1997 Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. *Am. Nat.* **149**, 933–954.

Wedell, N. & Cook, P. A. 1998 Determinants of paternity in a butterfly. *Proc. R. Soc. Lond.* B **265**, 625–630.

Winge, O. 1937 Succession in broods of *Lebistes reticulatus*. *Nature* **140**, 467.

Zane, L., Nelson, W. S., Jones, A. G. & Avise, J. C. 1999 Microsatellite assessment of multiple paternity in natural populations of live-bearing fish, *Gambusia holbrooki*. *J. Evol. Biol.* **12**, 61–69.