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SHORT COMMUNICATION

Geographic variation in sperm traits reflects predation risk and natural rates of multiple paternity in the guppy

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sexual selection;
sperm competition.

Abstract

Guppies (*Poecilia reticulata*) are models for understanding the interplay between natural and sexual selection. In particular, predation has been implicated as a major force affecting female sexual preferences, male mating tactics and the level of sperm competition. When predation is high, females typically reduce their preferences for showy males and engage more in antipredator behaviours, whereas males exploit these changes by switching from sexual displays to forced matings. These patterns are thought to account for the relatively high levels of multiple paternity in high-predation populations compared to low-predation populations. Here, we assess the possible evolutionary consequences of these patterns by asking whether variation in sperm traits reflect differences in predation intensity among four pairs of Trinidadian populations: four that experience relatively low levels of predation from a gape-limited predator and four that experience relatively high levels of predation from a variety of piscivores. We found that males in high-predation populations had faster swimming sperm with longer midpieces compared to males in low-predation populations. However, we found no differences among males in high- and low-predation populations with respect to sperm number, sperm head length, flagellum length and total sperm length.

Introduction

The impressive diversity in sperm number, velocity and morphology across species is thought to be primarily a result of strong evolutionary pressure from sperm competition, the competition between the ejaculates of two or more males for the fertilization of a single set of ova (Parker, 1970; Birkhead & Moller, 1998; Simmons, 2001; Pizzari & Parker, 2009). The probability of encountering sperm competition (i.e. sperm competition risk; *sensu* Parker *et al.*, 1997) and the average number of ejaculates competing for each fertilization (i.e. sperm competition intensity; *sensu* Parker *et al.*, 1996) varies broadly among species and ecological gradients. Ecological variables can shape mating behaviour, which in turn affects sperm competition. For example, the frequency of extrapair

paternity, and hence sperm competition risk, in birds can be affected by breeding density (Richardson & Burke, 2001), territory quality (Rubenstein, 2007) and food availability (Vaclav *et al.*, 2003).

One ecological variable that has received little attention in studies of sperm competition is predation. It has long been recognized that particular reproductive behaviours can increase the risk of predation (Lima & Dill, 1990; Magnhagen, 1991; Sih, 1994). As a result, predation risk can shape reproductive strategies (Magurran & Seghers, 1990; Sih, 1994; Candolin, 1998), the frequency and duration of reproduction (Sih *et al.*, 1990; Candolin, 1997; Rohr & Madison, 2001) and operational sex ratios (Lodé *et al.*, 2004), all of which can affect sperm competition risk and intensity (Birkhead & Moller, 1998; Simmons, 2001). For example, in the agile frog (*Rana dalmatina*), elevated levels of predation lead to a reduced probability of multiple mating by females, and thus a lower risk of sperm competition (Lodé *et al.*, 2004).

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Reproductive behaviour and predation intensity are closely integrated in the guppy, *Poecilia reticulata* (Luyton & Liley, 1985; Magurran & Seghers, 1990; Darden & Croft, 2008). Guppies are small, live-bearing fish found in streams throughout the northern mountainous range of Trinidad (Houde, 1997). These streams vary in ecological conditions and are isolated from one another, making this system ideal for studying the effects of various ecological factors on behaviour (Liley & Seghers, 1975; Magurran, 2005). For example, in lowland streams guppies typically face high levels of predation pressure because they co-exist with a number of large piscivores. These include members of the Characidae family, such as *Hoplias malabaricus* and species belonging to the Cichlidae, such as *Crenicichla frenata* (Liley & Seghers, 1975). However, waterfalls and rapids can act as barriers that preclude the upstream migration of these large predators into smaller tributaries. Consequently, guppies that inhabit upstream streams typically face low levels of predation because they co-exist only with a smaller gape-limited predator, *Rivulus hartii* of the family Cyprinodontidae, which is incapable of consuming large adult guppies, but is a significant predator of juveniles (Liley & Seghers, 1975).

Variation in the level of predation has been implicated as an important determinant of male behavioural and morphological traits, including mating behaviour (reviewed in Endler, 1995; Houde, 1997). Individual male guppies employ two distinct mating tactics interchangeably, a courtship behaviour called the sigmoid display, and a form of sneaky mating called the gonopodial thrust (Houde, 1997). Under elevated risk of predation, females become increasingly unresponsive to male courtship (Godin & Briggs, 1996; Gong, 1997), typically increase their vigilance and antipredator behaviours (Magurran, 2005) and actively select areas of high-predation risk to reduce sexual harassment by males (Darden & Croft, 2008). For their part, males exploit these changes in female behaviour by switching from courtship to forced sneaky mating attempts (Evans *et al.*, 2002). Consequently, sneaky mating is more prevalent in high-predation populations than in low-predation ones (Luyton & Liley, 1985; Magurran & Seghers, 1990), and these differences in the relative levels of forced matings among populations are thought to account for the relatively higher levels of multiple paternity in high-predation locations (as measured by the mean number of sires per brood) (Neff *et al.*, 2008). Consequently, the level of sperm competition is thought to be higher in populations experiencing high levels of predation (Neff *et al.*, 2008).

One possible mechanism of sperm competition, the 'fair raffle' model, occurs when the male who enters the most tickets (i.e. contributes the most sperm) is most likely to succeed in fertilizing the majority of a female's ova (Parker *et al.*, 1990). The fair raffle principle predicts that males will respond to sperm competition by increasing ejaculate size and overall sperm production, and this

has been supported in comparative (e.g. Stockley *et al.*, 1997) and intraspecific studies (e.g. Gage *et al.*, 1995). Nevertheless, previous work on guppies indicates that populations inhabiting high-risk locales had lower sperm reserves (as estimated by manually stripping ejaculates) than their low-risk counterparts (Evans & Magurran, 1999), which, although based on a relatively small sample ($n = 5$ populations) is inconsistent with the idea that selection imposed through sperm competition favours elevated sperm production. Instead, selection may favour the evolution of traits that influence the quality of ejaculates, independent of numbers. For example, in many species, there are several sperm traits in addition to number that can influence fertilization efficiency, creating a 'loaded raffle' (Parker *et al.*, 1990; Snook, 2005; Pizzari *et al.*, 2008a). These sperm traits are collectively referred to as 'sperm quality' and may include traits such as viability (e.g. García-González, 2005), velocity (e.g. Birkhead *et al.*, 1999; Gage *et al.*, 2004) and morphology (e.g. LaMunyon & Ward, 1998; Oppliger *et al.*, 2003). In guppies, sperm competition appears to follow such a 'loaded raffle' mechanism (Neff & Wahl, 2004). Indirect evidence linking sperm swimming velocity to competitive fertilization success comes from the association between relative paternity success and the area of orange in the male's colour pattern (Evans *et al.*, 2003a) – a trait positively correlated with sperm velocity (Locatello *et al.*, 2006; Pitcher *et al.*, 2007; but see Skinner & Watt, 2007). There also appears to be a positive relationship between sperm velocity and the combined length of the sperm's head and midpiece (Pitcher *et al.*, 2007; Skinner & Watt, 2007). Overall, variation in morphology and velocity appears to vary among individuals, and these traits are all implicated in mediating competitive fertilization success. These sperm traits therefore presumably face distinct selection pressure because of variation in the ecological conditions that occur among populations of guppies.

In this study, we exploited the variation in predation intensity among natural guppy populations to determine whether sperm quality exhibits consistent variation across eight populations differing in the level of predation. We predicted that because of the expected asymmetry in the level of sperm competition between high- and low-predation populations (Neff *et al.*, 2008), coupled with the recently established direct (C. Boschetto, C. Gasparini & A. Pilastro, personal communication) and indirect (Evans *et al.*, 2003a; Locatello *et al.*, 2006; Pitcher *et al.*, 2007) associations between sperm quality and competitive fertilization success, that populations of guppies facing high-predation intensity would have higher sperm velocity (and consequently relatively longer sperm heads and midpieces, because these measures are correlated with sperm velocity; Pitcher *et al.*, 2007) than populations experiencing lower predation intensity. We also compare sperm counts (as estimated from stripped ejaculates) among populations to compare

our results with those obtained from a more limited sample by Evans & Magurran (1999).

Materials and methods

Fish collections

We collected male guppies from both downstream and upstream populations of four river systems (Aripo, Tacarigua, Quare and Turure) in Trinidad's Northern Mountain Range. For Tacarigua, the Tunapuna stream was the upstream sampling site. As is typical of rivers in the Northern Mountain Range, upstream locations exhibited low-predation intensity, and downstream locations exhibited high-predation intensity (Houde, 1997; Magurran, 2005). All sampling sites have been extensively surveyed for predators (Magurran & Seghers, 1994; Endler & Houde, 1995) and subsequently monitored by other researchers (e.g. Evans *et al.*, 2003b, T.E. Pitcher, personal observations). We collected 25 males from each population; however, for some males, sperm degraded prior to analysis or the video recordings were not of sufficient quality for velocity analysis (see below) so fewer individuals were used.

Sperm trait assessment

Males were isolated for 3 days after capture from the wild to allow sperm reserves to replenish (Kuckuck & Greven, 1997). We collected sperm from males from each of the eight populations, following Matthews *et al.* (1997). Individuals were placed under a dissecting microscope, and sperm were then extracted by swinging the gonopodium forwards and applying pressure to the side of the abdomen with a blunt probe until all spermatozeugmata (i.e. sperm bundles) were released. Initially, a fixed number of sperm (25 bundles) were drawn up in a pipette and added to 250 μ L Courtland's saline, which contained bovine serum albumin at 1% v/v (hereafter saline solution) (Evans *et al.*, 2003a). The resulting solution was drawn repeatedly into the pipette to break the sperm bundles and activate the sperm for velocity analysis (see below). Remaining sperm bundles were then collected and diluted in saline solution, as outlined earlier, for sperm number and sperm morphology analyses (see below). Finally, we took a digital photograph of the left side of each male, which was later used to measure total body length (from the tip of the mouth to the base of the caudal fin, along the central axis) using IMAGEJ software (available at <http://rsbweb.nih.gov/ij/>).

Sperm numbers at rest (hereafter sperm number) were calculated by counting sperm cells in an 'improved Neubauer chamber' haemocytometer under 400 \times magnification (see Pitcher *et al.*, 2003). The distribution of sperm cells across the haemocytometer was checked visually for evenness before counts commenced. If the sperm were unevenly distributed across the haemo-

cytometer then the count was discarded and started over. Sperm numbers are expressed as the total number of sperm in a male's stripped ejaculate.

Video recordings for sperm velocity analyses were made using a CCD B/W video camera module at 50 Hz vertical frequency, mounted on a digital compound microscope (magnification 400 \times , Olympus BX60). We suspended 25 sperm bundles in 8 μ L of saline solution on a haemocytometer, covered with a cover slip. To minimize the likelihood of sperm sticking to the glass, the glass slide and cover slip were precoated by immersion in 1% bovine serum albumin followed by a rinse in distilled water (see Billard *et al.*, 1995). Video recordings were analysed using the HTM-CEROS sperm tracking package (CEROS version 12, Hamilton Thorne research, Beverly, MA, USA), an objective tool for studying sperm motility in fish (see Kime *et al.*, 2001; Rurangwa *et al.*, 2004; Pitcher *et al.*, 2009). The following parameters were included in our analysis: number of frames = 25; minimum contrast = 15; minimum cell size = 5 pixels. We assessed average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell) at 5 s post-activation (i.e. breaking of the bundle). Because the variables describing sperm velocity (VAP, VSL and VCL) were highly correlated, we performed a Principle Component Analysis on these variables, which yielded one informative PC axis (hereafter referred to as sperm velocity) that explained 66.3% of the variation (PC loadings: VAP = 0.99, VSL = 0.94, VCL = 0.32). The sperm velocity estimates used in the final analyses corresponds to the mean velocity of all motile cells analysed for each male. Sperm that were stuck to one another or the glass slide and those whose movement beneath the cover slip was caused by convection currents were excluded from analyses. Between 19 and 378 sperm were measured for velocity per male (mean \pm SE = 90.1 \pm 7.45).

Sperm morphology assessment followed Pitcher *et al.* (2007). Briefly, we placed 20 μ L of preserved sperm (sperm suspended in a 2.5% glutaraldehyde) in saline solution and applied it to a glass slide, from which we took digital images at \times 1000 magnification using a light microscope and oil immersion. From these photographs, we used IMAGEJ software to measure the length of the head, midpiece and flagellum of 11–15 undamaged sperm for each male (mean \pm SE = 13.94 \pm 0.70). The head was measured along the midline from the forward apex to the neck. The midpiece was measured in the same manner from the neck to the insertion of the flagellum. The flagellum was measured from the insertion point to the terminal filament. Total length was obtained by combining the lengths of all three components. Analyses were carried out using mean values for each male.

Statistical analyses

We analysed variation in sperm traits (log transformed sperm number and sperm morphology metrics) between predation regimes using linear mixed effects models fitted via restricted maximum likelihood (*nlme* package, R Development Core Team, 2009). In these analyses, sperm-related traits were entered as response variables, with predation as a fixed factor (2 levels, high- and low-predation). However, as predation level varied between upstream and downstream locations within rivers, river and predation level within river were treated as random effects (see Schielzeth & Forstmeier, 2009). Thus, our analyses incorporate the random effects of river (treated as random intercepts) and predation (treated as random slopes) to account for variation in the effect of predation both within and between rivers. To test the between-group effect of predation within streams, we calculated intraclass correlation coefficients (ICC), reflecting the proportion of variance attributable to each level of the model (see Raudenbush & Bryk, 2002). For example, when modelling sperm velocity as a function of predation, the estimated ICC was < 1%, indicating minimal between-group effect (i.e. negligible variation in the effect of predation on sperm velocity between rivers) suggesting that there is no need to retain the random effect of predation in the model. However, for additional models in our analyses, estimates of ICC were considerably higher (e.g. for the model reflecting the effect of predation on midpiece length, the ICC was considerably higher at ~ 35%). Therefore, to avoid overconfident estimates from our mixed models (see Schielzeth & Forstmeier, 2009), we retained the random effect of predation within each model.

To compare sperm number across predation levels, we first tested the relationship between sperm number and adult body size (i.e. body length), which are often found to covary within populations (see Pitcher & Evans, 2001). In this study, we found that sperm number was indeed correlated with body length ($F_{1, 157} = 17.10$, $r^2 = 0.10$, $P < 0.001$), so we added body size as a covariate to

analyses involving sperm number. To ensure that we constructed models that produce unambiguous and unbiased estimates of within- and between-subject effects when covariates are employed, we applied group centering to evaluate whether the modelled relationship captured the effect both within subjects (stream section within rivers) and between subjects (rivers) (see van de Pol & Wright, 2009). In our case, when comparing sperm number across predation levels, the effect of the centred value was not significant ($P = 0.46$) indicating the modelled relationship was similar within and between groups.

We also used linear mixed effects models (*nlme* package, R Development Core Team, 2009) to examine whether potential trade-offs existed between sperm number and sperm size metrics. Here, as earlier, our analyses incorporated the random effects of river and predation but did not include the covariate of adult body length.

Results

Our analyses revealed significant differences among populations in male sperm traits (see Table 1 for descriptive statistics), which in turn were attributable to variation in the level of predation at each site (Table 2). High-predation males possessed significantly faster sperm (Fig. 1) and sperm with longer midpieces than their low-predation counterparts (Table 2). Finally, sperm number, flagellum length and total sperm length did not differ significantly between high- and low-predation populations (Table 2).

We found a weak but significant negative relationship between sperm number and sperm head length ($t_{55} = 5.46$, $P = 0.04$). There was no relationship between sperm number and midpiece length ($t_{55} = 2.11$, $P = 0.15$), flagellum length ($t_{55} = -0.93$, $P = 0.35$) or total sperm length ($t_{55} = -1.19$, $P = 0.24$). Finally, there was no relationship between sperm number and sperm size metrics when we examined high- ($n = 27$ males) and low-predation ($n = 35$ males) populations separately (all $P > 0.10$).

Table 1 Mean \pm standard deviation and sample sizes for body size, sperm number, sperm velocity, sperm head length, midpiece length, flagellum length and total sperm length of guppies (*Poecilia reticulata*). Values presented for sperm morphology traits are back-transformed from log-transformed values used in the analyses (see Materials and methods).

Population	Predation intensity	Body length (mm)	Sperm number ($\times 10^6$)	<i>n</i>	Sperm velocity (PC1)	<i>n</i>	Head length (μ m)	Midpiece length (μ m)	Flagellum length (μ m)	Total sperm length (μ m)	<i>n</i>
Lower Aripo	High	16.14 \pm 1.06	3.19 \pm 1.50	18	0.58 \pm 1.24	14	4.26 \pm 1.02	6.15 \pm 1.05	44.75 \pm 1.03	55.18 \pm 1.02	8
Upper Aripo	Low	18.34 \pm 1.05	4.13 \pm 1.75	20	-0.33 \pm 0.70	14	4.18 \pm 1.02	5.64 \pm 1.05	45.60 \pm 1.01	55.44 \pm 1.01	10
Tacarigua	High	15.91 \pm 1.06	2.02 \pm 0.73	20	-0.10 \pm 0.44	14	4.28 \pm 1.03	6.22 \pm 1.03	43.49 \pm 1.01	53.99 \pm 1.01	10
Tunapuna	Low	18.04 \pm 1.07	3.83 \pm 1.86	20	-0.99 \pm 0.46	11	4.38 \pm 1.05	6.03 \pm 1.12	44.05 \pm 1.02	54.50 \pm 1.01	10
Lower Quare	High	17.22 \pm 1.06	5.84 \pm 3.15	19	1.22 \pm 0.88	6	4.20 \pm 1.03	6.00 \pm 1.08	43.27 \pm 1.02	53.49 \pm 1.02	10
Upper Quare	Low	16.60 \pm 1.05	3.91 \pm 1.72	22	0.55 \pm 1.33	14	3.91 \pm 1.04	4.75 \pm 1.09	41.82 \pm 1.02	50.50 \pm 1.02	8
Lower Turure	High	16.86 \pm 1.05	2.75 \pm 1.05	18	0.10 \pm 0.61	14	4.37 \pm 1.02	6.03 \pm 1.06	44.03 \pm 1.04	54.45 \pm 1.03	10
Upper Turure	Low	17.78 \pm 1.07	3.11 \pm 1.38	22	-0.42 \pm 0.65	18	4.33 \pm 1.02	5.81 \pm 1.07	45.56 \pm 1.02	55.73 \pm 1.01	10

Table 2 Population comparisons of guppy (*Poecilia reticulata*) sperm traits.

Sperm trait	Predation Intensity		Mean \pm SE*	n	Δt	P-value
	High	Low				
Sperm number ($\times 10^6$)	High	Low	3.81 \pm 0.80	75	-0.09	0.93
	High	Low	3.82 \pm 0.75	84		
Sperm velocity (PC1)	High	Low	0.73 \pm 0.17	48	4.37	< 0.001
	High	Low	-0.29 \pm 0.31	57		
Head length (μm)	High	Low	4.28 \pm 1.05	38	0.96	0.34
	High	Low	4.20 \pm 1.03	38		
Midpiece Length (μm)	High	Low	6.10 \pm 1.10	38	2.0	0.048
	High	Low	5.54 \pm 1.05	38		
Flagellum length (μm)	High	Low	44.10 \pm 1.01	38	-0.57	0.57
	High	Low	44.24 \pm 1.02	38		
Total length (μm)	High	Low	54.25 \pm 1.04	38	0.25	0.80
	High	Low	54.00 \pm 1.02	38		

Mean values (estimated from the mixed model) and their standard errors (\pm SE) for sperm number, velocity, head length, midpiece length, flagellum length and total length. *n* denotes number of individuals sampled.

*Values presented are back-transformed from log-transformed values used in the analyses (see Materials and methods).

Discussion

Our findings support the prediction that some aspects of sperm quality reflect the level of predation and therefore possibly the level of sperm competition among natural populations of Trinidadian guppies. Guppy sperm were faster and had longer midpieces in high-predation populations compared to those inhabiting low-predation populations. However, sperm numbers and other sperm morphological traits did not differ between low-predation and high-predation populations.

We found that on average sperm velocity was greater in high-predation populations than in low-predation populations. There is now increasing evidence that sperm number does not fully explain sperm competition success, and that instead of a 'fair raffle' mechanism, in many species sperm competition proceeds on the basis of a 'loaded raffle' mechanism, where traits other than sperm number influence competitive fertilization success (Parker *et al.*, 1990; Snook, 2005). In guppies, there is increasing evidence for such a 'loaded raffle' mechanism. For example, Neff & Wahl (2004) used Pitcher *et al.*'s (2003) paternity estimates to evaluate the mechanistic basis of sperm precedence in guppies. Their mathematical model incorporated Pitcher *et al.*'s estimates of relative ejaculate size to examine the relationship between fertilization success and the number of sperm competing for fertilization. A nonlinear relationship between paternity and relative ejaculate size would be indicative of a loaded raffle mechanism. Their analysis hinted at last-male sperm precedence with diminishing reproductive returns for increased sperm production, thereby supporting a loaded raffle mechanism. This conclusion has been

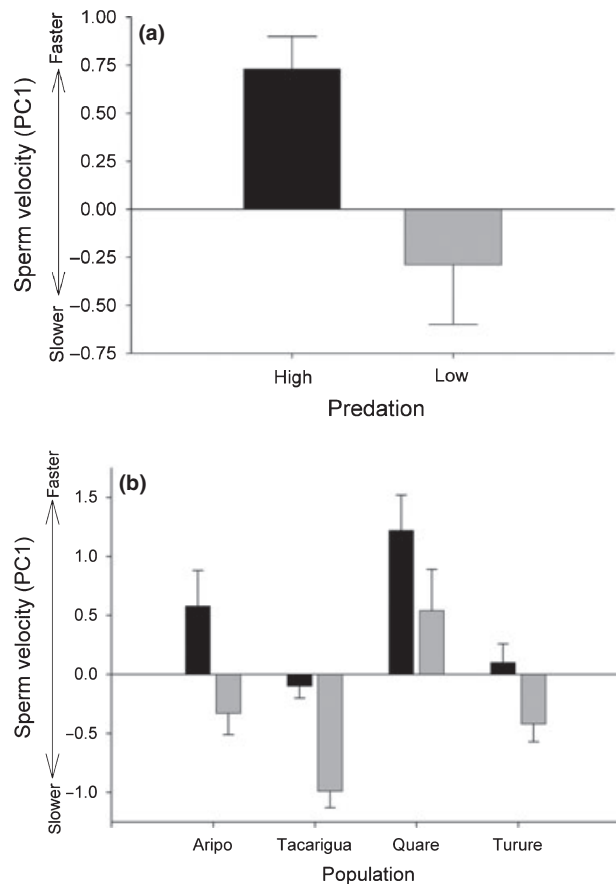


Fig. 1 (a) Estimated means (\pm SE) from the mixed model of sperm velocity across eight populations of guppies (*Poecilia reticulata*) in relation to predation intensity. (b) Mean (\pm SE) sperm velocity across eight populations of guppies paired by river, comparing high-predation (black bars) and low-predation (grey bars) regions.

supported by studies documenting nonrandom paternity biases when the number of sperm from each male is regulated through artificial insemination. For example, in a recent unpublished study, Boschetto, Gasparini & Pilastro (personal communication) used artificial insemination to transfer controlled amounts of sperm to female guppies and found that both relative ejaculate size and sperm velocity were the primary determinants of paternity. This supports findings from a similar experiment conducted by Evans *et al.* (2003a), who found that male paternity was related to the amount of orange colouration, a trait which has been linked to sperm velocity (Locatello *et al.*, 2006; Pitcher *et al.*, 2007; but see Skinner & Watt, 2007).

Functionally, the increase in velocity in high-predation compared to low-predation populations may be because of differences in midpiece length, which were significantly longer in high-predation populations compared to low-predation populations. Pitcher *et al.* (2007) previously demonstrated that in guppies, sperm head length

(a measure that included the midpiece) was positively correlated with sperm velocity. Skinner & Watt (2007) found a similar pattern using sperm midpiece area. The midpiece is the component of the sperm containing the mitochondria, which provide energy for the beating of the flagellum (Baccetti & Afzelius, 1976). Thus, a larger midpiece could potentially provide more energy for locomotion, resulting in faster sperm, although this possibility has yet to be thoroughly investigated and other components of sperm morphometry, including scaling relationships between the sperm's head and flagellum (see Humphries *et al.*, 2008), need to be incorporated into future analyses that link sperm swimming velocity to sperm size.

Sperm competition is predicted to select for greater sperm number through a 'fair raffle' mechanism, whereby males with greater numbers of sperm are able to enter more 'tickets' into the contest for fertilization and are therefore likely to outcompete males with fewer sperm (Parker *et al.*, 1990). It follows that males facing increasing risk or intensity of sperm competition invest in producing greater numbers of sperm (Gage *et al.*, 1995; Stockley *et al.*, 1997). Instead, unlike Evans & Magurran (1999), we did not find that sperm number (after controlling for body size) differed significantly between low-predation and high-predation populations. This finding may indicate that total sperm number at rest may not be a trait selected for by sperm competition, perhaps because total sperm number is not representative of ejaculate size. Alternatively, investment in sperm production may be constrained by investment in sperm quality. Interestingly, in a recent multivariate analysis of sexual selection on precopulatory and post-copulatory traits in guppies, Head *et al.* (2008) reported that selection actually favoured males with lower sperm reserves, prompting them to speculate that components of sperm quality may be traded off against sperm production. Our results similarly suggest that sperm quality may be traded off to some extent against sperm quantity, because we found a very weak but significant negative relationship between sperm number and sperm head size, albeit not when we examined high- and low-predation populations separately. Nevertheless, experimental and/or quantitative genetic approaches are needed to properly test the basis for such relationships.

Our comparative analyses revealed variation in sperm traits reflect differences in predation intensity and concordant rates of multiple paternity among populations of Trinidadian guppies. We found that males in high-predation populations had faster swimming sperm with longer midpieces, but there were no differences in sperm number, flagellum length and total sperm length among high- and low-predation populations. Although our results appear to be consistent with the idea that predation and the concordant rates of multiple paternity account for the variation in sperm traits we observed, we interpret this relationship cautiously

because in addition to sperm competition, other variables that differ among the high- and low-predation populations may also explain some of the variation we found in sperm traits. For example, effective population size, sex ratio, food availability, population density and longevity differ between high- and low-predation populations (reviewed in Houde, 1997; Magurran, 2005). Although all of these variables may in theory affect sperm production to some extent, the most biologically relevant variable is possible age differences between the males in the high- and low-predation populations. Because of high levels of predation on adult guppies, the males we sampled from high-predation populations were likely younger on average compared to low-predation populations. It is therefore plausible that, rather than sperm competition *per se*, senescence might account for some of the results we report in this study (Radwan, 2003; Pizzari *et al.*, 2008b). For example, the result that males from high-predation populations produce sperm of higher velocity might be explained by the fact that a random sample of these males will be closer to their reproductive prime than a random sample of males from low-predation populations, which are expected to be older on average. Although we do not possess any data to directly test this hypothesis, it seems unlikely that age differences among males from high- and low-predation populations could explain our main result because Gasparini *et al.* (2010) found that older male guppies actually produced slower sperm, more sperm and longer sperm compared to younger males.

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