

Ejaculate traits in relation to male body size in the eastern mosquitofish *Gambusia holbrooki*

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In the present study, the correlation between sperm number, sperm quality (speed, viability, longevity and length), sperm bundles quality (size and dissolving rate) and male body size has been tested in the eastern mosquitofish *Gambusia holbrooki* a poeciliid species characterized by coercive mating tactics where males do not possess obvious ornaments, and the body size is the key determinant of pre-copulatory male mating success. The results do not tally with theoretical predictions. Indeed, no correlation between male body size and either sperm or sperm-bundle traits has been found, evidencing the lack of the theoretically expected trade-off between the investment in characters involved in mate acquisition and the investment in ejaculate quality. An explanation for the observed pattern comes from the extremely dynamic mating system of *G. holbrooki*, characterized by variable size-related male mating success and strong post-copulatory selective pressure, with all males facing a similar high level of sperm competition. In this situation, a higher investment in growth and maintenance at the expense of ejaculate quality is not expected. These results underscore the necessity to comprehend detailed information on species' reproductive biology and reproductive environment to understand both the evolution of ejaculate characteristics and possible deviations from theoretical predictions.

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INTRODUCTION

In species where females mate promiscuously, a male's reproductive success depends not only on its ability to acquire mates but also on the ability of its ejaculate to compete with those of other males. The competition among ejaculates, usually called sperm competition, is nowadays deemed one of the most powerful evolutionary forces shaping male behaviour, morphology and physiology (Birkhead & Møller, 1998; Birkhead & Pizzari, 2002). An almost universal adaptation to sperm competition in males is represented by an increased sperm

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production in response to increased competition, and sperm competition success is frequently determined by the relative number of sperm released by rival males (Birkhead & Møller, 1998). Variation in sperm number is, however, only one part of a complicated situation. Indeed, empirical studies have recently shown that, in addition to sperm number, sperm quality traits such as size, longevity, viability and speed influence fertilization efficiency, hence playing a role in paternity contests (Snook, 2005). Sperm quality traits appear to vary among species, but how they vary intraspecifically in response to different levels of sperm competition is just beginning to be investigated (Snook, 2005; Locatello *et al.*, 2006; Rudolfson *et al.*, 2006).

Since the costs of ejaculate production are often not trivial and the resources available to reproduction may be limited (Dewsbury, 1982; Shapiro *et al.*, 1994; Olsson *et al.*, 1997), a trade-off is expected between traits involved in sperm competition and traits playing a role in mate acquisition (Parker, 1998). Experimental evidence, however, shows that the correlation between the development of male ornaments and ejaculate quality may be positive (Malo *et al.*, 2005; Locatello *et al.*, 2006), null (Parker *et al.*, 2006) or negative (Froman *et al.*, 2002). Exploring the disparities among different species and relating them to differences in reproductive biology and the reproductive environment may yield important insights into the evolution of ejaculate characteristics.

In fishes, a wide array of studies on sperm competition have focused on externally fertilizing species exhibiting alternative male mating tactics (Snook, 2005). In these species, dominant males usually exhibit larger body size and are more aggressive, whereas subordinate males are smaller in size, less aggressive and adopt opportunistic mating tactics (Taborsky, 1994). In most of these species, a negative correlation has been found between male investment in traits involved in mate acquisition and either sperm number and sperm quality traits since subordinate males are smaller and exhibit less conspicuous sexual characters than dominant ones but have a higher sperm production and a better ejaculate quality (Uglem *et al.*, 2001; Rasotto & Mazzoldi, 2002; Rudolfson *et al.*, 2006).

Empirical studies on sperm competition in internally fertilizing fish species almost exclusively concern poeciliids (Constantz, 1984; Magurran, 2005), a family of freshwater fishes in which male anal fins are modified into a copulatory organ called gonopodium. Males of this family can adopt two different mating tactics: court the female and obtain her co-operation during mating or attempt forced insemination through gonopodial thrusting. The two tactics coexist in most species but one of the two is usually prevalent and, in about a half of the species, only gonopodial thrusting has been reported (Constantz, 1989; Bisazza, 1993). In species in which males court the female, females generally show a mating preference for large male body size (Rosenthal & Evans, 1998; Bisazza *et al.*, 2001; Basolo, 2004), courtship rate (Bischoff *et al.*, 1985; Kennedy *et al.*, 1987), area and brightness of colour spots on the body (Houde, 1997), tail length (Basolo, 1990) and gonopodium length (Langerhans *et al.*, 2005). The larger and more ornamented males usually court the females, whereas the smaller and less ornamented ones mainly or exclusively adopt sneaky mating tactics (Constantz, 1989; Zimmerer & Kallman, 1989; Bisazza, 1993). Overall, sexual selection seems to operate in two distinct directions: female choice and male–male competition (where present) usually favours large

(and often ornamented and courting) males, whereas sexual coercion favours small males (Bisazza, 1993; Bisazza & Pilastro, 1997).

While the behavioural and morphological outcomes of pre-copulatory sexual selection are widely documented, research on the association between male phenotypic characteristics and ejaculate traits (in terms of sperm number and quality) has until now almost solely focused on unravelling the relation between sperm numbers and body size (Constantz, 1984; Aspbury & Gabor, 2004; Schlupp & Plath, 2005). The relationship between phenotypic characteristics and other ejaculate traits, however, has so far only been investigated in the guppy *Poecilia reticulata* Peters. In *P. reticulata*, the expression of male sexual secondary characters, such as courtship rate and area of colour spots, positively co-varies with sperm production (Matthews *et al.*, 1997; Pilastro & Bisazza, 1999; Pitcher & Evans, 2001), sperm quality traits [Locatello *et al.*, 2006; Pitcher *et al.*, 2007; although Skinner & Watt (2007) found no association between male colouration and sperm quality] and sperm competition success (Evans *et al.*, 2003a). By contrast, body size appears to be negatively related to success in sperm competition (Evans *et al.*, 2003a), suggesting that there may be a trade-off between sperm quality and body growth and maintenance. A negative phenotypic correlation between sperm quality and body size has been found in other fishes (Vladic & Järvi, 2001; Urbach *et al.*, 2007), suggesting that this may be a common pattern.

Here, the analysis of the relationship between ejaculate characteristics and body size has been extended to another poeciliid species, the eastern mosquitofish *Gambusia holbrooki* Girard, distributed in brackish and fresh waters in the south-eastern U.S. As in other poeciliids, fertilization is internal: the male gonopodium is inserted into the female's genital pore conveying sperm in the form of spermatozeugmata (sperm bundles) (Constantz, 1989). Unlike *P. reticulata*, males neither possess obvious ornaments nor exhibit courtship behaviour and undergo copulation mainly through gonopodial thrusts (McPeck, 1992; Bisazza, 1993). Furthermore, male-male competition is an important component of male pre-copulatory success in *G. holbrooki*, as large males are usually dominant over other males and limit the access of small males to females (Bisazza & Marin, 1991, 1995; Zulian *et al.*, 1993). Male sexual harassment is intense (Evans *et al.*, 2003b), and it has been estimated that a female is inseminated 0.25–0.50 times per day (Pilastro *et al.*, 1997). Females can store sperm and fertilize their eggs for months after a single copulation (Dulzetto, 1928) and sperm competition is therefore intense (Zane *et al.*, 1999). Post-partum and male-deprived females have been shown to preferentially associate with large males (Bisazza *et al.*, 2001), suggesting a latent female preference for large body size. In contrast, small males are favoured during gonopodial thrusts because they have a greater chance to approach females from behind without being detected and have greater manoeuvrability when inserting the gonopodium into the female's gonoduct (Pilastro *et al.*, 1997). Large males are therefore expected to obtain relatively more matings 2 to 3 days after parturition (Pilastro *et al.*, 1997), when females exhibit a preference for large males (Bisazza *et al.*, 2001) and ovulation occurs (Constantz, 1989). In contrast, small males are likely to obtain relatively more matings during pregnancy, when they can use their greater manoeuvrability to obtain successful forced copulation (Pilastro

et al., 1997). The time between insemination and fertilization will therefore be longer, on average, in these matings than in matings occurring soon after parturition. Collectively, these results suggest that in *G. holbrooki*, the selective pressure on the ejaculate quality traits may be stronger on small males than on larger males. Conversely, large males may be constrained to allocate more resources to body growth and maintenance at the expense of the ejaculate quality. If so, a negative correlation between male body size and sperm quality traits would be expected. In the present study, this prediction has been tested by examining the sperm reserves, the sperm traits (length, speed, viability and longevity) and the sperm bundle traits (size and dissolving rate) and in relation to male body size in *G. holbrooki*.

MATERIALS AND METHODS

The fish used for the analyses were collected from an introduced population in Valle Averno, a system of brackish water ponds in the Venetian lagoon basin, northern Italy, at the beginning of June 2004 and June 2005. The fish were maintained in captivity in mixed-sex 30 l aquaria (c. 1:1 sex ratio), at 25° C, range $\pm 1^\circ$ C and 14L:10D photoperiod. Fish were fed a mixed diet of brine shrimp nauplii *Artemia* sp. and commercial food flakes. Males were transferred to transparent individual containers that allowed visual but no physical contact with females for 3 days before sperm collection to allow for replenishment of their sperm reserves.

A total number of 110 males was used for the analyses: 63 in 2004 and 47 in 2005. In summer 2004, sperm longevity, percentage of dissolved spermatozeugma and sperm length were measured in 31 males. Sperm count was calculated in another 32 males and spermatozeugma size was measured on a sub-sample (23) of these males. In summer 2005, sperm speed was estimated in 25 males and sperm length was measured on a sub-sample (16) of these males. Sperm viability was then evaluated in another 22 males and sperm length was measured on a sub-sample (eight) of these males. Prior to the analyses, each male was anaesthetized in a water solution of MS 222 (0.15 g l^{-1}) and a digital photograph was taken of the male alongside a reference ruler (Nikon CoolPix 4500, Tokyo, Japan). The standard length (L_S , *i.e.* distance between the snout and the base of the tail) was estimated from the digital images using image analysis software (Image Tool, <http://ddsdx.uthscsa.edu/dig/itdesc>). The anaesthetized male was then placed on a microscope slide and viewed under $\times 4$ magnification with his gonopodium swung forward. Gentle pressure was applied to the side of his abdomen, just anterior to the base of the gonopodium, to release spermatozeugmata. All males were used for a single trial and none of them died as a consequence of the treatment.

SPERM COUNT

The total number of sperm produced was calculated for 32 males (L_S range: 15.83–24.38 mm). All the spermatozeugmata obtained from the stripping of the males were broken up in 20 ml of KCl 0.9% and the sperm were stained with drops of 1% Rose Bengal. After 20 min to allow stain absorption, 4 ml of a 30% formalin solution was added to fix the sperm. The solution was then filtered with a Millipore system (Billerica, MA, U.S.A.; 0.22 μm pore size) under vacuum. The filters were dried and sperm were then counted in 10 different areas of the filter under a microscope ($\times 400$ magnification) fitted with a reticule eyepiece. The mean of 10 counts was then multiplied by a conversion factor corresponding to the area represented by the grid on the microscope and the total area of the filter. The number of spermatozeugmata stripped, and thus the average number of sperm contained in each spermatozeugma, was also counted for 19 of the 32 males.

SPERM LENGTH

The mean head length and total sperm length were measured in 55 males (L_S range: 18.51–26.71 mm). Fifty microlitre aliquots of each male's sperm sample were incubated in a solution of 10 μl of 1% Rose Bengal for 20 min and dyed samples were then viewed under $\times 1000$ magnification and photographed with a digital camera. Using these photographs, 15 sperm per male were measured using image analysis software (Image Tool).

SPERM SPEED

Sperm speed was estimated in 25 males (L_S range: 19.21–27.18 mm). A two-step procedure was followed to ensure simultaneous activation of all sperm cells (Billard & Cosson, 1992). First, 50 spermatozeugmata were placed into 5 μl of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl_2 , 0.49 mM MgCl_2 , 0.41 mM MgSO_4 , 10 mM Tris, pH 7.5) in which sperm remain quiescent (Gardiner, 1978). The sample was maintained at 3–5° C until required for the motility analyses (within 2 h of collection), at which point it was warmed to 26° C and activated with a 20 μl solution of 150 mM KCl and 2 mg ml^{-1} of bovine serum albumin (BSA). Immediately after adding the activating solution, the sperm bundles were gently broken by drawing and expelling each sample 100 times using a micropipette to induce motility. The resultant samples (3 μl) were placed individually in disposable 12 μm deep microcell chambers and analysed using an IVOS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, U.S.A.). Sperm speed measurements were based on an mean \pm s.d. of 122.8 ± 46.0 sperm tracks per sample (range 56–272). These measurements included: (1) average path speed (V_{AP}), which estimates the average speed of sperm cells over a smoothed cell path, (2) straight line speed (V_{SL}), the average speed on a straight line between the start and the end point of the track and (3) curvilinear speed (V_{CL}), the actual speed along the trajectory. The threshold values defining static cells were predetermined at 20 $\mu\text{m s}^{-1}$ for V_{AP} and V_{CL} , and 15 $\mu\text{m s}^{-1}$ for V_{SL} . These three motility measurements provide an estimate of progressive speed and have been shown to correlate well with fertilization rates in various vertebrate species (Froman & Feltmann, 2000; Rurangwa *et al.*, 2004). The dilution used in this study resulted in an mean \pm s.d. of 32.46 ± 7.93 sperm per field-of-view (range 12.13–49.44). The motility analyses were performed on two sub-samples of the ejaculate for each male and the mean was used in the final analysis. The within-sample repeatability was 0.64, 0.64 and 0.61 for V_{AP} , V_{SL} and V_{CL} , respectively (Lessells & Boag, 1987).

SPERM VIABILITY

The proportion of living sperm immediately after stripping and 3 h after stripping was estimated using the eosin-Y staining test (Lin *et al.*, 1998) in 22 males (L_S range: 18.51–27.71 mm). This stain works by penetrating the head membrane of dead cells, which appear pink (live cells appear colourless). One hundred spermatozeugmata from each male were diluted in 20 μl of 0.9% NaCl solution and broken up. Five microlitre of the sample were mixed on a microscope slide with 5 μl of eosin-Y stain (0.5% mass: volume). After 2 min, the slide was covered with a cover slip and examined under a light microscope ($\times 1000$ magnification with oil immersion). The proportion of live sperm was calculated per 100 sperm per slide for each male. This procedure was repeated after 3 h using a second aliquot of the sperm sample (which was maintained at 26° C in a water-bath until used).

SPERM LONGEVITY

Sperm longevity was estimated in 31 males (L_S range: 19.51–25.27 mm). Twenty spermatozeugmata were collected from each male, diluted in 40 μl of 0.9% NaCl activating solution and broken up. Every 30 min, a 10 μl sample from each male was placed on

a glass slide and observed under a light microscope ($\times 400$ magnification) at room temperature. Sperm longevity was estimated as the time from activation until the last observation at which *c.* 90% of sperm within the visual field were no longer motile (showing head or tail movements).

PERCENTAGE OF DISSOLVED SPERMATOZEUGMATA

The percentage of intact and dissolved spermatozeugmata 30 min after stripping was measured in 31 males (L_S range: 19.51–25.27 mm). Each male was stripped on a Petri dish half filled with 0.9% NaCl solution. The sample was then observed under a light microscope ($\times 50$ magnification) and the number of spermatozeugmata in the visual field was counted. The observation was repeated after 30 min to determine the percentage of spermatozeugmata that were still intact (regular and intact borders) and the percentage dissolved (no delimited borders).

SPERMATOZEUGMATA SIZE

The mean spermatozeugmata area, minor diameter and major diameter were measured in 23 males (L_S range: 15.87–24.38 mm). Fifteen spermatozeugmata for each male were viewed under $\times 5$ magnification and photographed with a digital camera. The photographs were then analysed with image analysis software (Image Tool).

RESULTS

Overall, male L_S ranged from 15.8 to 28.0 mm [mean \pm s.d. = 21.3 ± 2.7 ; coefficient of variation (c.v.) = 12.63]. Males showed a large phenotypic c.v. in all the traits considered with the exception of sperm size, which showed little variation among individuals (Table I). A significant positive correlation was found between male L_S and the total number of stripped sperm per male, the number of sperm bundles and the mean number of sperm per bundle (Table I and Fig. 1). Sperm speed variables (V_{AP} , V_{SL} and V_{CL}) were all highly correlated (all $r > 0.89$, all $P < 0.001$). Sperm and sperm bundle traits, such as speed, longevity, viability and the percentage of dissolved spermatozeugmata did not significantly correlate with male L_S (all $r < 0.28$, all $P > 0.05$) (Table I). A significant positive correlation was found between sperm longevity and the percentage of intact sperm bundles 30 min after stripping ($r = 0.61$, $n = 31$, $P < 0.001$; Fig. 2). No correlation was found between male L_S and sperm length variables (all $r < 0.21$, all $P > 0.05$) or between male size and sperm bundle size measures ($r < 0.26$, all $P > 0.05$; Table I).

DISCUSSION

The present results showed a relatively large variation in phenotypic variance in most ejaculate traits, with the sole exception of sperm size. In particular, a large phenotypic variation was observed in sperm speed and viability, in the size of sperm reserves and in the mean size of sperm bundles. As predicted, the size of sperm reserves increased with body size. This result is not surprising as a positive association between body size and sperm reserves has been documented in other poeciliid species such as *Poecilia latipinna* (Lesueur) (Aspbury & Gabor, 2004) and *P. reticulata* (Matthews *et al.*, 1997; Pilastro & Bisazza,

TABLE I. Correlations between male *Gambusia holbrooki* standard length (L_S) and ejaculate traits. The mean \pm s.d. and the coefficient of variations (c.v.) are reported for each variable. Significant values are in bold

	n	Mean \pm s.d.	c.v.	r	P
Sperm longevity (min)	31	272.45 \pm 88.76	32.58	0.19	>0.05
Intact sperm bundles (%)	31	33.8 \pm 25.0	74.0	0.21	>0.05
Sperm head length (μm)	55	4.24 \pm 0.17	3.97	0.21	>0.05
Total sperm length (μm)	55	62.81 \pm 3.11	4.94	0.07	>0.05
Total sperm number ($\times 10^6$)	32	5.89 \pm 3.66	62.11	0.67	<0.001
Number of sperm bundles	19	1704 \pm 895	52.50	0.64	<0.01
Number of sperm per bundle	19	3638 \pm 786	21.61	0.48	0.047
Bundle area (μm^2)	23	3198 \pm 418	13	-0.17	>0.05
Sperm V_{AP} ($\mu\text{m s}^{-1}$)	25	91.4 \pm 18.3	20.0	-0.10	>0.05
Live sperm at stripping (%)	22	94.4 \pm 3.5	3.8	0.04	>0.05
Live sperm after 3 h (%)	22	72.9 \pm 17.1	23.4	0.37	>0.05

n , sample size; V_{AP} , average path speed.

1999; Pitcher & Evans, 2001). In contrast, no relationship between male L_S and the ejaculate quality traits was found, indicating that, in this species, the theoretically predicted trade-off between the investment in traits involved in mate acquisition (*i.e.* male body size) and ejaculate quality is not present. Considering the high c.v. of all the examined sperm traits excepting length (see Table I), the lack of correlation with male L_S cannot be attributed to a lack of variability. The low phenotypic variability observed in sperm length parallels findings in other species, both at intraspecific and interspecific level (Gage *et al.*, 2004; Calhim *et al.*, 2007; Locatello *et al.*, 2007), suggesting that sperm morphology might be more strongly constrained by sperm competition than other ejaculate traits.

An explanation for the observed patterns in sperm characteristics, and the lack of correlation between them and body size, might be explained by *G. holbrooki* mating system and reproductive environment. Indeed, this species is characterized

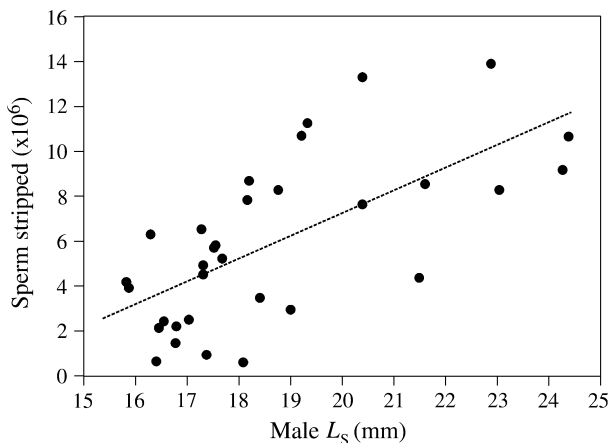


FIG. 1. Relationship between male *Gambusia holbrooki* body size (L_S) and total number of sperm produced.

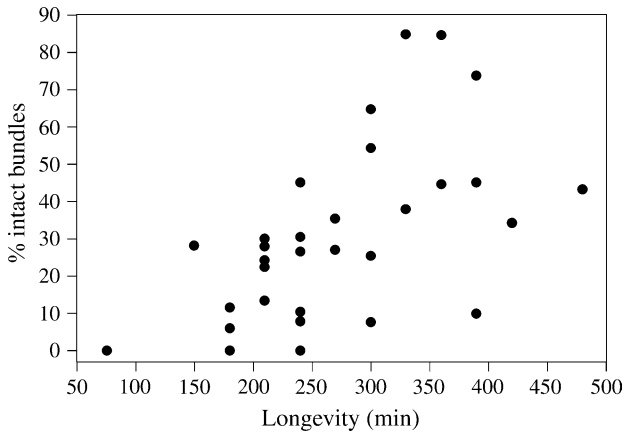


FIG. 2. Relationship between *Gambusia holbrooki* sperm longevity and the percentage of intact bundles 30 min after stripping.

by an almost exclusively coercive mating system (McPeck, 1992; Bisazza *et al.*, 2001) in which all the males have to deal with a high and probably similar level of sperm competition during each spawning, irrespective of the selective pre-copulatory pressures faced (Zane *et al.*, 1999). When all the males experience a high level of sperm competition, the selective pressure for a high sperm quality might act equally on all males, irrespective of their body size. Consequently, sperm quality would not vary consistently among males of different body size, although there is some evidence that males respond to the perceived risk of sperm competition allocating more sperm to matings in *G. holbrooki* (Evans *et al.*, 2003b) and in other poeciliids (Aspbury, 2007). Moreover, in *G. holbrooki*, the mean size of mature males varies in relation to population density (Zulian *et al.*, 1995) and the reproductive success of males depends on the size at which they mature in response to the social environment (Bisazza *et al.*, 1996). Small size is favoured in populations with low density or female-biased sex ratios. At high densities, the influence of intra-sexual and inter-sexual selection leads males to delay maturation and attain larger sizes (Bisazza & Marin, 1995; Zulian *et al.*, 1995). Male dominance, largely influenced by body size, can therefore rapidly change according to the size of the males in a shoal. In such a dynamic system, influenced by intense post-copulatory selective pressures on males (Zane *et al.*, 1999), a higher investment in growth and maintenance at the expense of sperm quality may not be favoured. A similar lack of relationship between sperm characteristics and body size has recently been documented in a frog species with intense sperm competition and where alternative male mating tactics plastically occur in relation to population density (Hettley & Dale Roberts, 2007). Furthermore, decreasing sperm quality in large males may be advantageous if they were able to inseminate more sperm than their small-sized counterparts. Sperm transfer, however, is likely to be, at least partly, under female control (Pilaastro *et al.*, 1997, 2004) and males may therefore be selected to always produce high-quality ejaculates.

The finding here of a negative correlation between sperm longevity and the sperm-bundle dissolving rate may prove important for investigating the

evolution of ejaculate characteristics in poeciliids. The result may indicate that the bundles compactness could protect the sperm from immediate activation allowing them to live longer once the activation occurs, as suggested by the negative association between sperm longevity and sperm-bundle dissolving rate. In *P. reticulata*, artificial inseminations are more successful when the bundles are intact upon insertion into the female genital tract (J. P. Evans, pers. comm.), suggesting a general adaptive value of this positive relation between sperm quality and sperm-bundle compactness in poeciliids. Again, however, the data did not provide evidence of any influence of male body size on these quality traits suggesting that whatever the basis for the phenotypic correlation between bundle dissolving rate and sperm longevity, these two traits are not traded-off against body size. Due to logistic constraints, however, in most cases different sperm traits have been measured in different individuals and it cannot be excluded that other sperm traits are positively or negatively correlated to each other. Whether correlations between sperm traits within ejaculate may reveal undetected trade-offs with body size warrants further investigation.

The lack of correlation between male body size and sperm quality traits in the *G. holbrooki* seems to be due to the high sperm level of competition (Zane *et al.*, 1999) experienced by males irrespectively of their body size. Indeed, small males are at an advantage in sneaky matings (Bisazza & Pilastro, 1997; Pilastro *et al.*, 1997); in contrast, large males are at an advantage in male–male competition but are probably unable to monopolize the females under most common population density and sex ratio conditions (Bisazza & Marin, 1995). Laboratory experiments simulating a varying sperm competition risk (by modification of sex ratio) reported, in response to increasing sperm competition, a higher number of coercive mating attempts (Evans *et al.*, 2003b). Together, all these results suggest that the *G. holbrooki* male strategy may be to assess the average level of competition in the population and adjust their mating effort in consequence rather than modify their ejaculate quality.

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