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Colourful male guppies produce faster and more viable sperm

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Abstract

In guppies (*Poecilia reticulata*) precopulatory sexual selection (via female choice) and post-copulatory selection (via sperm competition) both favour males with relatively high levels of carotenoid (orange) pigmentation, suggesting that colourful males produce more competitive ejaculates. Here we test whether there is a positive association between male orange pigmentation and sperm quality. Our analysis of sperm quality focused on sperm swimming speeds (using CASA: computer-assisted sperm analysis to estimate three parameters of sperm velocity *in vitro*), sperm viability (proportion of live sperm per stripped ejaculate) and sperm lengths. We found that males with relatively large areas of orange pigmentation had significantly faster and more viable sperm than their less ornamented counterparts, suggesting a possible link between dietary carotenoid intake and sperm quality. By contrast, we found no relationship between sperm length (head length and total sperm length) and male phenotype. These findings, in conjunction with previous work showing that highly ornamented male guppies sire higher quality offspring, suggest that female preference for colourful males and sperm competition work in concert to favour intrinsically higher quality males.

Introduction

In species where females mate with several males during a single reproductive episode a male's reproductive success will depend not only on his ability to secure matings but also on the ability of his sperm to compete for fertilization (Birkhead & Møller, 1998; Birkhead, 2000). In such polyandrous species, sperm competition can be mediated by differences in the relative number of sperm ejaculated by rival males (e.g. Parker, 1970, 1990) and/or by the relative competitive abilities of the competing ejaculates (Snook, 2005). As sperm production is costly for males (e.g. Dewsbury, 1982; Van Voorhies, 1992; Pitnick, 1993; Shapiro *et al.*, 1994; Olsson *et al.*, 1997; Wedell *et al.*, 2002), reproductive traits that mediate the outcome of sperm competition will often be traded-off against those involved in mate acquisition. For example, in the feral fowl (*Gallus gallus*), socially subordinate males produce superior quality sperm, despite the fact that they are less successful in obtaining copulations (Froman *et al.*, 2002). Similarly, in many fish species in which males exhibit alternative mating tactics (e.g. sneaks, parentals and satellites), negative associations have been reported between the expression of male ornaments and sperm traits, including ejaculate size (e.g. black goby Rasotto & Mazzoldi, 2002) and quality (e.g. salmon: Vladic & Jarvi, 2001; corkwing wrasse: Uglem *et al.*, 2001; bluegill sunfish: Burness *et al.*, 2004, but see Neff *et al.*, 2003). By contrast, several other studies have reported no significant association between male ornaments and sperm traits (birds: Birkhead & Petrie, 1995; Birkhead *et al.*, 1997, 1998; fish: Liljedal *et al.*, 1999; Kortet *et al.*, 2004).

Guppies (*Poecilia reticulata*) are among the few species in which it has been shown that the expression of overt sexually selected characteristics *positively* covaries with ejaculate traits (see also Malo *et al.*, 2005b). Guppies are live-bearing, sexually dimorphic fish with internal fertilization and a polyandrous, nonresource based mating

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system in which female choice plays an important role (Constantz, 1984; Houde, 1997). Females typically base their mating preferences on the proportion of orange (carotenoid) spots in male colour patterns, which are revealed to females during elaborate 'sigmoid' courtship displays. The area of orange pigmentation in these colour patterns, and consequently male attractiveness, is highly heritable in guppies (Winge, 1927; Houde, 1992; Brooks, 2000), although males must obtain carotenoids from their diet to express these pigments in their colour patterns (but see Grether *et al.*, 2001). Several studies have revealed that a number of phenotypic traits, including orange pigmentation, the rate of sigmoid display, and body size, positively covary with sperm load, as estimated by counting the number of sperm per stripped ejaculate (Matthews *et al.*, 1997; Pilastro & Bisazza, 1999; Pitcher & Evans, 2001; Evans *et al.*, 2002). More recently, studies have revealed that males with relatively high levels of orange pigmentation are more likely to be favoured during post-copulatory episodes of sexual selection (Evans & Magurran, 2001; Pilastro *et al.*, 2002, 2004; Pitcher *et al.*, 2003), even when the relative contribution of sperm from competing males is controlled through artificial insemination (Evans *et al.*, 2003).

Here, we determine whether the congruence between traits selected via pre- and post-copulatory sexual selection in guppies is because of the association between male attractiveness and sperm quality. We examine whether components of the male colour patterns and body size, which are known to predict sperm competition success in guppies (Evans *et al.*, 2003), are correlated with sperm performance (viability and motility) and sperm morphology. Sperm velocity or viability has been shown to be associated with fertilization success in sea urchins (Levitan, 2000), fish (Burness *et al.*, 2004; Gage *et al.*, 2004; Rurangwa *et al.*, 2004), birds (e.g. Birkhead *et al.*, 1999) and mammals (e.g. Malo *et al.*, 2005a). Evidence that sperm morphology affects fertilization success is controversial (see Snook, 2005), but the observation that sperm length is often positively correlated with sperm competition in internal fertilizers (e.g. Briskie & Montgomerie, 1992; Gomendio *et al.*, 1998; Anderson *et al.*, 2005) suggests that sperm size may influence fertilization success in a competitive context. We therefore focus on these sperm traits in three captive guppy populations (including the one used by Evans *et al.*, 2003) descended from natural populations in Trinidad.

Materials and methods

General methods

The guppies used in this experiment were descendents of fish collected in 2002 from the Middle Tacarigua (abbreviated hereafter to MT) (grid coordinates: N10° 40.736′ W061° 19.168), Lower Aripo (LA: N10° 39.036′ W061°13.380′) and Upper Aripo (UA: N10° 41.743′ W061° 12.406′) Rivers in Trinidad. Males were reared in several mixed-sex 150 L aquaria (*c.* 1 : 1 sex ratio) until required (at $T = 25$–27 °C and 12 : 12 h light/dark cycle). Fish were fed a mixed diet of brine shrimp nauplii and commercial flake food. Before sperm collection, males were isolated from females for 3 days to allow the full replenishment of their sperm reserves (Kuckuck & Greven, 1997). Each male was then anaesthetized in a water solution of MS 222 (0.15 g L^{-1}) and a digital photo was taken of the male alongside a reference ruler (Nikon CoolPix 4500, Nikon, Tokyo, Japan). We measured the distance between the snout and the base of the tail (standard length, SL), the total area of the body (including head and caudal fin) and the area of carotenoid spots from the digital images using image analysis software (IMAGE TOOL, http://ddsdx.uthscsa.edu/dig/itdesc). The relative area of carotenoid spots was calculated as the ratio between the area of spots and total body area (Evans *et al.*, 2003) (our results did not change when we used the residuals from a regression of carotenoid area on total body area). To collect spermatozeugmata (sperm bundles) the anaesthetized male was placed on a microscope slide and viewed under ×4 magnification with his gonopodium (the intromittent organ) swung forward. Gentle pressure was then applied to the side of his abdomen, just anterior to the base of the gonopodium, to release sperm bundles. The stripped ejaculates from 158 males were used in this study ($n = 45$ for the sperm viability trials and $n = 113$ for sperm motility).

Sperm viability assays

The proportion of live sperm immediately after stripping and 3 h after stripping was estimated using the eosin-Y staining test (Lin *et al.*, 1998). This stain works by penetrating the head membrane of dead cells which appear pink (live cells appear colourless). Fifty spermatozeugmata from each male ($n = 15$ males per population) were diluted in 50 μL of 0.9% NaCl solution and broken up by gently drawing and expelling each sample 100 times using a micro-pipette. Five microlitres of the sample was mixed on a microscope slide with 5 μL of eosin-Y stain (0.5% w/v). After 2 min, the sample was covered with a coverslip and examined under a light microscope (×1000 magnification with oil immersion). The proportion of live sperm in a sample of 100 sperm cells was estimated 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 velocity

Sperm velocity was estimated in 105 males (UA, $n = 37$; LA, $n = 30$; MT, $n = 38$). A two-step procedure was

followed to ensure simultaneous activation of all sperm cells (Billard & Cosson, 1992): first, we placed 20 spermatozeugmata into 10 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 40 μL solution of 150 mM KCl and 2 mg L^{-1} bovine serum albumin (Billard & Cosson, 1990). Immediately after adding the activating solution, the sperm bundles were gently broken apart (see above) 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, USA). Sperm velocity measures were based on an average of 123.8 ± 83.0 sperm tracks per sample (range 10–339, $n = 105$). These measures included: (1) average path velocity (VAP), which estimates the average velocity of sperm cells over a smoothed cell path; (2) straight line velocity (VSL), the average velocity on a straight line between the start and the end point of the track and (3) curvilinear velocity (VCL), the actual velocity along the trajectory. The threshold values defining static cells were predetermined at 20 μm s^{-1} for VAP and VCL, and 15 μm s^{-1} for VSL. These three motility measures provide an estimate of progressive velocity 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 average of 35.6 ± 9.61 sperm per field-of-view (range 18.6–72.6). For each male the motility analyses were performed on two sub-samples of the ejaculate and the mean was used in final analysis. The within-sample repeatability was 0.81, 0.80 and 0.75 for VAP, VSL and VCL respectively (Lessells & Boag, 1987).

Sperm length

To measure sperm lengths we incubated 50 μL aliquots of each male's sperm sample in a solution of 10 μL of 1% Rose Bengal for 20 min. Dyed samples were then viewed under ×1000 magnification and photographed with a digital camera. Using these photographs we estimated the mean head length and total sperm length from 15 sperm per male using image analysis software (IMAGE TOOL).

Analysis

Statistical tests were performed using SPSS v. 13. (SPSS Inc., Chicago, IL, USA) Data were checked for normality and proportions were arcsine square-root transformed prior to analysis. Correlations between variates were tested using Pearson product–moment correlation tests. The overall significance of the association between sperm motility measures and percentage of carotenoids was first tested using a multivariate analysis of covariance in which the three sperm velocity measures were the dependent variables, percentage of carotenoids and sperm concentration the covariates, and population origin a fixed factor (MANCOVA procedure, SPSS). Subsequently, the association between each sperm motility measure and percentage of carotenoids was analysed separately using ANCOVA. The association between sperm viability at stripping and after 3 h and the proportion of carotenoids was tested using a repeated measure ANCOVA. All probabilities are two-tailed.

Results

There was no significant difference in body size among the three populations (mean SL in mm ± SD: UA = 18.17 ± 1.32; LA = 18.11 ± 1.06; MT = 18.46 ± 1.57; $F_{2,158} = 1.06$, $P = 0.35$). Likewise, the sperm viability measures (i.e. proportion of live sperm at stripping and after 3 h) did not significantly differ among the three populations (mean proportion of live sperm ± SD at stripping: UA = 0.89 ± 0.03; LA = 0.89 ± 0.05; MT = 0.90 ± 0.04; after 3 h: UA = 0.70 ± 0.11; LA = 0.72 ± 0.09; MT = 0.75 ± 0.09; $F_{2,44} = 0.26$, $P = 0.77$; after 3 h: $F_{1,44} = 1.25$, $P = 0.30$); we therefore pooled these data (i.e. $n = 45$) in the subsequent analyses. However, we did find that the proportion of orange pigmentation varied among the populations (mean % carotenoids ± SD: UA = 5.30 ± 2.76; LA = 5.34 ± 3.07; MT = 7.08 ± 2.91; $F_{2,157} = 7.20$, $P < 0.01$). We therefore entered % carotenoids as a covariate in our subsequent analysis. The proportion of live sperm was high at stripping (mean proportion of live sperm ± SD: 0.89 ± 0.04) and significantly declined after 3 h (proportion of live sperm after 3 h: 0.72 ± 0.10), but this decline was less pronounced in the most colourful males (repeated measures ANOVA with time as the factor and % carotenoids entered as a covariate; time: $F_{1,43} = 29.42$, $P < 0.001$; interaction between time and carotenoids: $F_{1,43} = 6.11$, $P < 0.018$; Fig. 1).

Sperm movement was nearly rectilinear and therefore VAP and VSL, which were highly correlated ($r = 0.99$, $P < 0.001$, $n = 105$), better represented the actual velocity of sperm movement in the two-dimensional space than did VCL. In contrast, VCL was influenced by sperm concentration and showed lower variation compared with the other two sperm velocity measures (Table 1). Sperm motility measures were significantly associated with the proportion of carotenoids and did not differ among populations (MANCOVA, covariates = carotenoids: $F_{3,98} = 3.96$, $P = 0.01$, sperm concentration: $F_{3,98} = 1.90$, $P = 0.13$; fixed factor = population: $F_{6,198} = 1.26$, $P = 0.29$). After removing the nonsignificant factors (population origin and sperm concentration), the proportion of carotenoids significantly predicted sperm velocity ($F_{3,101} = 4.90$, $P = 0.003$). Univariate tests

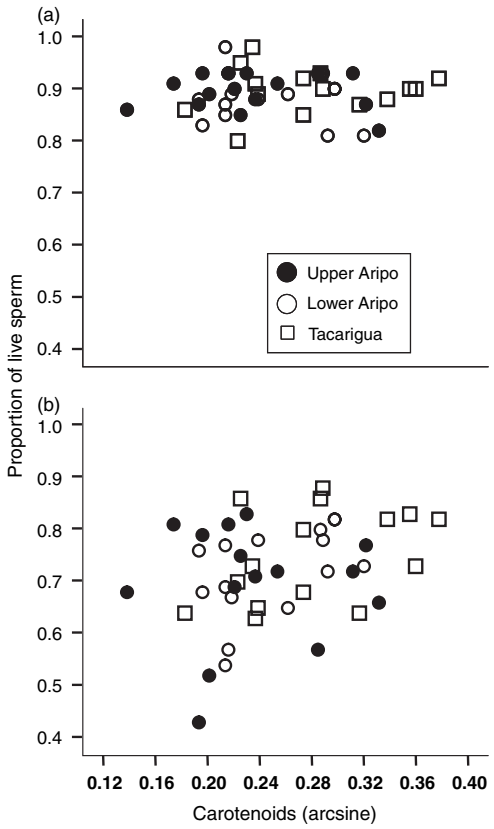

Fig. 1 Proportion of live sperm at stripping (a) and 3 h after stripping (b) in relation to the area of carotenoid spots (percentage of body area with carotenoid spots).

indicated that the two measures of sperm velocity, VAP and VSL, were significantly correlated with the relative area of carotenoid spots, whereas VCL was not (Table 1, Fig. 2). Body size (SL) was not correlated with any of the sperm velocity measure (all $r < |0.05|$, all $P > 0.62$).

Sperm length did not differ between populations (mean head length in μm ± SD: 3.91 ± 0.14; mean total length in μm ± SD: 54.19 ± 1.32; MANOVA, $F_{4,202} = 0.45$, $P = 0.77$; univariate ANOVAS, $F_{2,104} = 0.07$, $P = 0.93$ and $F_{2,104} = 0.62$, $P = 0.54$, respectively). Neither measures of sperm length was correlated with male phenotype (percentage of carotenoids and SL) or with sperm motility (all $r < |0.15|$, all $P > 0.24$).

Discussion

We found that sperm swimming speed was significantly correlated with the size of male sexual secondary characters (relative area of carotenoid spots), but not with body size in the three populations. In contrast, sperm length was not significantly associated with male phenotype or the other sperm traits. The observation that these patterns persisted in all three populations strongly suggests the relationship between sperm performance and the expression of secondary sexual traits in males is a general phenomenon in guppies. Moreover, the significant association between sperm performance (*in vitro*) and male phenotype provides a possible mechanism for previous findings that males with high levels of orange pigmentation perform well in sperm competition (Pitcher *et al.*, 2003), even when the number of sperm from rival males is controlled using artificial insemination (Evans *et al.*, 2003).

Our results also revealed that sperm longevity (as detected through our viability assays) was related to the size of carotenoid spots. A similar association between sperm longevity and male ornamentation was reported in the roach *Rutilus rutilus* (Kortet *et al.*, 2004). Interestingly, in our study this relationship between sperm viability and carotenoids was absent in the fresh samples and was only apparent in samples that were kept for 3 h after stripping. In the samples tested immediately after stripping, the proportion of live sperm was uniformly high in all subjects (range = 0.80–0.98, CV = 4.6%), whereas after 3 h we found more variation (range = 0.43–0.88, CV = 13.5%), suggesting that colourful males are able to produce sperm that live longer. This finding is likely to have important biological significance for guppies because sperm remain in the female's gonoduct for several days prior to ovulation (Constantz, 1984, 1989; Pilastro & Bisazza, 1999) or before they are stored in the

Table 1 Sperm motility measures (mean ± SD) for the three guppy populations.

Population	VAP	VSL	VCL
Upper Aripo ($n = 37$)	45.96 ± 19.99	86.42 ± 16.42	41.27 ± 20.12
Lower Aripo ($n = 30$)	42.79 ± 13.87	87.4 ± 10.02	37.93 ± 14.76
Lower Tacarigua ($n = 38$)	52.26 ± 17.57	92.46 ± 15.32	47.74 ± 17.92
Total	47.34 ± 17.81	88.89 ± 14.57	42.66 ± 18.21
Coefficient of variation	37.6%	42.7%	16.4%
ANCOVA			
Population	$F_{2,104} = 1.23$, $P = 0.30$	$F_{2,104} = 1.21$, $P = 0.30$	$F_{2,104} = 1.98$, $P = 0.14$
Carotenoids	$F_{1,104} = 7.60$, $P = 0.007$	$F_{1,104} = 8.39$, $P = 0.005$	$F_{1,104} = 0.09$, $P = 0.77$
Sperm concentration	$F_{1,104} = 3.91$, $P = 0.051$	$F_{1,104} = 3.52$, $P = 0.064$	$F_{1,104} = 4.78$, $P = 0.031$

VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

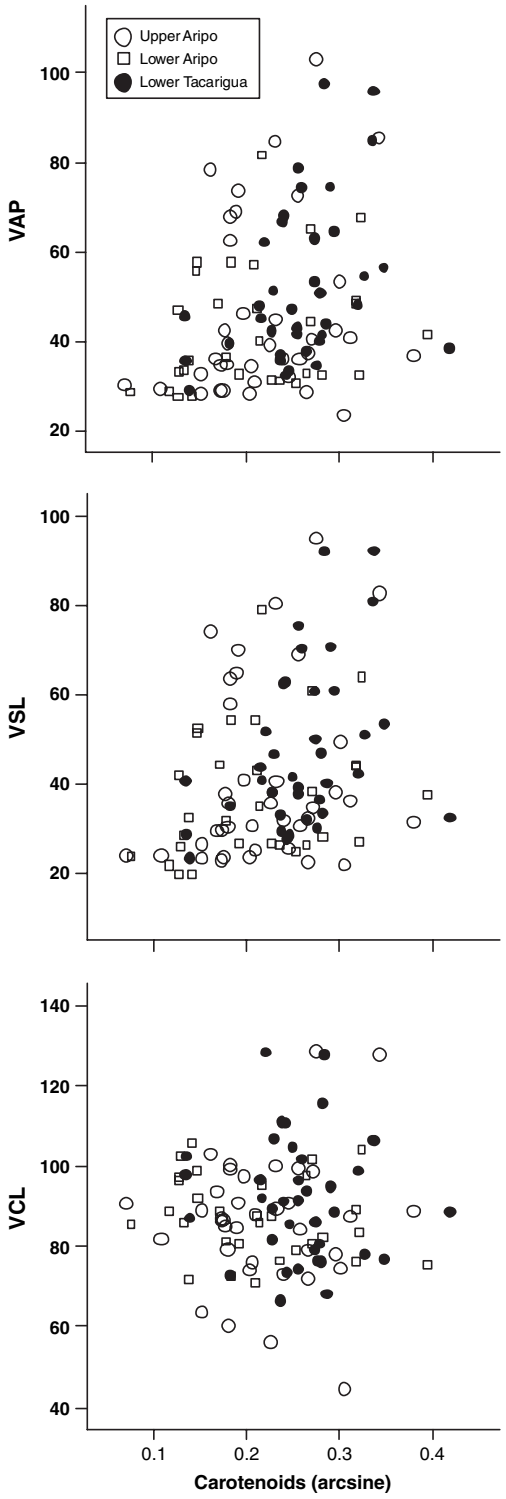

Fig. 2 Sperm motility index (average path velocity, VAP; curvilinear velocity, VCL and straight line velocity, VSL) and male phenotype (proportion of body area with carotenoid spots, arcsine transformation) in three guppy populations.

ovary where they remain viable for several months (Winge, 1937; Hildemann & Wagner, 1954). We suggest that sperm longevity is likely to be a crucial determinant of sperm competition success in guppies and eagerly await experimental tests that evaluate paternity over successive brood cycles following heterospermic (artificial) inseminations.

A positive association between sperm function and the expression of secondary sexual traits is predicted by the phenotype-linked fertility hypothesis (Sheldon, 1994). This hypothesis predicts that a male's ornaments reliably signal his fertilizing efficiency, and selection acts directly on females to choose mates that are capable of fertilizing all of their eggs. An assumption of this hypothesis, therefore, is that male infertility can limit female fecundity. Although our results are consistent with the main prediction of the PLFH, we argue that in guppies sperm limitation is unlikely to be a factor governing female mating decisions. This is because female guppies store sperm for several months and produce several consecutive broods following a single copulation (Winge, 1937; Houde, 1997 and citations therein). Nevertheless, female guppies typically mate with more than one male during each brood cycle (e.g. Pitcher *et al.*, 2003) and are able to manipulate the number of sperm transferred during solicited copulations so that relatively attractive males inseminate higher numbers of sperm (Pilastro *et al.*, 2002, 2004). Thus, rather than being limited by available sperm, female guppies actually limit the number of sperm transferred during solicited copulations and constantly avoid unsolicited (i.e. forced) copulations (Magurran & Seghers, 1994; Matthews & Magurran, 2000), during which large ejaculates can be delivered (Pilastro & Bisazza, 1999; Pilastro *et al.*, 2002).

Another possible explanation for our finding that sperm swimming velocity and longevity positively covary with the area of orange pigmentation is that male ornamentation and sperm quality both depend on a high dietary intake of antioxidants in the diet (Blount *et al.*, 2001). According to this hypothesis, diets rich in antioxidants will mitigate the deleterious effects of free radical damage (due to high rates of metabolic activity in sperm) and contribute towards the expression of antioxidant-dependent (i.e. carotenoid-rich) male display traits (Blount *et al.*, 2001). However, in the current study the possibility that male ornamentation reflected male foraging ability seems unlikely for two reasons. First, since the males used in this experiment were fed *ad libitum* with the same diet carotenoid intake was unlikely to be limited. Secondly, we measured carotenoid spot area, which has a strong genetic component (Houde, 1992; Brooks, 2000; Brooks & Endler, 2001), whereas condition mainly affects the brightness of orange spot (Kodric-Brown, 1989; Houde & Torio, 1992).

Our results, in conjunction with the recent observation that specific male pairs exhibit highly repeatable patterns of sperm precedence across different (unrelated) females

(J.P. Evans, unpublished), are more consistent with the idea that intrinsically (i.e. genetically) high quality males produce better quality sperm (Yasui, 1997). Male quality correlates with the size of orange spots in guppies (e.g. Nicoletto, 1991; van Oosterhout *et al.*, 2003; Evans *et al.*, 2004, but see Brooks, 2000) and colourful males may be able to both allocate more carotenoids to sexual secondary characters and produce better quality sperm. Future work would be necessary to determine whether the supply of carotenoids in the diet, which is known to influence male attractiveness (Grether, 2000), influences both sperm quality and competitive fertilization success following heterospermic artificial insemination (e.g. Evans *et al.*, 2003).

In conclusion, to the extent that ejaculate features such as motility and viability are heritable and reflect male condition, we suggest that indirect selection may favour females who mate with relatively attractive males via post-copulatory processes (e.g. Harvey & May, 1989; Birkhead *et al.*, 1993; Keller & Reeve, 1995; Yasui, 1997; Pizzari & Birkhead, 2002; Hosken *et al.*, 2003). This scenario is consistent with the recent finding that relatively colourful males sire offspring with enhanced survival skills (Evans *et al.*, 2004), although it is still necessary to demonstrate an explicit (genetic) link between variation in ejaculate features and male quality.

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References

Anderson, M.J., Nyholt, J. & Dixson, A.F. 2005. Sperm competition and the evolution of sperm midpiece volume in mammals. *J. Zool.* **267**: 135–142.

Billard, R. & Cosson, M.P. 1990. The energetics of fish sperm motility. In: *Controls of Sperm Motility: Biological and Clinical Aspects* (C. Gagnon ed.), pp. 155–173. CRC Press, Boca Raton, Florida.

Billard, R. & Cosson, M.P. 1992. Some problems related to the assessment of sperm motility in freshwater fish. *J. Exp. Zool.* **261**: 122–131.

Birkhead, T.R. 2000. *Promiscuity: An Evolutionary History of Sperm Competition and Sexual Conflict*. Harvard University Press, Cambridge.

Birkhead, T.R. & Møller, A.P. 1998. *Sperm Competition and Sexual Selection*. Academic Press, London.

Birkhead, T.R. & Petrie, M. 1995. Ejaculate features and sperm utilization in peafowl *Pavo cristatus*. *Proc. R. Soc. Lond. B* **261**: 153–158.

Birkhead, T.R., Møller, A.P. & Sutherland, W.J. 1993. Why do females make it so difficult for males to fertilize their eggs? *J. Theor. Biol.* **161**: 51–60.

Birkhead, T.R., Buchanan, K.L., Devoogd, T.J., Pellatt, E.J., Székely, T. & Catchpole, C.K. 1997. Song, sperm quality and testes asymmetry in the sedge warbler. *Anim. Behav.* **53**: 965–971.

Birkhead, T.R., Fletcher, F. & Pellatt, E.J. 1998. Sexual selection in the zebra finch *Taeniopygia guttata*: condition, sex traits and immune capacity. *Behav. Ecol. Sociobiol.* **44**: 179–191.

Birkhead, T.R., Martinez, J.G., Burke, T. & Froman, D.P. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proc. R. Soc. Lond. B* **266**: 1759–1764.

Blount, J.D., Møller, A.P. & Houston, D.C. 2001. Antioxidants, showy males and sperm quality. *Ecol. Lett.* **4**: 393–396.

Briskie, J.V. & Montgomerie, R. 1992. Sperm size and sperm competition in birds. *Proc. R. Soc. Lond. B* **247**: 89–95.

Brooks, R. 2000. Negative genetic correlation between male sexual attractiveness and survival. *Nature* **406**: 67–70.

Brooks, R. & Endler, J.A. 2001. Direct and indirect sexual selection and quantitative genetics of male traits in guppies (*Poecilia reticulata*). *Evolution* **55**: 1002–1015.

Burness, G., Casselman, S.J., Schulte-Hostedde, A.I., Moyes, C.D. & Montgomerie, R. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). *Behav. Ecol. Sociobiol.* **56**: 65–70.

Constantz, G.D. 1984. Sperm competition in poeciliid fishes. In: *Sperm Competition and the Evolution of Animal Mating Systems* (R.L. Smith ed.), pp. 465–485. Academic Press, London.

Constantz, G.D. 1989. Reproductive biology of poeciliid fishes. In: *Ecology and Evolution of Livebearing Fishes (Poeciliidae)* (G. K. Meffe & F. F. Snelson, eds), pp. 33–50. Prentice Hall, Englewood Cliffs.

Dewsbury, D.A. 1982. Ejaculate cost and male choice. *Am. Nat.* **119**: 601–610.

Evans, J.P. & Magurran, A.E. 2001. Patterns of sperm precedence and predictors of paternity in the Trinidadian guppy. *Proc. R. Soc. Lond. B* **268**: 719–724.

Evans, J.P., Pitcher, T.E. & Magurran, A.E. 2002. The ontogeny of courtship, colour and sperm production in male guppies. *J. Fish Biol* **60**: 495–498.

Evans, J.P., Zane, L., Francescato, S. & Pilastro, A. 2003. Directional postcopulatory sexual selection revealed by artificial insemination. *Nature* **421**: 360–363.

Evans, J.P., Kelley, J.L., Bisazza, A., Finazzo, E. & Pilastro, A. 2004. Sire attractiveness influences offspring performance in guppies. *Proc. R. Soc. Lond. B* **271**: 2035–2042.

Froman, D.P. & Feltmann, A.J. 2000. Sperm mobility: phenotype in roosters (*Gallus domesticus*) determined by concentration of motile sperm and straight line velocity. *Biol. Repr.* **62**: 303–309.

Froman, D.P., Pizzari, T., Feltmann, A.J., Castillo-Juarez, H. & Birkhead, T.R. 2002. Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, *Gallus gallus domesticus*. *Proc. R. Soc. Lond. B* **269**: 607–612.

Gage, M.J.G., Macfarlane, C.P., Yeates, S., Ward, R.G., Searle, J.B. & Parker, G.A. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the

primary determinant of fertilization success. *Curr. Biol.* **14**: 44–47.
Gardiner, D.M. 1978. Utilization of extracellular glucose by spermatozoa of two viviparous fishes. *Comp. Biochem. Physiol. A* **59A**: 165–168.
Gomendio, M., Harcourt, A.H. & Roldan, E.R.S. 1998. Sperm competition in mammals. In: *Sperm Competition and Sexual Selection* (T. R. Birkhead & A. P. Møller eds), pp. 667–756. Academic Press, London.
Grether, G.F. 2000. Carotenoid limitation and mate preference evolution: A test of the indicator hypothesis in guppies (*Poecilia reticulata*). *Evolution* **54**: 1712–1724.
Grether, G.F., Hudon, J. & Endler, J.A. 2001. Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). *Proc. R. Soc. Lond. B* **268**: 1245–1253.
Harvey, P.H. & May, R.M. 1989. Copulation dynamics – out for the sperm count. *Nature* **337**: 508–509.
Hildemann, W.H. & Wagner, E.D. 1954. Intraspecific sperm competition in *Lebistes*. *Am. Nat.* **88**: 87–91.
Hosken, D.J., Garner, T.W.J., Tregenza, T., Wedell, N. & Ward, P.I. 2003. Superior sperm competitors sire higher-quality young. *Proc. R. Soc. Lond. B* **270**: 1933–1938.
Houde, A.E. 1992. Sex-linked heritability of a sexually selected character in a natural population of *Poecilia reticulata* (Pisces: Poeciliidae) (guppies). *Heredity* **69**: 229–235.
Houde, A.E. 1997. *Sex, Color, and Mate Choice in Guppies*. Princeton University Press, Princeton, New Jersey.
Houde, A.E. & Torio, A.J. 1992. Effect of parasitic infection on male color pattern and female choice in guppies. *Behav. Ecol.* **3**: 346–351.
Keller, L. & Reeve, H.K. 1995. Why do females mate with multiple males – the sexually selected sperm hypothesis. *Adv. Stud. Behav.* **24**: 291–315.
Kodric-Brown, A. 1989. Dietary carotenoids and male mating success in the guppy: an environmental component to female choice. *Behav. Ecol. Sociobiol.* **25**: 393–401.
Kortet, R., Vainikka, A., Rantala, M.J. & Taskinen, J. 2004. Sperm quality, secondary sexual characters and parasitism in roach (*Rutilus rutilus* L.). *Biol. J. Linn. Soc.* **81**: 111–117.
Kuckuck, C. & Greven, H. 1997. Notes on the mechanically stimulated discharge of spermiozeugmata in the guppy, *Poecilia reticulata*: a quantitative approach. *Z. Fischk.* **4**: 73–88.
Lessells, C.M. & Boag, P.T. 1987. Unrepeatable repeatabilities: a common mistake. *Auk* **104**: 116–121.
Levitan, D.R. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. *Proc. R. Soc. Lond. B – Biol. Sci.* **267**: 531–534.
Liljedal, S., Folstad, I. & Skarstein, F. 1999. Secondary sex traits, parasites, immunity and ejaculate quality in the Arctic charr. *Proc. R. Soc. Lond. B* **266**: 1893–1898.
Lin, M.H., Morshedi, M., Srisombut, C., Nassar, A. & Oehninger, S. 1998. Plasma membrane integrity of cryopreserved human sperm: an investigation of the results of the hypoosmotic swelling test, the water test, and eosin-Y staining. *Fert. Steril.* **70**: 1148–1155.
Magurran, A.E. & Seghers, B.H. 1994. A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proc. R. Soc. Lond. B* **258**: 89–92.
Malo, A.F., Garde, J.J., Soler, A.J., Garcia, A.J., Gomendio, M. & Roldan, E.R.S. 2005a. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biol. Repr.* **72**: 822–829.
Malo, A.F., Roldan, E.R.S., Garde, J., Soler, A.J. & Gomendio, M. 2005b. Antlers honestly advertise sperm production and quality. *Proc. R. Soc. Lond. B* **272**: 149–158.
Matthews, I.M. & Magurran, A.E. 2000. Evidence for sperm transfer during sneaky mating in wild Trinidadian guppies. *J. Fish Biol* **56**: 1381–1386.
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.
Neff, B.D., Fu, P. & Gross, M.R. 2003. Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). *Behav. Ecol.* **14**: 634–641.
Nicoletto, P.F. 1991. The relationship between male ornamentation and swimming performance in the guppy, *Poecilia reticulata*. *Behav. Ecol. Sociobiol.* **28**: 365–370.
Olsson, M., Madson, T. & Shine, R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. R. Soc. Lond. B* **264**: 455–459.
van Oosterhout, C., Trigg, R.E., Carvalho, G.R., Magurran, A.E., Hauser, L. & Shaw, P.W. 2003. Inbreeding depression and genetic load of sexually selected traits: how the guppy lost its spots. *J. Evol. Biol.* **16**: 273–281.
Parker, G.A. 1970. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev. Cambr. Phil. Soc.* **45**: 525–567.
Parker, G.A. 1990. Sperm competition games: raffles and roles. *Proc. R. Soc. Lond. B* **242**: 120–126.
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.
Pilastro, A., Evans, J.P., Sartorelli, S. & Bisazza, A. 2002. Male phenotype predicts insemination success in guppies. *Proc. R. Soc. Lond. B* **269**: 1325–1330.
Pilastro, A., Simionato, M., Bisazza, A. & Evans, J.P. 2004. Cryptic female preference for colorful males in guppies. *Evolution* **58**: 665–669.
Pitcher, T.E. & Evans, J.P. 2001. Male phenotype and sperm number in the guppy (*Poecilia reticulata*). *Can. J. Zool.* **79**: 1891–1896.
Pitcher, T.E., Neff, B.D., Rodd, F.H. & Rowe, L. 2003. Multiple mating and sequential mate choice in guppies: females trade up. *Proc. R. Soc. Lond. B* **270**: 1623–1629.
Pitnick, S. 1993. Operational sex-ratios and sperm limitation in populations of *Drosophila pachea*. *Behav. Ecol. Sociobiol.* **33**: 383–391.
Pizzari, T. & Birkhead, T.R. 2002. The sexually-selected sperm hypothesis: sex-biased inheritance and sexual antagonism. *Biol. Rev.* **77**: 183–209.
Rasotto, M.B. & Mazzoldi, C. 2002. Male traits associated with alternative reproductive tactics in *Gobius niger*. *J. Fish Biol.* **61**: 173–184.
Rurangwa, E., Kime, D.E., Ollevier, F. & Nash, J.P. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture* **234**: 1–28.
Shapiro, D.Y., Marconato, A. & Yoshikawa, T. 1994. Sperm economy in a coral-reef fish, *Thalassoma bifasciatum*. *Ecology* **75**: 1334–1344.
Sheldon, B.C. 1994. Song rate and fertility in the chaffinch. *Anim. Behav.* **47**: 986–987.

Snook, R.R. 2005. Sperm in competition: not playing by the numbers. *Trends Ecol. Evol.* **20**: 46–53.

Uglem, I., Galloway, T.F., Rosenqvist, G. & Folstad, I. 2001. Male dimorphism, sperm traits and immunology in the corkwing wrasse (*Symphodus melops* L.). *Behav. Ecol. Sociobiol.* **50**: 511–518.

Van Voorhies, W.A. 1992. Production of sperm reduces nematode life-span. *Nature* **360**: 456–458.

Vladic, T.V. & Jarvi, T. 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. *Proc. R. Soc. Lond. B* **268**: 2375–2381.

Wedell, N., Gage, M.J.G. & Parker, G.A. 2002. Sperm competition, male prudence and sperm-limited females. *Trends Ecol. Evol.* **17**: 313–320.

Winge, O. 1927. The location of eighteen genes in *Lebistes reticulatus*. *J. Genetics* **18**: 1–43.

Winge, O. 1937. Successions of broods in *Lebistes*. *Nature* **140**: 467.

Yasui, Y. 1997. A ''good-sperm'' model can explain the evolution of costly multiple mating by females. *Am. Nat.* **149**: 573–584.

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