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.

Keywords: cryptic female choice; genetic benefits; good genes; good sperm; sexy sperm; sperm competition.

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 & Möller, 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. sneak, 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
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 spermatozoa into 10 μL of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49 mM MgCl2, 0.41 mM MgSO4, 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 mL−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-μL 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 variables 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; F3,98 = 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; F2,44 = 0.26, P = 0.77; after 3 h: F1,44 = 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; F2,157 = 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: F1,43 = 29.42, P < 0.001; interaction between time and carotenoids: F1,43 = 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: F3,98 = 3.96, P = 0.01, sperm concentration: F3,98 = 1.90, P = 0.13; fixed factor = population: F6,198 = 1.26, P = 0.29). After removing the nonsignificant factors (population origin and sperm concentration), the proportion of carotenoids significantly predicted sperm velocity (F3,101 = 4.90, P = 0.003). Univariate tests
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 $\mu$m ± SD: 3.91 ± 0.14; mean total length in $\mu$m ± SD: 54.19 ± 1.32; MANOVA, $F_{4,202} = 0.45$, $P = 0.77$; univariate ANOVA, $F_{2,104} = 0.07$, $P = 0.93$ 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

![Fig. 1](image.png)

**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).

<table>
<thead>
<tr>
<th>Population</th>
<th>VAP</th>
<th>VSL</th>
<th>VCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper Aripo ($n=37$)</td>
<td>45.96 ± 19.99</td>
<td>86.42 ± 16.42</td>
<td>41.27 ± 20.12</td>
</tr>
<tr>
<td>Lower Aripo ($n=30$)</td>
<td>42.79 ± 13.87</td>
<td>87.4 ± 10.02</td>
<td>37.93 ± 14.76</td>
</tr>
<tr>
<td>Lower Tacarigua ($n=38$)</td>
<td>52.26 ± 17.57</td>
<td>92.46 ± 15.32</td>
<td>47.74 ± 17.92</td>
</tr>
<tr>
<td>Total</td>
<td>47.34 ± 17.81</td>
<td>88.89 ± 14.57</td>
<td>42.66 ± 18.21</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>37.6%</td>
<td>42.7%</td>
<td>16.4%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANCOVA</th>
<th>$F_{2,104}$</th>
<th>$F_{2,104}$</th>
<th>$F_{2,104}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population</td>
<td>1.23, $P = 0.30$</td>
<td>1.21, $P = 0.30$</td>
<td>1.98, $P = 0.14$</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>7.60, $P = 0.007$</td>
<td>8.39, $P = 0.005$</td>
<td>0.09, $P = 0.77$</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>3.91, $P = 0.061$</td>
<td>3.52, $P = 0.064$</td>
<td>4.78, $P = 0.031$</td>
</tr>
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VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.


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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.

Acknowledgments

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