Attractive males have faster sperm in three-spined sticklebacks *Gasterosteus aculeatus*

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**Abstract** Recent studies have revealed that sexually selected traits may signal sperm quality and hence male fertilisation ability. There is also evidence that the expression of male sexual ornamentation and associated sperm characteristics depend on an individual’s ability to cope with oxidative stress. Carotenoids are known for their antioxidant properties and carotenoid-based ornaments might represent honest signals as these pigments can be traded off between the investment in sexual ornamentation, sperm function as well as immune response. In this study, we examined the relationship between sexual ornamentation (breeding coloration) and sperm characteristics (e.g., velocity and morphology) in the three-spined stickleback *Gasterosteus aculeatus*, an externally fertilising fish species, in which sperm competition commonly occurs. During the breeding season males are sperm limited and develop a conspicuous carotenoid-based coloration, which is under strong pre-copulatory sexual selection due to female mate choice and male-male competition. The results of the present study show that the expression of stickleback male breeding coloration is significantly positively associated with the linearity of sperm movement, whereas sperm morphology (head length to tail length ratio) is significantly negatively related to the trajectory of sperm movement. Moreover, there is some support for the phenotype-linked fertility hypothesis as the intensity of male red breeding coloration is significantly positively correlated with sperm velocity, which is supposed to be an important determinant of fertilisation success in external fertilisers, indicating the honesty of the sexually selected nuptial red coloration [Current Zoology 59 (6): 761–768, 2013].

**Keywords** Mate choice, Sperm competition, Carotenoids, Oxidative stress, Reproductive success, CASA ImageJ

In many animal species, males develop conspicuous secondary sexual ornaments, which often signal male quality that otherwise cannot be communicated to females directly and honestly (see Andersson, 1994 for examples). The costs underlying ornament expression are often assumed to maintain signal honesty so that females might gain indirect (i.e. genetic) as well as direct (i.e. resources and/or parental care) benefits by choosing a “high quality” male (Kirkpatrick and Ryan, 1991). Another direct benefit for females is the fertilisation ability of males. Egg production is energetically costly and it should be advantageous for a female to receive sufficient and/or high quality sperm to ensure a high probability of fertilisation.

The phenotype-linked fertility hypothesis (PLFH) predicts a positive relationship between sexual ornaments and male functional fertility (the success of ejaculates in fertilising eggs), and that females select highly ornamented males to guarantee increased fertilisation success (see Sheldon, 1994). For example, a study by Janhunen et al. (2009) showed that in male Arctic charr *Salvelinus alpinus*, the expression of the carotenoid-based red breeding coloration is positively correlated with sperm velocity and hence provides useful information to females concerning a male’s sperm fertilisation ability. The relationship between male attractiveness (e.g., orange coloration) and sperm traits (e.g., velocity and morphology) has been well studied in guppies *Poecilia reticulata*, although these studies gave contradictory results; some findings support the PLFH (Locatello et al., 2006; Pitcher et al., 2007; resulting in a greater parentage of more colourful males: Evans et al., 2003), whereas others failed to show a positive relationship between sexually selected ornamentation and sperm quality traits (Skinner and Watt, 2007; Evans, 2010). Also Pilastro et al. (2008) did not find support for PLFH in guppies as female fecundity was not influenced by male phenotype. In birds for instance the relationship between sexual ornaments and sperm quality traits is equally ambiguous. For example, Peters et al.
(2004) found a positive relationship between a male’s carotenoid-based coloration and sperm velocity in mallards Anas platyrhynchos, whereas Rowe et al. (2010) found a negative relationship between pre- and postcopulatory traits in the red-backed fairy-wrens Malurus melanocephalus.

We used the three-spined stickleback as study species. In this small fish, males develop a conspicuous carotenoid-based breeding coloration, which plays an important role in male-male competition and female mate choice (e.g., McLennan and McPhail, 1990; Bakker and Milinski, 1993; Bakker, 2010). During the breeding season (April–August), reproductively active males build a tunnel-shaped nest composed of filamentous algae. Once nest-building has been completed the male attempts to acquire clutches of several females over a period of three or more days after which only the male cares for the developing embryos (Wootton, 1984). The stealing of fertilisations (sneaking) is a widespread phenomenon in our study species (e.g., Largiadèr et al., 2001), resulting in a strong risk of sperm competition. This stresses the importance of producing high quality sperm, as especially in externally fertilising species it is expected that sperm with higher swimming abilities will have a higher probability to encounter an unfertilised egg more rapidly (reviewed in Pizzari and Parker, 2009).

In sticklebacks spermatogenesis is completed before the start of the breeding season (November–January) and sperm supply is not renewed during the reproductive phase (see Borg, 1982 for details), resulting in a larger number of mature sperm stored in testes of virgin males (early spring) compared to those of multiply mated males (late summer) (Zbinden et al., 2001). As stickleback males are sperm limited over the course of one breeding season, careful sperm allocation is a critical component of reproductive success (e.g., Zbinden et al., 2004). In addition, both the intensity of breeding coloration (Bakker and Milinski, 1993) and of gonadal investment (Cubillos and Guderley, 2000) have a positive influence on a stickleback male’s reproductive success and under non-competitive mating conditions the expression of male breeding coloration was found to be positively correlated with the proportion of fertilised eggs (Pike et al., 2010). The well-studied complex reproductive behaviour of sticklebacks makes this fish ideal to study the relationships among traits under pre- and post-copulatory sexual selection. More specifically, the aim of the present study was to test the relationships among male breeding coloration (trait under pre-copulatory sexual selection due to female mate choice), sperm motility traits (e.g., velocity) and sperm morphology (traits under post-copulatory sexual selection due to sperm competition).

## 1 Material and Methods

### 1.1 Experimental subjects

We conducted the experiments between May and August 2011, using both one-year-old laboratory-bred and wild-caught three-spined stickleback males from a large anadromous population (Texel, the Netherlands).

Laboratory-bred fish were the F1-progeny of randomly crossed sticklebacks caught in April 2010. In June 2010 one male was allowed to spawn with one female to achieve different full-sib groups, which were raised in holding tanks measuring 50 cm × 30 cm × 30 cm (length × width × height) under standardised laboratory conditions (light-dark cycle: 8L:16D, temperature: 17 ± 1°C). During the first month of life juveniles received Artemia nauplii and were fed daily with red mosquito larvae (Chironomus spp.) later on. In May 2011 the light regime was changed to summer conditions (16L:8D) because in sticklebacks sexual maturation is stimulated by long photoperiods (Borg et al., 2004). To avoid pseudo-replication only one male per family was used in the subsequent experiments (see below).

Wild-caught fish were collected during their spring migration in April 2011. They were fed daily with red mosquito larvae and kept in a large outdoor-tank (750 L) with a constant supply of fresh tap-water (3 L min⁻¹). However, during the experimental phase (see below) holding conditions were the same for both laboratory-bred and wild-caught males.

### 1.2 Experimental procedure

Males that showed initial signs of nuptial coloration (laboratory-bred n=15; wild-caught n=22) were isolated under standardised conditions (16L:8D, 17 ± 1°C) in single tanks 30 cm × 20 cm × 20 cm, each equipped with a sand-filled petridish (Ø 9 cm) and 2 g of green cotton threads (length 30 ± 10 mm; Gütermann, colour 8065) for standardised nest-building. Males were visually isolated from each other by grey opaque partitions and during the experiments they were fed with red mosquito larvae daily ad libitum. Naturally occurring red mosquito larvae contain a.o. lutein (Czeczuga, 1970). This is one of the major carotenoid pigments for the development of male breeding coloration in stickleback males (e.g., Wedekind et al., 1998). It is known that animals cannot synthesise carotenoids de novo
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(Goodwin, 1984), so that the given food supply was the only carotenoid source. Nest-building was stimulated by presenting a gravid female daily for 15 min in a transparent container in front of the tanks (e.g., Mehlis et al., 2010).

1.3 Measurement of breeding coloration

Shortly after nest-building was finished (mean ± standard deviation: 3.08 ± 1.66 days after isolation) the expression of male breeding coloration, which is then at its peak, was quantified by measuring spectral reflectance of the skin part located centrally on the red-coloured cheek directly below the eye (breeding coloration, see Fig. 1). In most stickleback populations females use male red breeding coloration in mate choice (e.g., McLennan and McPhail, 1990), which can also be assumed for females from our study population as they show an increased behavioural sensitivity to visual signals in the 'red' part of the spectrum (Rick et al., 2011).

Fig. 1  Mean reflectance ± standard error (%) between 300 and 700 nm of the cheek region (A) of reproductively active males

To determine the expression of breeding coloration males were stimulated with a receptive female for 15 min as described above. Thereafter, males were immediately placed on a piece of black fabric outside the water and spectral reflectance scans of the left red cheek were taken below the eye relative to a 98% Spectralon white standard with a spectrophotometer (Avantes AvaSpec-2048) connected to a deuterium-halogen light source (Avantes DH-S). For each male twenty replicate measurements were made in succession without changing probe contact (Avantes AvaSoft 7.5), imported into Microsoft Excel and averaged for further analysis. The whole procedure did not harm the fish and took about one minute so that short-term colour changes could be ruled out (see Rick et al., 2013). As a reliable measure of carotenoid pigmentation (see also Rick et al., 2011) we determined the colorimetric variable red chroma, which is defined as the amount of light reflected in the range of 575-700 nm relative to the total amount of light in the range of 300-700 nm.

1.4 Measurement of sperm quality traits

Sperm motility was quantified on average two weeks after isolation (mean ± standard deviation: 14.84 ± 4.51 days). Therefore, males were quickly killed by decapitation and testes were removed because sperm stripping is not possible in three-spined sticklebacks. Testes of three-spined stickleback males contain almost exclusively spermatozoa during the breeding season (see Borg, 1982 for details). Consequently, sperm motility measurements were solely performed on mature sperm. All males used in the present study were about one year old and released sperm twice in the two weeks after isolation as they were used in another study as well (M. Mehlis in prep.). Nevertheless, all individuals were handled the same way so that the effect for the current study is identical for all individuals.

The right testis of each male was pestled in 500 µl artificial ‘ovarian fluid’ (3.0 g NaCl, 0.1g KCl, 0.07 g CaCl2 in 1 L aqua dest.; after Elofsson et al., 2006), which was set at a constant temperature (16 °C; Thriller, PEQLAB_V0410E). Exactly two minutes later a Leja counting chamber (depth 12 µm) was loaded with 3 µl of the mixed sperm suspension and sperm movement was tracked (25 fps) at 320 x magnification (Motie microscope B3 Professional Series; VIDO camera CC540X via iMovie). Sperm were filmed at ten different positions for three seconds each. These randomly selected positions followed a sigmoid pattern, which was equally distributed across the whole chamber. Bakker et al. (2006) showed that in sticklebacks it takes about ten minutes to completely fertilise one clutch, which implies that the time point at which sperm movement was measured in the current study is relevant for fertilisation under natural conditions.

Sperm motility (i.e. velocity and linearity) determination was based on six randomly chosen one-second-lasting sequences for each male, which were analysed using the CASA plug-in in ImageJ. Accordingly, we achieved the mean values of the following variables, which were based on the movement of about hundred single sperm (mean ± standard deviation: 95.62 ± 58.17 sperm) for each male: (1) velocity curvilinear (VCL [µm s⁻¹]), (2) velocity average path (VAP [µm s⁻¹]), (3) velocity straight line (VSL [µm s⁻¹]), (4) straightness (STR=VSL/VAP × 100 [%]), (5) linearity (LIN=VSL/VCL × 100 [%]) and (6) wobble (side to side movement of the sperm head; WOB=VAP/VCL × 100 [%]).
The threshold values for excluding immotile sperm were specified as 10 µm s\(^{-1}\) for VCL, 5 µm s\(^{-1}\) for VAP and 2 µm s\(^{-1}\) for VSL.

The measured variables that describe sperm velocity (VCL, VAP and VSL) were significantly positively correlated with each other (Pearson correlations: \(n=37, r_p=0.602, \text{all } P<0.001\)), which was also true for the values that represent sperm linearity, i.e. the trajectory of sperm movement (LIN, STR (exponentially transformed to achieve normal distribution) and WOB; Pearson correlations: \(n=37, r_p=0.362, \text{all } P<0.028\)). Since no information is available on which are the best predictors of sperm movement in sticklebacks two principal components analyses (Kaiser criterion: eigenvalues > 1) were conducted in order to obtain a one-dimensional variable for sperm velocity (eigenvalue: 1.59; proportion of variance: 83.9%) and for sperm linearity (eigenvalue: 1.52; proportion of variance: 76.6%). There was a significant positive relationship between PC1 of sperm velocity and VCL, VAP and VSL (Pearson correlations: \(n=37, r_p=0.811, \text{all } P<0.001\)) as well as between PC1 of sperm linearity and LIN, STR and WOB (Pearson correlations: \(n=37, r_p=0.681, \text{all } P<0.001\)).

Sperm concentration was not standardised during sperm motility measurements; the total number of stored sperm differed between males (range: \(1.74 \times 10^7 – 6.72 \times 10^7\)). Thus, we additionally recorded the number of sperm cells that were detected by CASA and the results revealed that during the motility measurements sperm density was not related to sperm velocity (Pearson correlations: \(n=37, r_p=-0.107, P=0.527\)) nor sperm linearity (Pearson correlations: \(n=37, r_p=0.136, P=0.422\)).

Sperm morphology variables (head length, hl, including mid-piece, and tail length, tl) were determined measuring 30 sperm per male via ImageJ. Therefore, 5 µl of the sperm suspension that was used for motility determination were fixed on a glass slide with a cover slip using nail polish. Afterwards, we photographed sperm at 1000 × magnification via cell\(^D\) 5.1 (Olympus) with a camera (Olympus ColorView IIIu) mounted on a microscope (Olympus BX51). We calculated the repeatability of the measurements of sperm morphology traits and its standard error as described in Mehlis et al. (2012). Three randomly chosen sperm were measured twice for each male (10 % of total sample size), resulting in high repeatability values (head length: 85.03% ± 0.54%; tail length: 98.92% ± 0.04%).

### 1.5 Statistical analysis

For statistical analyses linear mixed effect models (“lme”) were conducted using the ‘lme’ command of the ’nlme’ library (Pinheiro et al., 2009) of the R 2.9.1 statistical package. We used both sperm velocity and sperm linearity as dependent variables. Red chroma as a measure of intensity of the male red breeding coloration (see Rick et al., 2011) and sperm head to tail length ratio ((hl/tl); see Humphries et al., 2008) were used as explanatory variables (see Table 1 for an overview of all conducted models). Furthermore, we included male origin (laboratory-bred or wild-caught) as random factor and never removed it from the final model to control for the different rearing conditions prior to the experimental trials. As the use of “lme” requires that the dependent variable is normally distributed, we checked the residuals of the best explaining model for normal distribution according to Kolmogorov-Smirnov tests. All reported p-values are two-tailed.

## 2 Results

Sperm velocity was significantly explained by breeding coloration (red chroma) (“lme”: \(n=37, \chi^2=4.391, P=0.036\); Table 1) with more intensely red coloured males having significantly faster sperm (Pearson correlation: \(n=37, r_p=0.335, P=0.043\); Fig. 2A), whereas sperm morphology (head to tail length ratio) was not significantly associated with sperm velocity (“lme”: \(n=37, \chi^2=0.694, P=0.405\); Table 1). In addition, sperm linearity was significantly explained by red chroma (“lme”: \(n=37, \chi^2=6.631, P=0.010\); Table 1) as well as sperm morphology (“lme”: \(n=37, \chi^2=11.206, P<0.001\); Table 1). The relationship between red chroma and sperm linearity was significantly positive, with more intensely coloured males having sperm that swim in a

| Dependent variable | Explanatory variable, breeding coloration (red chroma) | Random factor, origin of males (laboratory-bred or wild-caught) | Explanatory variable, sperm morphology (head to tail length ratio) | Random factor, origin of males (laboratory-bred or wild-caught) |
|--------------------|-------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| sperm velocity     | \(4.391\) \(\text{P-value} = 0.036\)                 | \(<0.001\) \(\text{P-value} = 0.999\)                        | \(0.694\) \(\text{P-value} = 0.405\)                        | \(<0.001\) \(\text{P-value} = 0.999\)                        |
| sperm linearity    | \(6.631\) \(\text{P-value} = 0.010\)                 | \(<0.001\) \(\text{P-value} = 0.999\)                        | \(11.206\) \(\text{P-value} = <0.001\)                      | \(<0.001\) \(\text{P-value} = 0.999\)                        |

Significant results are printed in bold.
more linear path (Pearson correlation: \( n=37, r_p=0.406, P=0.013; \) Fig. 2B). The head to tail length ratio was significantly negatively correlated with sperm linearity (Pearson correlation: \( n=37, r_p=-0.512, P=0.001; \) Fig. 2C), which means that sperm with a longer tail in relation to the head swam in a more linear path. However, there seems to be no link between the investment in breeding coloration and sperm morphology (Pearson correlation: \( n=37, r_p=-0.096, P=0.572).\) Moreover, faster sperm also showed a more linear swimming path (Pearson correlation: \( n=37, r_p=0.345, P=0.037).\)

![Fig. 2 Relationship between (A) sperm velocity and breeding coloration (red chroma); (B) sperm linearity and breeding coloration (red chroma); (C) sperm linearity and sperm morphology (head to tail length ratio)](image)

3 Discussion

The results of the present study revealed that in three-spined sticklebacks sperm velocity is positively correlated with sperm linearity as well as with a sexually selected male trait, the intensity of red breeding coloration. Thus, we showed correlative evidence for pre-copulatory sexual selection on sperm characteristics in sticklebacks. The positive relationship between male ornamentation and sperm swimming ability is in accordance with theoretical predictions of the phenotype-linked fertility hypothesis (Sheldon, 1994). There are two explanations for the observed positive association. First, males that can afford to invest a high amount of carotenoids in the development and maintenance of their breeding coloration may also have better protected sperm (e.g., Blount et al., 2001). Second, there may be a genetic coupling between carotenoid-based coloration and sperm quality traits (e.g., Chargé et al., 2013). Further investigations are necessary in order to elucidate which of these explanations apply to the stickleback breeding system.

In general, the PLFH is controversially discussed since recent studies found no or even a negative relationship between pre- and postcopulatory selected traits (Kortet et al., 2004; Pitcher et al., 2009). A negative correlation is predicted by sperm competition theory because disfavoured males should invest more in spermatogenesis than in sexual ornamentation to compensate their limited access to females (Parker, 1990). In addition, a recent meta-analysis found little support for a direct benefit to females by choosing a more attractive male at least as to fertility assurance (Mautz et al., 2013). However, a recent study on sticklebacks supported the PLFH (Pike et al., 2010). By manipulating dietary access to carotenoids the authors showed that the fertilisation success of male sticklebacks under non-competitive conditions is positively related to the expression of the red breeding coloration. Male coloration may thus act as an indicator of functional fertility (Pike et al., 2010). Pike et al. (2010) measured the proportion of fertilised eggs without focussing on potential underlying mechanisms, such as sperm quality traits. The results of the present study reveal that an enhanced sperm velocity and linearity may act as a proxy for the increased functional fertility (proportion of fertilised eggs) observed by Pike et al. (2010).

A recent study on great tits Parus major showed that the expression of male sexual ornamentation and associated sperm characteristics are determined by an individual’s ability to cope with oxidative stress (Helfenstein et al., 2010), revealing a potential proximate mechanism underlying the observed positive relationship between ornaments and sperm characteristics in
our study (see also von Schantz et al., 1999; Garratt and Brooks, 2012). It is known that allocation trade-offs of carotenoid antioxidants, which represent the main component of stickleback male breeding coloration (Wedekind et al., 1998; Pike et al., 2011), are capable of mediating the expression of condition-dependent sexual ornaments (Blount et al., 2003; Pike et al., 2007; but see Hartley and Kennedy, 2004).

Sperm cells are highly prone to suffer from oxidative stress leading to negative impacts on males’ fertilisation abilities (Blount et al., 2001; Velando et al., 2008; Dowling and Simmons, 2009). For instance, sperm plasma membrane and DNA can be impaired by oxidative stress, resulting in reduced fertilisation efficiency and competitiveness of sperm (Aitken and Baker, 2006; Poiani, 2006). Moreover, the antioxidant capacity of seminal fluid can have an impact on sperm motility and viability, thereby serving as a protective agent against oxidative damage (den Boer et al., 2010; Simmons et al., 2011). However, seminal and plasma oxidative status as well as sperm DNA damage were not the focus of the present study. Further research on the underlying mechanisms is required in order to strengthen the role of oxidative stress in the relationship between male sexual traits observed in the present study.

Sperm competition (post-copulatory sexual selection) is a widespread and powerful selective force, which affects sperm quantity and/or sperm quality in such a way that competitive fertilisation success is maximised (Snook, 2005; Rowe and Pruett-Jones, 2011). Sperm velocity can be a determinant of male fertility and sperm competitive ability. Especially in breeding systems with external fertilisation, it is expected that sperm with a higher swimming ability will have a higher probability to encounter an unfertilised egg more rapidly (see Pizzari and Parker, 2009). Thus, the intensity of stickleback male breeding coloration may act as an honest signal, which provides females with important information on a male’s fertilisation ability. Three-spined stickleback males face an increased risk of sperm competition (e.g., offspring quality) and the mechanisms underlying the linkage of the studied traits is required in order to make safer assumptions about whether the phenotype-linked fertility hypothesis accounts for sticklebacks.

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