

Sexually attractive males produce relatively more sons in a fish with external fertilisation

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Abstract

According to the mate attractiveness hypothesis of biased progeny sex-ratio, sexually attractive males should produce relatively more sons. We tested this hypothesis with three-spined sticklebacks *Gasterosteus aculeatus*, a fish species with external fertilisation, in which the intensity of red breeding coloration is a sexually selected trait. We used reflecto-spectrophotometry and visual modelling to assess red breeding coloration from a stickleback's point of view. More intensely red-coloured males produced relatively more sons as assessed with molecular sexing three weeks after hatching of 34 clutches from different parents. Confounding factors like mortality rate and maternal and paternal effects could largely be ruled out. We discuss potential mechanisms for the role of fathers in sex-ratio allocation.

Introduction

Fisher's sex ratio theory (Fisher 1930) assumes equal resource allocation to daughters and sons resulting in a 1:1 female:male progeny sex ratio. There are various theoretically predicted exceptions to this rule (see e.g. Davies et al. 2012), the most appealing is the Trivers-Willard hypothesis that predicts a male-biased offspring sex-ratio when mothers would produce above-average attractive sons that would have a benefit in sexual selection via male-male competition or female mate choice (Trivers and Willard 1973). The hypothesis was initially developed for mammals where sons' attractiveness (condition) is regulated via the condition of the mother. Red deer are a classical empirical example of the Trivers-Willard hypothesis (Clutton-Brock et al. 1984, 1986). The Trivers-Willard hypothesis was extended to the mate attractiveness hypothesis by considering male traits that are generally under sexual selection (Burley 1981, 1986). Recent meta-analyses of tests of the mate attractiveness hypothesis in songbirds support the hypothesis (Booksmith et al. 2017; Szász et al. 2019). The relationship between mate attractiveness and brood sex-ratio was independent of the proportion of extra-pair paternity (Szász et al. 2019). Thus, mate quality dependent sex-ratio adjustment may have evolved by direct rather than indirect genetic benefits (Szász et al. 2019). The ultimate causes of sex-ratio variation in vertebrates, especially in relation to paternal attractiveness, are still poorly understood (Krackow 1995; Pen and Weissing 2000; Fawcett et al. 2007).

Examples of a skewed sex-ratio are bird biased and proximate causes usually restricted to offspring sex-ratio manipulation by the mother, which is the heterogametic sex. In taxa where males are the heterogametic sex, males can theoretically manipulate offspring sex-ratio by producing unequal amounts of X- and Y-bearing sperm (Malo et al. 2017; Douhard 2018). Here we focus on offspring sex-ratio in a species with external fertilization and male parental care, the three-spined stickleback, *Gasterosteus aculeatus* L. It is well-studied and recognised as a supermodel species for evolutionary biology (Gibson 2005; Barber and Nettleship 2010). We were interested in whether attractive males produce a male-biased offspring sex-ratio as predicted by Trivers and Willard (1973) for mammals and Burley (1981, 1986) for birds. Attractiveness in sticklebacks is among others related to the intensity of

male red breeding coloration (e.g. Milinski and Bakker 1990; Rick and Bakker 2008). We would therefore expect that redder males would produce relatively more sons.

Material and Methods

Three-spined stickleback, *Gasterosteus aculeatus* L., parents originated from an anadromous population. They were caught during spring migration in April 2008 on the island of Texel, the Netherlands (see for details Rick et al. 2011; Mehlis and Bakker 2013). Under standardised summer laboratory conditions (day length 16L:8D, temperature $17 \pm 1^\circ\text{C}$) isolated, randomly sampled males were allowed to build a nest and to reproduce with randomly sampled females of the same population. Male body-size and -mass were determined both before nest building and right after spectrophotometrical measurements that were made two hours after fertilization (see below). Female body-size and -mass were determined before and after spawning. Body condition (CF) was calculated after Bolger and Connolly (1989), i.e. $\{\text{body mass (g)} * 100 / \text{body size (cm)}^3\}$. Fish were fed daily with defrosted *Chironomus* sp. larvae in excess. Clutches were removed from the nest two hours after fertilization, the mass of 10 eggs was quantified, and the number of eggs was counted 24 h after fertilisation. Clutches were split into two halves, and each half was put into a 1 l vial with tap water and air supply. Water was replenished daily. After hatching, each clutch was raised in two separate full-sib groups per parental pair. Initially, juveniles were fed daily with *Artemia* nauplii. At the age of three weeks, group sizes were reduced to 50 randomly chosen individuals per tank and used in other experiments (Rick et al. 2011; Mehlis and Bakker 2013). Of the surplus of juveniles, 30 per parental pair (about 15 of each full-sib group) were randomly chosen and conserved in 96% ethanol for sexing. If there were less than 30 left, all remaining juveniles were conserved. In this way we collected juveniles (mean 28.47 ± 0.67 SE) from 34 different parental pairs for sex-ratio analysis. For assessing mortality rate, we distinguished between mortality rate before hatching, that is, the relative number of fertilized eggs that did not hatch, and mortality rate in the first three weeks after hatching. The latter was assessed by daily removing dead juveniles in the vials. For analyses, we summed the mortalities and used % total mortality.

Colour measurement

Male coloration of the orange-red cheek region below the eye was quantified two hours after fertilisation by reflecto-spectrophotometry (see for details Rick et al. 2011). In short, 15 reflectance scans between 300 and 700 nm were made in quick succession with a 200 μm probe held at a 90° angle to the body. Measurements were taken from the orange-red cheek region directly below the eye. The intensity of the male's red breeding coloration as viewed through the visual system of stickleback females was calculated with a physiological model based on stickleback cone absorbance maxima (Rowe et al. 2004). Red chromaticity was calculated as an estimate of red intensity (Endler and Mielke 2005). Details and assumptions of the eye model are given in Rick et al. (2011).

Testis and sperm traits

Right after the quantification of male coloration, males were quickly killed by decapitation and their testes excised. The testes were weighed to the nearest milligram in order to determine the gonadosomatic index (GSI) after de Vlaming et al. (1982), i.e. $\{\text{testes mass (mg)} * 100 / \text{body mass (mg)}\}$. The left testis was used to estimate male sperm number, the right testis to measure sperm morphology using scanning electron microscopy (for details see Mehlis and Bakker 2013). Per male 20 sperm were measured (median 20, quartiles 18, 21). Sperm variables that were measured included sperm head length (hl), sperm head width (hw), sperm mid-piece length (ml), sperm mid-piece width (mw), and sperm tail length (tl). The variables were used to calculate head-to-tail-length ratio $\{(hl + ml) / tl\}$ and mid-piece volume $\{\pi * (mw / 2)^2 * ml\}$ in μm^3 as proxies of sperm motility (see Bakker et al. 2014).

Molecular sexing

Sexes of the progeny were determined with a PCR-based sex test developed by Bakker et al. (2017). In short, genomic DNA was extracted from whole juveniles by using a Chelex resin. PCR amplifications were done using the forward primer N2-1 (ACC TGT GAG TGT CAG AGA TG) and the reverse primer Alt-1 rev (GTC GTT GCC ATC GCC CCG). On a mini agarose gel, males are characterised by two bands (95 bp and 335 bp) whereas females only show the 95 bp band (for details see Bakker et al. 2017).

Statistics

Statistical analyses were performed using R 4.5.1 (R Core Team 2025). Data were tested for normality (Shapiro-Wilk normality test) and homogeneity of variances (Levene test). Red chromaticity was Box-Cox transformed to the power of -5.0428 and % total mortality to the power of 0.1692 to meet normality assumptions. Correlations between male and female traits were calculated using Pearson correlation coefficients. Deviation from a 0.5 sex ratio in the population was tested with a chi-square test. We constructed a linear model with sex ratio as dependent variable and various father traits (red chromaticity, CF, body size, GSI, head-to-tail-length ratio, mid-piece volume), mother traits (CF after spawning, number of eggs, size of the eggs) and relative mortality of the offspring as explaining variables. We used backward elimination to perform a stepwise model reduction where nonsignificant variables were removed in the order of their statistical relevance. The alpha level of statistical tests was set at 0.05.

Results

We analysed 34 stickleback pairs and their offspring (Table 1). Offspring sex-ratio of the sample population did not significantly deviate from 50% (chi-square test, $\chi^2 = 38.55$, $df = 33$, $p = 0.233$; Table 1).

More intensely red-coloured males produced offspring with a more male biased sex-ratio (lm: $p = 0.0329$; Table 2, Fig. 1, regression line: $r = -0.385$, $p = 0.0245$). Male traits other than red chromaticity (body condition, GSI, various sperm morphology traits) had no significant effect on offspring sex-ratio (Table S1, 2, all $p > 0.05$). Mother traits (egg size, egg number, body condition) also had no significant effect on offspring sex-ratio (Table S1, 2, all $p > 0.05$).

lm(sex.ratio ~ chromaticity + headtotail.ratio + mass.10.eggs), multiple $R^2 = 0.272$, adjusted $R^2 = 0.248$, $F_{3,30}=3.30$, $p = 0.0337$

Offspring mortality was low (mean % \pm SE before hatching: 2.90% \pm 0.71, after hatching: 3.47% \pm 0.36, total 6.37% \pm 0.74; Table 1) and not correlated to the red intensity of the father (before hatching $r = -0.281$, $p = 0.108$; after hatching $r = -0.001$, $p = 0.955$; total $r = -0.275$, $p = 0.115$). None of the male traits that we measured were significantly correlated with any of the measured female traits (all $p > 0.05$).

Discussion

We found that three-spined stickleback males with a more intensive red breeding coloration produced relative more male offspring. The intensity of male breeding coloration is an important determinant of male attractiveness in three-spined sticklebacks (e.g. McLennan and McPhail 1989; Milinski and Bakker 1990; Bakker 1993; Bakker and Mundwiler 1994; Künzler and Bakker 2001; Rick and Bakker 2008; Pike 2018). Thus, our results are in agreement with the mate attractiveness hypothesis of biased offspring sex-ratio (Burley 1981, 1986). The hypothesis was mainly tested and supported in birds (Booksmith et al. 2017; Szász et al. 2019). In fishes, guppies, *Poecilia reticulata*, support the mate attractiveness hypothesis. In contrast to the present study species, this fish species has internal fertilization. Progeny of attractive guppy males (that is, males with extensive orange spots or long tails) was relatively male biased (Karino et al. 2006; Karino and Sato 2009; Sato and Karino 2011). Sex allocation was adaptive as sons of male-biased broods had higher reproductive success in large broods (Sato and Karino 2017). Females were responsible for the manipulation of offspring sex ratio (Sato and Karino 2010).

Confounding effect on the relationship between male attractiveness and offspring sex-ratio could largely be excluded in our study. Male and female juveniles may have different survival rates as established for instance in brown trout (Morán et al. 2016). We assessed sex ratio relatively shortly after hatching. Survival rates of juveniles were high and not significantly correlated to the red intensity of fathers' breeding coloration. Also confounding effects of female quality traits (mass of egg, egg number, body condition) were not significant. Cryptic female-choice through the effects of ovarian fluid surrounding the eggs, which can modify sperm and sperm competition (e.g. Gasparini et al. 2020; Hadlow et al. 2020, 2023; Pitnick et al. 2020; Cattelan et al. 2023; Nusbaumer et al. 2023), were largely ruled out as females were ready to spawn and mating was random.

In the present study, random mating of gravid females and external fertilization suggest that sex-ratio manipulation was driven by the father. Several mechanisms are possible (Douhard 2018). First, X- and Y-bearing sperm numbers in the ejaculate may be biased. Second, ejaculates of males may differ in the competitiveness of X- and Y-bearing sperm. Third, fertilisation and/or development may be biased with respect to sex through chemicals in the seminal fluid. Three-spined sticklebacks have a XY sex-determination system (Peichel et al. 2004). It is unknown whether the head-volume of Y-bearing sperm is smaller than that of X-bearing sperm as is the case in bovine sperm (van Munster et al. 1999). If we assume that this is the case, the head-to-tail-length ratio will probably be lower for Y-bearing sperm and

thereby the fertilisation success greater (Humphries et al. 2008). We have further to assume that attractive stickleback males have relatively longer (i.e., lower head-to-tail-length ratio) Y-bearing sperm than duller males. In sticklebacks, the sperm of more intensely red-coloured sticklebacks were faster and swam in a more linear path, which was related to the head-to-tail-length ratio (Mehlis et al. 2013). In a dietary experiment using sticklebacks, the intensity of male breeding coloration of well-fed males was positively correlated with sperm velocity, which was correlated with paternity in sperm competition experiments (Mehlis et al. 2015; see also Pike et al. 2010).

Haploid selection within ejaculates may be far more common than we have previously assumed (Kekäläinen 2022). In mice, partial deletions of the Y chromosome, caused that X- and Y-bearing sperm had differential motility and morphology but were equally able to fertilize the egg, thus resulting in a biased offspring sex ratio (Rathje 2019).

It is unknown whether there is non-parity of X- and Y-bearing sperm in sticklebacks let alone that the degree of non-parity would be related to male attractiveness. There are some cases of inequality of X- and Y-bearing sperm numbers, for instance ejaculates of male pygmy hippopotamus, *Choeropsis liberiensis*, contain about 43% Y-bearing sperm (Saragusty et al. 2012) whereas ejaculates of mice, *Mus musculus*, contained on average about 52% Y-bearing sperm with large variation between males (range 45–58%) (Edwards et al. 2016). In the white-footed mouse, *Peromyscus leucopus*, high-quality fathers (males with relatively low inbreeding) produced a higher proportion of sons via changes in the proportion of X- and Y-bearing sperm (Malo et al. 2017).

Post-release conditions can exert significant selection on existing intra-male phenotypic variation of sperm (Marshall 2015; Reinhardt et al. 2015; Hadlow et al. 2023) and thus potentially also on the fertilisation chances of X- and Y-bearing sperm. Regarding the mate attractiveness hypothesis of biased offspring sex-ratio, carotenoids in the testes and seminal fluid may be of particular interest. The red male breeding coloration of stickleback is made up of carotenoids (Wedekind et al. 1998; Pike et al. 2007, 2010, 2011; Mehlis et al. 2015; Pike 2018), which have an anti-oxidative capacity (e.g. Krinsky 1998; von Schantz et al. 1999). Stickleback males fed supplementary carotenoids had a more intense red breeding coloration and higher fertilization success in a non-competitive context (Pike et al. 2010). The anti-oxidant capacity of the testes of high-carotenoid diet males was significantly greater than those of low-carotenoid diet males (Pike et al. 2010). However, other stickleback studies found a trade-off between the male's red breeding coloration (in all these cases measured as the relative size of the red area) and the anti-oxidant capacity of the testes (and other tissues) as deduced from oxidative DNA damage in muscle, testes and sperm during the peak breeding season (Kim and Velando 2020), juvenile viability (Kim and Velando 2016) and reduced reproductive investment and a shorter lifespan when carotenoid intake is limited (Pike et al. 2007).

In conclusion, attractive stickleback males in terms of the intensity of red nuptial coloration produced relatively more male progeny. Differential embryo and post-hatching mortality and influences of the mother on the biased sex-ratio could largely be ruled out. The paternal sex-ratio manipulation opens new

research avenues concerning the underlying mechanisms ranging from biased numbers of X- and Y-bearing sperm, performance of X- and Y-bearing sperm to genetic associations between attractiveness and sex ratio. Intriguing genetic effects had been detected in stalk-eyed flies, *Teleopsis dalmanni*, in which male eyespan, a sexually selected trait, is a signal of meiotic drive (Cotton et al. 2014) and genomic regions that influence sterility also influence sperm length and sex ratio (Wilkinson et al. 2014).

Declarations

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Authors' contributions

TCMB and MM-R conceived and designed the study, MM-R and BM collected the data, MM-R and TCMB analysed the data, TCMB and MM-R wrote the manuscript, all authors approved the final draft.

Data availability

The datasets generated during this study are available in the supplementary files.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the use of animals were followed. Animal care and experimental procedures were in accordance with the legal requirements of Germany. No additional license was required for this study.

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Figures

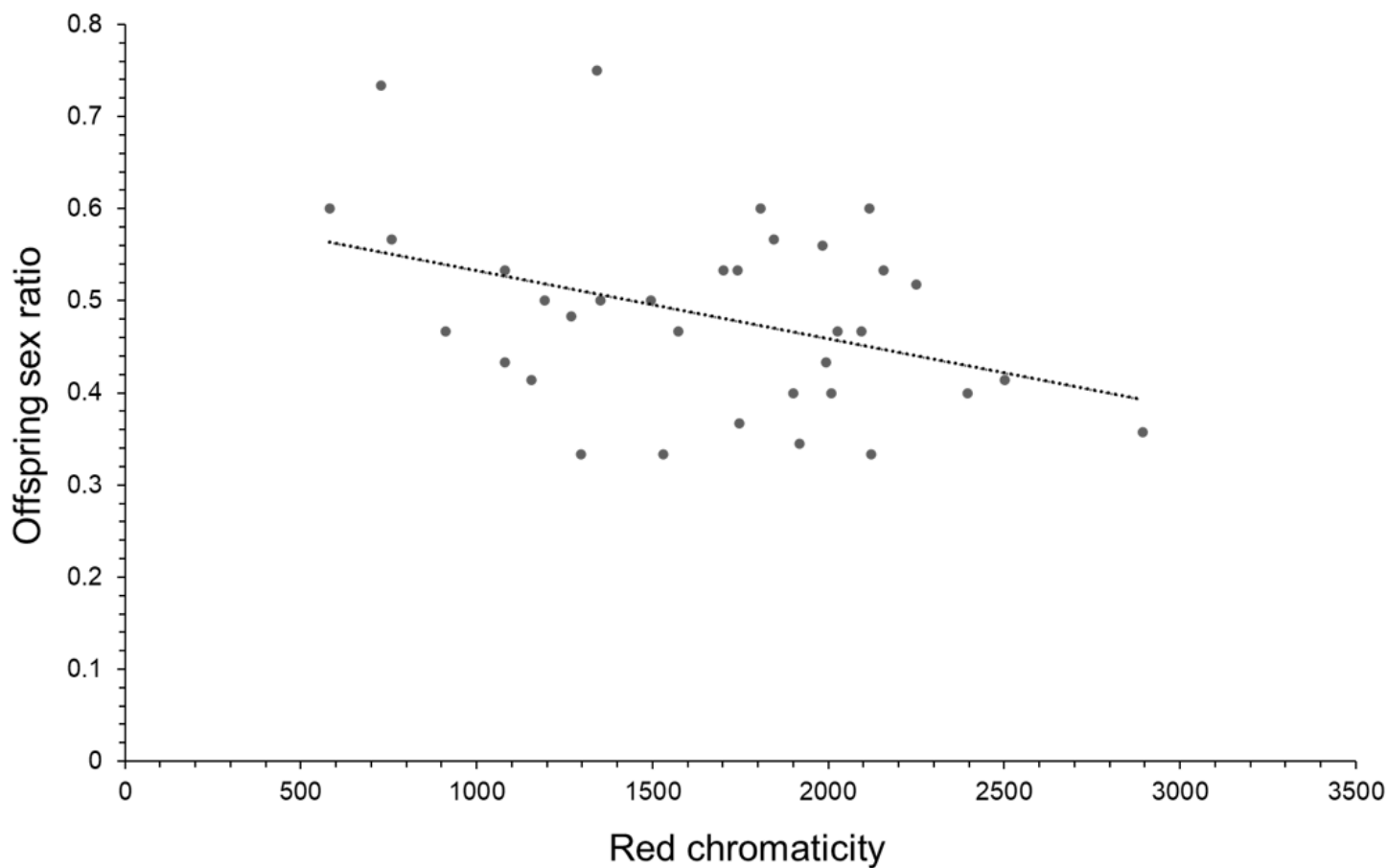


Figure 1

Relationship between the intensity of male red breeding coloration (chromaticity, Box-Cox transformed; greater values indicate more intense red coloration) and offspring sex-ratio (proportion of males). The line is the least-square linear regression

Supplementary Files

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