

Male coloration signals direct benefits in the European bitterling (*Rhodeus amarus*)

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Abstract Female mating preferences are frequently associated with exaggerated male sexual traits. In the European bitterling, *Rhodeus amarus*, a fish with a resource-based mating system, male coloration is not associated with indirect genetic benefits of female mate choice, and does not reliably signal spawning site quality. We tested a link between the extent of male carotenoid-based coloration and testis size and number of spermatozoa stripped from the testes. Male body size predicted spermatozoa number, but less reliably than the extent of male coloration. Male color was a highly significant predictor of spermatozoa number, with approximately 26 % of variance in the number of spermatozoa stripped from males predicted from male color after controlling for male body size. Body size, but not coloration, predicted teste size. Female bitterling often risk sperm limitation, especially during pair spawnings, and male nuptial coloration may be under direct selection through female mate choice as a signal of male fertilization efficiency.

Keywords Oviposition · Mate choice · Mating system · Nuptial coloration · Sexual selection · Spermatozoa

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Introduction

Expression of exaggerated traits is a feature of the mating system of a wide range of taxa. These traits include bright coloration, ornaments, odors, calls and elaborate behaviors. They are typically, though not exclusively, associated with males and have evolved through selection on mate preferences in the opposite sex, and are thus distinct from weapons, which evolve through intra-sexual selection (Andersson 1994). Ornaments potentially signal a range of benefits to prospective mates, and these can be categorized as either direct or indirect. Direct benefits of mate choice are those that promote female welfare or reproductive productivity. Examples include better access to food, assistance with parental care, access to oviposition sites, and protection from harassment or predation. In addition, and more controversially, selection may act on female preferences for “indirect” benefits, based on the genetic quality of potential mates (Kirkpatrick 1982; Eberhard 1996). “Good genes” (more correctly “good allele”) models predict additive genetic benefits of mate choice, while “compatible genes” (“compatible allele”) models predict that genetic benefits are non-additive and only a particular combination of complementary parental alleles generating fitness benefits in the offspring (Neff and Pitcher 2005; Kempenaers 2007). Both direct and indirect benefits to mate choice may operate in the same mating system, and they may be advertised by single or multiple signals. A key direct benefit of mate choice for females is male fertility, particularly for taxa with external fertilization (Levitan 1998). There is evidence that sexually selected ornaments signal fertility in some taxa (Mjelstad 1991; Matthews et al. 1997; Peters et al. 2004).

Both direct and indirect selection act on female mate preferences in the mating system of the European bitterling, *Rhodeus amarus*, a small freshwater fish that spawns and incubates its eggs in the gills of freshwater mussels. Male *R. amarus* compete for territories around mussels and court females (Smith et al. 2004; Konečná et al. 2010). Males that control access to mussels enjoy high reproductive success (Reichard et al. 2004, 2005). In *R. amarus*, and the closely related rose bitterling, *Rhodeus ocellatus*, male dominance is determined by size (Reichard et al. 2008; Casalini et al. 2009), with smaller males adopting alternative mating tactics, although these roles are not fixed and male mating behavior is opportunistic in both species (Kano 2000; Candolin and Reynolds 2001; Smith et al. 2002). Females have long ovipositors that they use to place one to eight eggs in the gill chamber of a mussel in a single spawning act, with females capable of multiple spawnings each day. Males release sperm over the inhalant siphon of the mussel and fertilized eggs develop inside the mussel gill where they are protected and ventilated. After completing development, juvenile bitterling emerge from mussels after approximately 1 month (Smith et al. 2004). The mortality rate of developing eggs and embryos in mussel gills varies with density, with eggs suffocating at high densities (Smith et al. 2001; Reichard et al. 2006, 2007a). Female oviposition decisions represent a key mating decision with direct fitness consequences for the female (Smith et al. 2000, 2001). Female bitterling are choosy about which mussels they use for oviposition and prefer mussels that do not already host eggs and embryos, thereby ensuring higher egg and embryo survival (Smith et al. 2000). Dissolved oxygen concentration in the exhalant current is the probable cue used by females to assess mussel quality (Smith et al. 2001).

Female bitterling are also choosy about mates. Mate choice decisions are based on olfactory cues that identify male genetic compatibility (*sensu* Neff and Pitcher 2005), such that females spawn preferentially with males with alleles complementary to their own. Female mate preferences are adaptive, with significant differences in embryo survival rates between embryos fathered by genetically compatible and incompatible males (Agbali et al. 2010; Reichard et al. 2012). Sperm competition is also a key feature of the mating system (Smith et al. 2009; Pateman-Jones et al. 2011; Spence et al. 2013). Notably, female *R. amarus* risk sperm-limitation during spawning, with some eggs sometimes going unfertilized when a female mates with only a single male (Smith and Reichard 2005).

Females show an unusual “skimming” behavior in which they perform a spawning action over a mussel, but without inserting their ovipositor. This behavior elicits male ejaculatory behavior (Smith et al. 2007), and encourages additional males to participate in a mating (Smith and Reichard 2005; Reichard et al. 2007b; Spence et al. 2013).

Male bitterling express striking carotenoid-based nuptial coloration that influences female behavior in both *R. amarus* and *R. ocellatus* (Candolin and Reynolds 2001; Casalini et al. 2009). Despite a considerable number of studies on the mating system of the bitterling fishes the functional role of male coloration has proven elusive. Mate choice in the rose bitterling (*Rhodeus ocellatus*) was associated with non-additive genetic benefits (Agbali et al. 2010), with female mating preferences correlated with the degree of functional MHC dissimilarity between males and females (Agbali et al. 2010; Reichard et al. 2012). The cues used by females in mate choice decisions appeared to be primarily olfactory (Agbali 2011), though male courtship vigor and color were also associated with mating preferences (Casalini et al. 2009). In European bitterling the intensity of red coloration was associated with female inspection of mussels, though not spawning (Candolin and Reynolds 2001). Male coloration did not accurately reflect the quality of mussels guarded by a male (Candolin and Reynolds 2001; Casalini 2012), and mussel quality as a site of incubation for her eggs is believed to be a key determinant of the decision by females to spawn (Smith et al. 2001; Reichard et al. 2005; Agbali et al. 2012). Male coloration may function in male-male interactions, for example as a signal of vigor in contests over territories. However, male success in territorial fights appears to be predominantly determined by male size (Reichard et al. 2005, 2008; Casalini et al. 2009, 2010), though Smith et al. (2002) found male size, color and mating success to be correlated in a natural population of *R. amarus*. Selection gradients for the extent of red eye coloration in *R. amarus* was under positive selection in experimental populations with a spatially clumped distribution of host mussels, though not with a regular distribution, indicating that male-male competition or male ability to monopolize resources, was associated with the extent of red in the eye, though no such effect was demonstrated for body color intensity (Reichard et al. 2008).

In the present study we investigated the role of male color signals in the European bitterling to evaluate whether color conveys information concerning male

reproductive quality. We tested whether male nuptial coloration functions as a signal of male fertility in the European bitterling, and predicted a relationship between the extent and intensity of male carotenoid coloration and 1. male testis size, and 2. spermatozoa number in the testes.

Materials and methods

Study subjects

Approximately 100 male European bitterling were collected by electrofishing from the River Kyjovka in the southeast of the Czech Republic, at the approximate centre of the range of this species in Europe (Zaki et al. 2008; Bryja et al. 2010). Collection took place during May 2012, at the peak of the spawning season. After collection, fish were transported to the Institute of Vertebrate Biology Brno, Czech Republic and stored in four 1,000 L outdoor tubs for 3 days. While housed in tubs they were fed with frozen chironomid larvae and frozen copepods and were also able to forage on algae and small invertebrates. They were exposed to females but were not provided with mussels as spawning sites.

Quantifying spermatozoa number

A random subsample of 25 males were individually killed with an overdose of 2-methylquinoline and immediately photographed using a Canon EOS 350D with a 60 mm macro lens under standard light conditions. After photography male body length (from the tip of the snout to the base of the caudal fin) was measured to the nearest 1 mm. Each fish was dried around the genital pore and stripped of all available sperm by pressing the abdomen towards the vent. Sperm was mixed in 10 ml of teleost saline (Yokoi et al. 2008) and a 1 ml subsample of this sperm solution was diluted with a further 9 ml of saline. The concentration of sperm in each diluted suspension was quantified by performing a count of individual spermatozoa. The sample was gently mixed and a subsample pipetted onto a haemocytometer (Neubauer improved, VWR International). A count was made of the number of spermatozoa in the sample using a binocular microscope at 40x magnification. Counts were made of sperm cells in five $1 \times 1 \times 0.1$ mm squares to obtain an estimate of mean spermatozoa density and thereby an

estimate of total spermatozoa stripped from each male. Although stripping sperm from males is not a lethal procedure it does demand extensive handling of subjects and fish were euthanized for welfare reasons.

Quantifying testis weight

An additional random subsample of 30 males were individually killed with an overdose of 2-methylquinoline and immediately photographed and measured. After photography, fish were individually fixed in 4 % buffered formaldehyde. After fixation of tissues the fish were carefully dissected under binocular microscope and the testes removed and weighed to the nearest 1 mg. The same males were not used to quantify spermatozoa number and testis weight lest stripping sperm had an impact on testis weight, and to ensure statistical independence of data.

Quantifying male coloration

The extent and saturation of nuptial coloration was scored blind from digital images using an ordinal 5-point scale by 3 independent observers using criteria for *R. ocellatus* from Casalini et al. (2009). Color ranking among the three experimenters was highly correlated (all $P < 0.001$). The median color score of each fish was used for analysis.

Statistical analysis

All data were normally distributed (Shapiro-Wilk test). Since color data were ordinal, correlations were calculated with Spearman's method. Multiple regression analysis was used to predict spermatozoa number and testis weight using male body length and coloration score as predictor variables. Semi-partial correlation was used to examine how variance in spermatozoa number and testis weight was partitioned between predictor variables. Statistical analysis was conducted using SPSS v.19.

Results

Spermatozoa number

There was a positive correlation between the two predictor variables of body length and color, though the correlation coefficient ($r_{24} = 0.54$) did not indicate high

multicollinearity between the two (Fig. 1). The overall multiple regression to predict spermatozoa number from body length and color gave an estimate of $R = 0.90$ (adjusted $R^2 = 0.80$). The regression model was highly significant ($F_{2,22} = 49.70, P < 0.001$).

Coloration was a highly significant predictor of spermatozoa number when body length was controlled statistically ($t_{22} = 5.58, P < 0.001$) (Fig. 2a). The squared semi-partial correlation coefficient ($sr^2 = 0.26$) indicated that approximately 26 % of variance in the number of spermatozoa stripped from males was uniquely predictable from male color after controlling for male body length. Body length was also significantly predictive of spermatozoa number when color was statistically controlled ($t_{22} = 3.98, P = 0.001$) (Fig. 2b). A relatively lower proportion of variance in spermatozoa number was explained by body length alone ($sr^2 = 0.13$).

Testis weight

Body length and coloration were weakly correlated ($r = 0.26$) suggesting low multicollinearity in the testis weight dataset (Fig. 3). The overall multiple regression to predict testis weight from male body length and coloration gave an estimate of $R=0.91$ (adjusted $R^2 = 0.82$), with the regression model highly significant ($F_{2,27} = 68.74, P < 0.001$). Color was not a significant predictor of testis weight after adjusting for body length ($t_{27} = 0.77, P = 0.450$) (Fig. 4a). Body length was a highly significant predictor of testis weight when coloration was statistically controlled ($t_{27} = 11.11, P < 0.001$) and body length explained a high proportion of variance



Fig. 1 Correlation between body length (mm) and median color rank for males used to measure number of spermatozoa stripped from testes

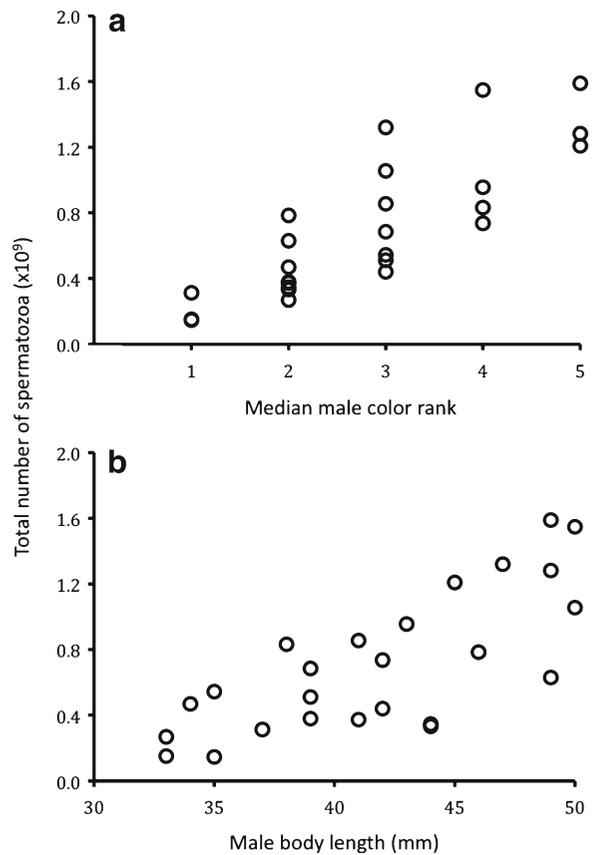


Fig. 2 Total number of spermatozoa stripped from males ($\times 10^9$) as a function of **a** median color rank **b** body length (mm)

in testis weight after controlling for the limited correlation with male color ($sr^2 = 0.75$) (Fig. 4b).

Discussion

The aim of this study was to examine whether male nuptial coloration functions as a signal of male fertility in the European bitterling. We predicted that the extent and intensity of male carotenoid coloration would correlate with testis size and number of spermatozoa stripped from the testes. Our results showed that male coloration reliably predicted number of spermatozoa, but not testis size. Male body size correlated strongly with testis size, and also significantly predicted number of spermatozoa, but less reliably than coloration alone.

The bitterling mode of reproduction is unusual, with fertilization taking place in the gill chamber of a living bivalve mollusk. In pair spawnings there is evidence that not all eggs are always fertilized (Smith and Reichard

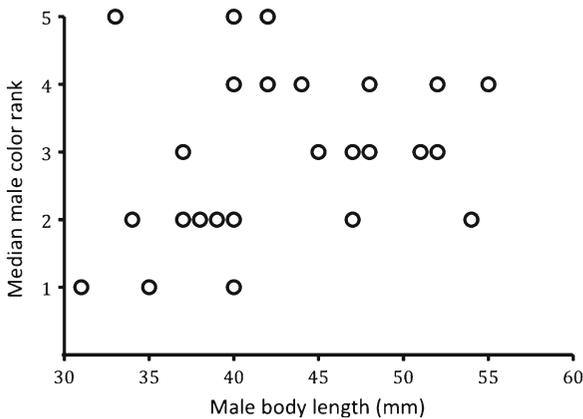


Fig. 3 Correlation between body length (mm) and median color rank for males used to measure testis weight

2005), raising the possibility that female reproductive success may be sperm limited and that mating with more extensively-colored males may enhance fecundity. Sperm limitation is believed to be a feature of many mating

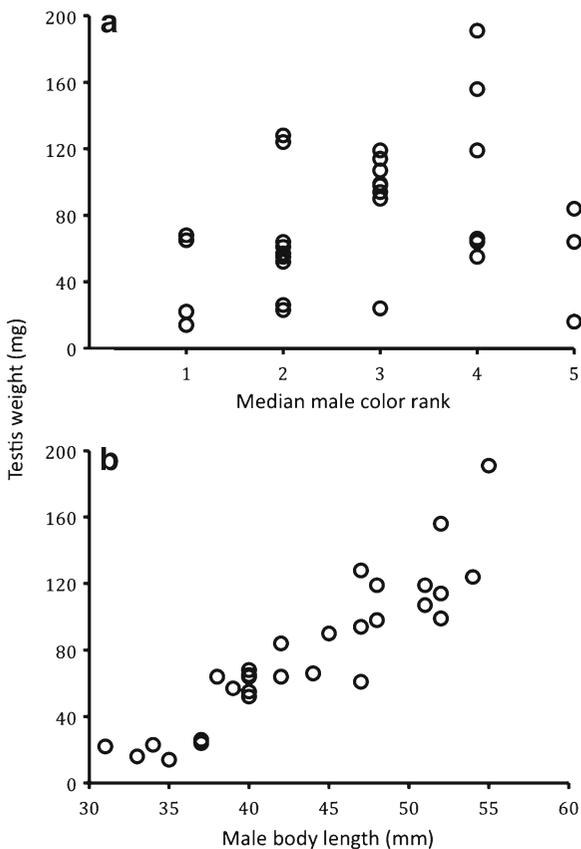


Fig. 4 Testis weight (mg) as a function of **a** median color rank **b** body length (mm)

systems (Eberhard 1996; Wedell et al. 2002), but may be a particular problem for bitterling since the spermatozoa must pass across the mussel gill surface in order to reach the eggs; the female deposits her eggs in the mussel exhalant siphon while the male releases sperm over the inhalant siphon (Smith et al. 2004). Territory-holding male bitterling risk sperm depletion over consecutive matings (Smith et al. 2009). However, males have the capacity to control ejaculate size (Pateman-Jones et al. 2011), and may modulate sperm release in order to conserve spermatozoa, potentially resulting in an intersexual conflict over ejaculate size (Smith et al. 2007, 2009). These conditions favor selection for an honest male signal of spermatozoa abundance, signaling a direct fitness benefit to females through fertilization efficiency. Our results showed male color and not body size to be the most reliable indicator of spermatozoa number, though body size independently of color did explain a significant proportion of variance in number of spermatozoa stripped from males. In a field study of European bitterling mating tactics, Smith et al. (2002) observed that females showed the highest rate of spawning with the largest and most colorful males, though mussel quality was the ultimate determinant of female oviposition choice.

The functional link between male nuptial coloration, which is typically carotenoid based, and male fertility has been proposed as arising through the antioxidant action of carotenoid pigments, which can have a protective effect on developing gametes (Blount et al. 2001). Carotenoids are obtained solely in the diet by vertebrates and have a number of critical physiological functions, making them vital, and possibly limiting, components of the diet. These features make carotenoid-based coloration a potentially honest indication of individual condition and foraging ability (Olson and Owens 1998). In the three-spined stickleback (*Gasterosteus aculeatus*) males that received a carotenoid-rich diet had higher fertilization success than controls, and expressed more intense red nuptial coloration (Pike et al. 2010). Similarly, in the guppy (*Poecilia reticulata*) sperm traits associated with fertility were significantly correlated with the extent of orange coloration in males (Locatello et al. 2006). Male color and body size have been linked to male fertility traits in a range of taxa (Peters et al. 2004; Skinner and Watt 2007; Janhunen et al. 2009; Helfenstein et al. 2010; Rasotto et al. 2010), also suggesting that overall male viability, including fertility, may be reflected by size and coloration.

A caveat to our conclusions is that we did not compare ejaculate size among males, but instead quantified the number of spermatozoa obtained from males by stripping sperm. Stripping sperm from males has been used routinely in comparable studies with fish (e.g. Gage et al. 1995; Janhunen et al. 2009), and appears to be a reliable index of the total volume of sperm available to a male for mating. While ejaculate size is a more direct measure for female fitness, it is extremely variable among ejaculations in the bitterling (Smith et al. 2009). Ejaculate size is affected by acute sperm depletion (Linklater et al. 2007), female quality, female behavior, and the presence of male rivals, potentially making ejaculate size an unreliable measure of sperm availability in some males (Wedell et al. 2002).

A second caveat concerns the approach adopted to assess bitterling coloration, since the method used in the present study ignores differences between human and bitterling perception of color. Human assessment of fish color in experimental studies is typically justified on the basis that human and teleost absorption spectra, while not identical, are similar (Frischnecht 1993; Rowe et al. 2006). While we measured high concordance among observers scoring fish for color, we were unable to judge whether the visual cues used by human observers agree with those of other bitterling. In a study of male throat redness in the three-spined stickleback (*Gasterosteus aculeatus*), Rowe et al. (2006) concluded that human and stickleback assessment of red coloration were comparable. Thus, without spectrometric measurements of bitterling color, the technique we have used to score male bitterling appears adequate. For greater reliability, future studies should comprise fish images acquired without chromatic bias, and integration of spectrometric measurements with bitterling photoreceptor sensitivities.

In conclusion, male nuptial coloration in the European bitterling was shown to correlate with the number of spermatozoa stripped from males. Body size also correlated with spermatozoa number, but explained less variance than color alone. Total testis weight was explained by male size, but not color. Male nuptial coloration may be under direct selection through female mating preferences as a signal of male fertilization efficiency. Thus male color, in combination with other traits that signal male genetic compatibility, may play a role in the oviposition decisions of female bitterling.

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