Dietary carotenoid availability, sexual signalling and functional fertility in sticklebacks

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In species where males express carotenoid-based sexual signals, more intensely coloured males may be signalling their enhanced ability to combat oxidative stress. This may include mitigating deleterious oxidative damage to their sperm, and so be directly related to their functional fertility. Using a split-clutch in vitro fertilization technique and dietary carotenoid manipulation, we demonstrate that in non-competitive fertilization assays, male three-spined sticklebacks (Gasterosteus aculeatus) that are fed higher (but biologically relevant) levels of carotenoids had a significantly increased fertilization success, irrespective of maternal carotenoid intake. Furthermore, within diet groups, a male’s fertilization success was positively related to the expression of his carotenoid-based nuptial coloration, with more intensely coloured males having higher functional fertility. These data provide, to our knowledge, the first demonstration that dietary access to carotenoids influences fertilization success, and suggest that females could use a male’s nuptial coloration as an indicator of his functional fertility.

Keywords: fertility; carotenoid coloration; antioxidants; oxidative stress

1. INTRODUCTION
It has been hypothesized that a male’s secondary sexual ornamentation may provide females with cues about his functional fertility (the success of ejaculates in fertilizing eggs; Sheldon 1994). This could potentially explain the maintenance of female preference for male ornaments as a result of direct selection in non-resource-based mating systems, where the indirect benefits have remained elusive (Kotiaho & Puurtinen 2007). A number of studies have presented correlative evidence for a link between secondary sexual signals and functional fertility (or, as a proxy, sperm characteristics) in various taxa (e.g. Malo et al. 2005; Pitcher et al. 2007; Polak & Simmons 2009). However, direct evidence linking functional fertility and ornamentation is lacking, although a number of potential physiological explanations for such a link have been proposed. Here, we test the hypothesis (Blount et al. 2001; Veloño et al. 2008) that antioxidant availability may be limited for the expression of sexual ornaments and the maintenance of fertility; if antioxidants are limited, allocation of antioxidants to sexual signals may trade off against antioxidant protection of developing gametes, an important factor affecting DNA damage in the male germ line (Lewis & Aitken 2005; Olsen et al. 2005; reviewed in Veloño et al. 2008). Carotenoids, with their dual role as both antioxidants and pigments involved in the formation of many sexual ornaments (Krinsky 1998; Olson & Owens 1998; von Schantz et al. 1999), have been proposed to play a key role in this process (Blount et al. 2001), leading us to predict a direct association between dietary carotenoid availability and functional fertility.

We tested this predication using a split-clutch in vitro fertilization (IVF) technique (Barber & Arnott 2000) in three-spined sticklebacks (Gasterosteus aculeatus). During the breeding season, male sticklebacks develop a region of red nuptial coloration based on carotenoids that are limited in the diet (Barber et al. 2000), and in mate-choice trials females generally prefer males expressing more intense, carotenoid-rich coloration (Wootton 1984). Males obtaining lower levels of carotenoids in the diet cannot maintain their red coloration over the breeding season and show a significantly higher susceptibility to oxidative stress, suggesting that the carotenoid coloration is a signal of somatic antioxidant defences (Pike et al. 2007a; Lindström et al. 2009).

2. MATERIAL AND METHODS
Juvenile three-spined sticklebacks were reared until sexual maturation on a diet based on antioxidant-free fish-feed pellets supplemented with either high (200 μg g−1) or low (10 μg g−1) levels of carotenoids (astaxanthin, lutein and zeaxanthin), but which were otherwise nutritionally identical. Rearing conditions and diet preparation were as described in Pike et al. (2007a). Twenty sexually mature males from each diet treatment were processed in a random order with respect to diet treatment. Eggs were obtained from a sample of gravid females (n = 10 from each diet treatment) that had recently given a ‘head-up’ response to a courting male (an indication of their readiness to spawn; Wootton 1984) by gently squeezing the abdomen. Each clutch was then carefully divided into two approximately equal halves (mean ± s.e.: 46.6 ± 8.5 eggs in each half; paired t-test: t6 = 0.83, p = 0.42) using fine tweezers and each half transferred into separate, narrow glass tubes, one containing sperm from a randomly selected male on a high-carotenoid diet and the other containing sperm from a randomly selected low-carotenoid diet male. Sperm and eggs were mixed in 1 ml aerated distilled water. Males were processed in a random order and the body condition index was calculated as mass/length6 (Bolger & Connolly 1989) and standardised reflectance scans of their nuptial coloration were taken (Pike et al. 2007b). These were used to calculate signal chroma (colour saturation; Pike et al. 2007b), which is known to predict the concentration of carotenoids that an individual invests in sexual ornamentation (R2 = 0.61; T. W. Pike 2005, unpublished data from biochemical analysis) and mating success (Pike et al. 2007b).

Following reflectance measurements, males were immediately killed with an overdose of anaesthesia (benzocaine) and dissected to remove the testes and seminal vesicles, which were transferred to sterilized watch glasses and macerated using fine forceps in 1 ml aerated distilled water. Males were processed in a random order and the body condition index was calculated as mass/length6 (Bolger & Connolly 1989) and standardised reflectance scans of their nuptial coloration were taken (Pike et al. 2007b). These were used to calculate signal chroma (colour saturation; Pike et al. 2007b), which is known to predict the concentration of carotenoids that an individual invests in sexual ornamentation (R2 = 0.61; T. W. Pike 2005, unpublished data from biochemical analysis) and mating success (Pike et al. 2007b).

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quantity of methylene blue (2.5 μg ml\(^{-1}\)) to reduce fungal infection (Barber & Arnott 2000); they were then incubated for 24 h at 15 ± 1 °C. The total number of eggs in each half clutch and the number of these that were fertilized (Swarup 1958) were then counted using a binocular microscope (Bakker et al. 2006). Functional fertility was defined as the proportion of fertilized eggs (Bakker et al. 2006). Tests were extracted from a further sample of males (n = 10 from each diet treatment), and tested for total antioxidant activity using the Trolox equivalent antioxidant capacity (TEAC) assay (Rice-Evans & Miller 1994). Higher scores indicate that an individual has a greater total antioxidant capacity (measured in millimoles of Trolox equivalents per length).

Complete methodological details are provided in the electronic supplementary material. The experiment was performed under licence from the UK Home Office.

3. RESULTS

Male sticklebacks fed with high levels of carotenoids had a significantly higher functional fertility than those fed with lower carotenoid levels (linear mixed-effects model, \(F_{1,17} = 6.73, p = 0.019\); figure 1), independent of maternal carotenoid intake (\(F_{1,17} = 0.99, p = 0.33\)) or male body condition (\(F_{1,17} = 0.02, p = 0.90\)). Moreover, the chroma of a male’s sexual ornamentation was significantly positively related to functional fertility (\(F_{1,17} = 7.03, p = 0.017\); figure 1), with males expressing more intense regions of nuptial coloration, in both diet treatment groups, fertilizing a greater proportion of eggs. Chroma also differed significantly between males on the two diet treatments (two-sample t-test, \(t_{38} = 3.21, p = 0.003\)); with low-carotenoid diet males actually developing more intense coloration than males on the high-carotenoid diet (figure 1). Body condition did not differ between treatment groups (mean ± s.e.: high, 14.1 ± 1.14; low, 12.8 ± 0.95; two-sample t-test, \(t_{38} = 0.91, p = 0.37\)).

The testes of high-carotenoid diet males had a significantly greater antioxidant capacity than those of males on the low-carotenoid diet (high: 0.12 ± 0.02 mmol l\(^{-1}\); low: 0.06 ± 0.01 mmol l\(^{-1}\); \(t_{18} = 2.46, p = 0.024\)).

4. DISCUSSION

The finding that males with a lower intake of carotenoids actually developed the more intense nuptial coloration might seem counterintuitive, but is actually in line with theoretical predictions: we have recently shown that stickleback males in poor condition should prioritize their investment in sexual ornaments at the start of the breeding season (as was the case in the present experiment), even at the expense of future survival, as this will maximize their chances of reproducing (Lindström et al. 2009). It is also consistent with the finding that breeding males on the low-carotenoid diet put a far greater proportion of their total body carotenoids into their throat patch than males on a high-carotenoid diet, but accumulate more oxidative damage and die sooner as a result (Pike et al. 2007a). The present study shows that this was also associated with an effect on male functional fertility, where the half of a female’s egg mass fertilized by males from the high-carotenoid diet treatment contained a significantly higher proportion of fertilized eggs than the half fertilized by low-carotenoid diet males.

![Figure 1. The relationship between sexual signal chroma and functional fertility (percentage of fertilized eggs) in males on high- (black points, solid line) and low-carotenoid (white points, dashed line) diet treatments. Least-squares regression lines are shown.](http://rsbl.royalsocietypublishing.org/Downloaded from http://rsbl.royalsocietypublishing.org/ on April 5, 2017)
for trade-offs using cross-sectional data will typically yield correlations in the opposite direction to those predicted by life-history theory (e.g. Hamel et al. 2009). Furthermore, honest signalling need not be a handicap (Getty 1998), and individuals with superior ability to acquire or assimilate dietary carotenoids may be able to simultaneously increase both nuptial coloration and functional fertility. The fact that males of poor quality, or in poorer condition as affected by carotenoid availability, cannot sustain their red coloration during the breeding season (Pike et al. 2007a; Lindström et al. 2009) suggests that, as time progresses, negative correlations between nuptial coloration and functional fertility may arise.

Female sticklebacks from most populations, including our own (Pike et al. 2007a), show a preference for redder males (Wootton 1984). Our data provide, to our knowledge, the first demonstration that dietary access to carotenoids influences functional fertility, consistent with Blount et al.’s (2001) hypothesis. Therefore, by selecting males with the most intense nuptial coloration, females may be able to select for mates with the greatest functional fertility (cf. Sheldon 1994).

The experiment was performed under licence from the UK Home Office.

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