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How integument colour reflects its carotenoid content: a stickleback's perspective

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Summary

1. Carotenoid-based signals typically vary in both the total concentration of carotenoids deposited and the relative quantities of different constituent carotenoids. As these constituents often have differing spectral properties, the relative and absolute concentrations of different carotenoids deposited in a signal can significantly affect the spectrum of light reflected. A critical but rarely tested assumption of hypotheses concerning the information content of carotenoid-based signals is that their colour directly reveals the concentration and composition of constituent carotenoids to intended recipients. Most previous studies have attempted to address this question using either photographic techniques or by analysing recorded reflectance spectra, neither of which take into account the specific properties of the receiver's visual system.

2. Here, we use psychophysical models of the visual system of three-spined sticklebacks (*Gasterosteus aculeatus*) to estimate their sensitivity to variation in the concentration and relative abundance of constituent carotenoids of males' carotenoid-based sexual signals.

3. We demonstrate that sticklebacks are acutely sensitive to variation in both the total concentration of carotenoids in the signal and the relative proportion of its constituents, and that the accuracy of these assessments is largely unaffected by the presence or absence of ultraviolet radiation in the illuminant. We discuss these findings in relation to the evolution, maintenance and information content of carotenoid-based sexual signals.

Key-words: carotenoid composition, sexual signal, spectral sensitivity

Introduction

Carotenoid pigments are responsible for much of the red, orange and yellow colouration seen in animals' skin, beaks and feathers (Fox & Vevers 1960; Goodwin 1984; Olson & Owens 1998). The considerable inter- and intraspecific variation seen in the expression of carotenoid-based colouration stems from variation in both the concentration and identity of constituent carotenoids, which can differ in their spectral properties (cf. Britton, Liaaen-Jensen & Pfander 2004). Pigmented feathers, for instance, typically contain several carotenoid pigments, and variation in the relative abundance of each carotenoid can markedly affect the feather's colour (Olson & Owens 1998; Stradi *et al.* 1998). Because vertebrates are incapable of biosynthesizing carotenoids *de novo*, many of these carotenoids are of direct dietary origin, although many animals are also capable of metabolically transforming ingested carotenoids into spectrally distinct forms (e.g. Goodwin 1984; Brush 1990; Stradi *et al.* 1998; Wedekind *et al.* 1998; McGraw, Adkins-Regan & Parker 2002a), potentially giving animals a fine degree of control over the appearance of carotenoid-based signals. However, how much of this variation is detectable by the intended receivers, and whether receivers are able to glean relevant information regarding carotenoid allocation strategies, is largely unknown (cf. Grether, Cummings & Hudon 2005).

The receivers of carotenoid-based ornaments are most likely to be conspecifics, as most if not all carotenoid-based signals are used for intraspecific communication (e.g. for sexual or offspring–parent signalling; Olson & Owens 1998). However, most studies that have tested for a direct relationship between signal colouration score and carotenoid content (e.g. Wedekind *et al.* 1998; Barber *et al.* 2000; Inouye *et al.* 2001; Saks, Ots & Hõrak 2003; Andersson, Prager & Johansson 2007; but see Grether, Cummings &

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Hudon 2005) have utilized colour metrics that do not take into account the specific visual capabilities of the species in question. Because there are often marked interspecific differences in the number, abundance and peak sensitivities of photoreceptors in animals' retinas, and in the subsequent neural processing of photoreceptor output, such metrics may not encompass all the subtle chromatic variation detectable by the species in question, or may overestimate the extent of discrimination (Endler & Mielke 2005). In particular, visual system-independent metrics do not allow us to explore what level of discrimination is possible by a receiver. Interpreting signal colour in terms of a conspecific's sensory capabilities may therefore allow us to better understand the costs and signal content of these secondary sexual traits (McGraw *et al.* 2002b).

During the breeding season male three-spined sticklebacks (*Gasterosteus aculeatus*) develop a region of intense, carotenoid-based colouration (Fig. 1) that is used by females when deciding on a mate and during male–male competition (Wootton 1984; Milinski & Bakker 1990). The signal is composed predominantly of astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), lutein (3,3′-dihydroxy-β,ε-carotene) and tunaxanthin (3,3′-dihydroxy-ε,ε-carotene) fatty acyl esters, with tunaxanthin probably being metabolically derived from dietary astaxanthin (Wedekind *et al.* 1998). As is typical of carotenoids, these have absorbance peaks in hexane of around 467, 445 and 438 nm (Hudon, Grether & Millie 2003) respectively; because long- and short-wave radiation are transmitted, the signals therefore appear red and yellow to human observers, but also have an ultraviolet (UV) component (Rick, Modarressie & Bakker 2004; Rowe *et al.* 2004) to which humans are not sensitive. As a result of variation in both the concentration and composition of carotenoids allocated to a signal, to human observers the sticklebacks' sexual colouration can appear red, orange or yellow, each at various intensities (Wedekind *et al.* 1998). However, whether differences in carotenoid allocation are detectable by conspecifics is unclear. The only previous studies to investigate carotenoid composition and perception in sticklebacks (Wedekind *et al.* 1998; Barber *et al.* 2000) used photographic techniques that probably introduced inherent biases into the colour estimates (Stevens *et al.* 2007) and limited analyses to the human-visible spectrum (400–700 nm), and so did not take into account visual differences between sticklebacks and humans. This is potentially important because sticklebacks have recently been shown to have an UV-sensitive cone (U), and the peak sensitivities of the other three cones (sensitive to long [L], medium [M] and short [S] wavelengths) differ from those in humans (Rowe *et al.* 2004; McLennan 2006). Although colour vision in sticklebacks is not well studied, there is evidence for tetrachromacy in other fish species (e.g. goldfish *Carassius auratus*, Neumeyer & Arnold 1989). In this study we compare the carotenoid content of the male's sexual signal with stickleback-specific measures of signal perception, in order to assess how well their visual system is suited to detect variation in the carotenoid composition and concentration of conspecific sexual signals.

Fig. 1. Two male three-spined sticklebacks in breeding condition, with differing expression of their carotenoid-based nuptial colouration.

Materials and methods

SOURCE OF FISH AND REARING CONDITIONS

The fish used in this study were captured as juveniles from the River Endrick, Scotland (56°04′N, 4°23′W), and held in the laboratory until the start of the breeding season under a simulated natural photoperiod and temperature regime. They were fed to satiation daily on a synthetic diet based on commercial fish feed pellets supplemented with astaxanthin, lutein and zeaxanthin (3,3′-dihydroxy-β,β-carotene), as described by Pike *et al.* (2007). This diet allowed males to produce nuptial colouration that was indistinguishable (both spectrometrically and to human observers) to that produced by wild-caught breeding males from the same population (TWP, personal observation) and the resulting levels of carotenoids in the nuptial colouration were within the range found in these wild males (Pike *et al.* 2007).

Sexually mature males were provided with a nesting dish (33 × 18 × 19 cm) filled with 1 cm sand and between 100 and 200 5-cm long strands of polyester thread as nesting material, and shown a gravid female enclosed in a plexiglas container for 5 min twice daily in order to stimulate nest building. Forty-nine males were selected from those that had developed breeding colouration in an attempt to encompass the full range of chromatic variation perceptible to humans (i.e. from red to yellow in hue and appearing 'bright' or 'dull' as described by Wedekind *et al.* 1998; see Fig. 1). Males were shown a gravid female for 10 min, immediately netted, and a standardized reflectance scan of their nuptial colouration obtained using an Ocean Optics USB2000 UV-visible spectrometer coupled with a deuterium–tungsten light source (Ocean Optics USB-DT). The tip of the fibre-optic probe was housed in a hollow, black plastic sheath with an angled tip that contacted the fish's skin below the jaw at 45° (following Uy & Endler 2004), and we used a spectrally flat 99% reflecting Spectralon standard (Labsphere, North Sutton, NH) and a dark current reading to standardize each scan. Measurements were taken from an ~3 mm diameter circle at 0·38 nm wavelength intervals, and converted to 1 nm intervals within the range 300–700 nm for analysis (Fig. 2a). Immediately following reflectance measurements, the fish were sacrificed with an overdose of anaesthetic (benzocaine) and the region of skin containing the nuptial colouration was immediately removed (Wedekind *et al.* 1998; Barber *et al.* 2000), weighed (±0·001 g), snap-frozen in liquid nitrogen and stored, under nitrogen gas, at −80 °C in the dark for up to 6 months until analysis of carotenoids (Schiedt & Liaaen-Jensen 1995). This work conforms to the legal requirements of the UK, and was carried out under licence from the home office.

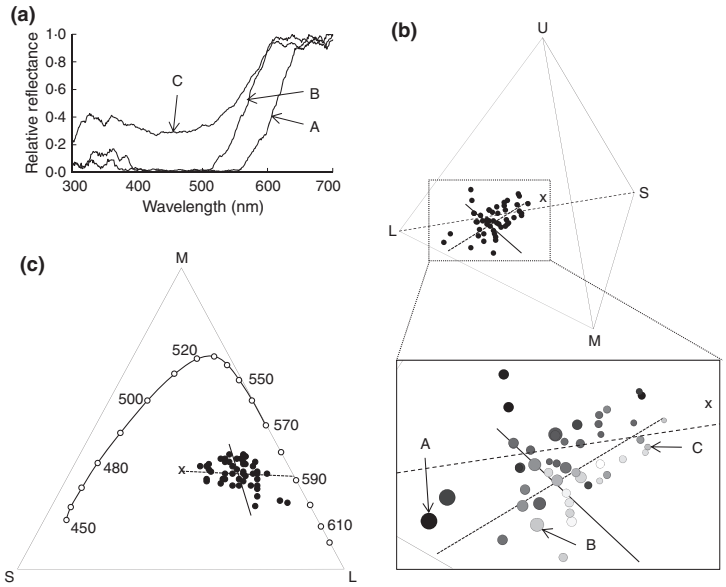

Fig. 2. (a) Representative reflectance spectra from three males' nuptial colouration, each differing in the allocation of carotenoids: high proportion of astaxanthin, high concentration (A); low proportion of astaxanthin, high concentration (B); low proportion of astaxanthin, low concentration (C). Spectra have been smoothed with a 10-point running mean. (b) Location of male sexual signals in tetrahedral colour space. The achromatic point (origin) is denoted by an 'x' at the centre of the tetrahedron, and the apices of the tetrahedron represent stimulation of a single one of the four cone classes. The empirical data all fell within an area that is shown as an enlarged rectangle. In this enlargement, point sizes are proportional to the concentration of carotenoids in the signal (largest, 381·2 μg g^{-1}; smallest, 12·8 μg g^{-1}) and shade is proportional to the proportion of astaxanthin (from 0·06, white, to 0·66, black); labelled data points correspond to the reflectance spectra in (a). Best-fit Least Sum of Euclidean Distances regression lines are shown, indicating linear trends in the colour space data in carotenoid concentration (dashed line) and the proportion of astaxanthin (solid line) (see Methods for details). (c) A triangular colour space representation of the data (from the horizontal, L, M, S, plane of the tetrahedron in (b) showing how colours would appear in a UV-deficient light environment. Details are as for (b). The monochromatic locus (solid line) is also plotted, with symbols placed at 10 nm intervals between 450 and 620 nm; numbers give the wavelength (nm) of some of these points.

MODELLING SIGNAL PERCEPTION

In order to derive stickleback-specific estimates of signal perception, reflectance spectra were processed to yield estimates of quantum catch by each of the four classes of stickleback photoreceptor cones, UV- (U; λ_{max} = 360), short- (S; λ_{max} = 445), medium- (M; λ_{max} = 530) and long-wavelength sensitive (L; λ_{max} = 605). Cone spectral sensitivity functions were calculated from published λ_{max} values (Rowe *et al.* 2004) using equations derived by Stavenga, Smits & Hoenders (1993), with β-peak positions set by an empirical formula by Palacios *et al.* (1998). The quantum catch, Q, for each cone class i, was estimated (Hudon, Grether & Millie 2003) as

$$Q_i = \sum_{\lambda=300}^{700} R(\lambda)I(\lambda)T(\lambda)S_i(\lambda),$$

where $R(\lambda)$ is the spectral reflectance of a male's carotenoid-based signal, $I(\lambda)$ is the irradiance spectrum, $T(\lambda)$ is the transmission spectrum across the pre-retinal media (Boulcott 2003), and $S_i(\lambda)$ is the spectral sensitivity of cone class i, across all stickleback-visible wavelengths (300–700 nm). The fish used in this study came from a fast-flowing riverine population so would generally breed in clear water and interact over short distances (Wootton 1984); the effects of absorption and scatter by water were therefore ignored (Rowe *et al.* 2004) and we used a standard daylight-simulating illumination spectrum (D65) in the model (Wyszecki & Stiles 1982).

Our estimates of cone catches were transformed to Cartesian (x, y, z) coordinates in three-dimensional tetrahedral colour space (Endler & Mielke 2005), using the following formulae

$$x = \frac{1 - 2q_S - q_M - q_U}{2}\sqrt{\frac{3}{2}},$$

$$y = \frac{-1 + 3q_M + q_U}{2\sqrt{2}},$$

$$z = q_U - \frac{1}{4},$$

where q_M, q_S and q_U denote normalized quantal catches for each cone class, such that $q_L + q_M + q_S + q_U = 1$. Each signal colour (i.e. male throat colour) is therefore represented by a point in the tetrahedron determined by the relative stimulation of the four cone types. If a colour stimulates only one cone type, then its coordinates lie at the appropriate tip of the tetrahedron, and when all four cone types are equally stimulated the point lies at the tetrahedron's centre (the origin of the graph and the stickleback achromatic point). A greater Euclidean distance between any two stimulus points in this colour space corresponds with a greater ability to discriminate between them (Endler & Mielke 2005). In sticklebacks, which cone(s) contribute to achromatic vision is unknown, and so here we assume that the perceived brightness (luminance) of a signal is proportional to the summed output of all four cone types (Endler & Mielke 2005).

Animals' perception of colour is achieved generally (if not universally) via opponent processing, where nervous systems subtract responses of photoreceptors with different spectral sensitivities (Endler & Mielke 2005). Such opponent mechanisms underpin colour

vision and the ability of animals to make chromatic discriminations. Based on the distribution of the data within tetrahedral colour space, we also consider putative opponent processing mechanisms that would allow sticklebacks to detect variation in signal composition. These are explained fully in the Results.

To allow comparison with previous studies, we also calculated two colour metrics (Andersson, Prager & Johansson 2007), both of which have been suggested to be sensitive to variation in carotenoid allocation, but which are independent of the receiver's visual system. The first, C_{CAR}, is the 'carotenoid chroma' (i.e. colour saturation in the red/orange region of the spectrum) and has been suggested to predict carotenoid concentration. The second, λ_{R50}, is defined as the wavelength at which reflectance is halfway between its minimum and its maximum (specifically, for the sigmoidal curves characteristic of pigmentary colouration). As this metric predicts variation in the redness/yellowness of a signal (Andersson, Prager & Johansson 2007), it may be sensitive to variation in the relative proportions of the constituent carotenoids. Both metrics only utilize variation in the human-visible (400–700 nm) region of the spectrum.

CAROTENOID ANALYSIS

Each skin sample (mean ± SE, 22·9 ± 1·1 mg) was homogenized in a mixture of methanol, distilled water and chloroform (1:1·5:3 mL). The solvent was evaporated from an aliquot of the chloroform-phase (1 mL) under a stream of nitrogen gas, and the sample re-dissolved in the HPLC mobile-phase. Carotenoids were quantified on an isocratic HPLC system using a LC-10 AS liquid chromatograph connected to a SPD-M10A VP photodiode array detector (detection wavelength at 470 nm), a SIL-10AD VP autoinjector and a SCL-10A VP system controller (Shimadzu, Kyoto, Japan). Chromatogram re-integrations were performed using the LC workstation Class-LC10 software (Shimadzu). The isocratic normal-phase system consisted of a H_3PO_4 modified silica gel column (Hibar LiChrosorb Si 60, length 125 mm, internal diameter 4 mm, 5 μ particle size; Merck, Darmstadt, Germany) as described by Vecchi *et al.* (1987). The mobile-phase was 14% (v/v) acetone in *n*-hexane and the flow rate was 1·2 mL min^{-1}. Carotenoids were quantified from chromatogram peak areas using an external standard of astaxanthin prepared from crystalline all-*E*-astaxanthin (DSM, Basel, Switzerland). The concentration of the standard solution was measured spectrophotometrically (UV-260, Shimadzu) using $E_{1\%,\ 1\ cm}$ = 2100 at absorbance maximum (λ_{max} = 470 nm). An $E_{1\%,\ 1\ cm}$-value of 2500 was used for quantification of lutein/tunaxanthin esters, and corrections were made for λ_{max} offset.

Carotenoids present in the nuptial colouration consisted predominantly of astaxanthin and lutein/tunaxanthin diesters (mean ± SE: 94·1 ± 0·9%); esters of lutein and tunaxanthin could not be separated using this procedure, and so only the total amount of these esters was determined (as in Wedekind *et al.* 1998). There were also smaller amounts of monoesters (5·8 ± 0·9%) and unesterified carotenoids (0·06 ± 0·04%) present, although the carotenoid composition of the di-, mono- and unesterified carotenoid fractions were similar. Here, we focus on the total concentration of diesters (hereafter 'total carotenoid concentration') and the proportion of diesters made up of astaxanthin diesters ('proportion of astaxanthin'). It should be noted that astaxanthin, lutein and tunaxanthin consist of 3, 8 and 10 optical *RS* isomers, and that their respective λ_{max}-values are similar. All optical *RS* isomers may be present in the stickleback in various proportions, but analyses of these isomers are beyond the scope of this work.

STATISTICAL ANALYSIS

Multivariate regression lines were fitted to the three-dimensional colour-space data using Least Sum of Euclidean Distances (LSED) regression (Kaufman *et al.* 2002; Endler & Mielke 2005; Mielke & Berry 2007), a multivariate analogue of Least Absolute Deviations regression. The significance of the fit was assessed by calculating a test statistic, T_0, as the proportionate reduction in the sums of absolute deviations between estimates for the full and reduced parameter models and determining *P* from a Monte Carlo approximation of the exact permutation distribution, where P = (number of $T \geq T_0 + 1$)/($m + 1$), for $m + 1 = 10^4$ permutations (Cade & Richards 1996). This provided a means to assess linear gradients in the colour-space data in relation to the carotenoid concentration and the proportion of astaxanthin in males' signals, and provided the predictive basis for subsequent statistical analyses.

As we were primarily interested in whether signal colour could allow conspecific receivers to predict patterns of carotenoid allocation, relationships between measures of signal allocation and metrics derived from the colour space analysis were subsequently analysed using linear models with either carotenoid concentration or the proportion of astaxanthin as dependent variables; the independent variables are described below.

Chromatic variation in the data was best explained by two metrics: distance from the achromatic point and the angular position of the point in colour space (see Results). These were obtained by converting the Cartesian coordinates of each data point into spherical coordinates (Stoddard & Prum 2008). In this coordinate system, distance from the origin is described by *r*, and the angular position in the three-dimensional colour space by two angles, θ and φ. θ explains variation around the horizontal (L, M, S cone) plane of the tetrahedron in Fig. 2b, where lower values of θ denote red hues and higher values yellower hues; φ describes variation around the vertical plane of the tetrahedron, where higher values of φ denote increased stimulation of the U cone. These two angles were significantly correlated ($r = -0{\cdot}59$, $n = 49$, $P < 0{\cdot}001$) – because carotenoids absorb most strongly in the middle of the stickleback's visible spectrum – and so we used a principal components analysis PCA to combine them into a single variable, PC1, which explained 79·6% of the variation in these measures. PC1 had a positive loading from θ (0·71) and a negative loading from φ (−0·71); signals with a low PC1 score are therefore those with a relatively red (rather than orange or yellow) hue in the human-visible spectrum and relatively rich in UV reflectance.

Results

SIGNAL COMPOSITION

The total carotenoid concentration and the proportion of astaxanthin in the nuptial region varied considerably between individuals (mean ± SE total carotenoid concentration: 147·9 ± 12·0 μg g^{-1}; mean ± SE proportion of astaxanthin: 0·39 ± 0·03, $n = 49$; Fig. 2). The concentration of astaxanthin in the signal did not correlate with the concentration of lutein/tunaxanthin (Pearson's correlation: $r = 0{\cdot}19$, $n = 49$, $P = 0{\cdot}20$) and the total carotenoid concentration was not

correlated with the proportion of astaxanthin ($r = 0{\cdot}21$, $n = 49$, $P = 0{\cdot}15$).

SIGNAL PERCEPTION

Figure 2b shows the throat colours represented as points in tetrahedral colour space with two LSED regression lines fitted, one (the dashed line) showing a linear gradient in the colour data as a function of the carotenoid concentration of a signal (permutation test, $P < 0{\cdot}001$), and the other (the solid line) showing a second, orthogonal gradient as a function of the proportion of astaxanthin in the signal (permutation test, $P < 0{\cdot}001$).

If the LSED regression line that best explains variation in carotenoid concentration is extended it would pass close to the (achromatic) origin (Fig. 2b), suggesting that the best measure of carotenoid concentration may therefore correspond to the distance from the achromatic point. Indeed, distance from the origin significantly predicted signal carotenoid concentration ($R^2 = 0{\cdot}58$, $F_{1,47} = 65{\cdot}75$, $P < 0{\cdot}001$; Fig. 3a) but not the proportion of astaxanthin ($F_{1,47} = 2{\cdot}78$, $P = 0{\cdot}10$). If we assume that the U cone receives no stimulation (e.g. as would occur under UV-deficient lighting; Fig. 2c), there is still a highly significant relationship between distance from the origin and carotenoid concentration ($R^2 = 0{\cdot}59$, $F_{1,47} = 68{\cdot}90$, $P < 0{\cdot}001$; Fig. 3a).

The LSED regression line best explaining variation in the proportion of astaxanthin allocated to the signal runs approximately parallel to the L-M axis of the tetrahedron (Fig. 2b). This suggests that the best measure of carotenoid composition may correspond to the angular position of the signal in colour space, and also demonstrates that the majority of the variation in the perceived proportion of astaxanthin lies in the human-visible region of the spectrum, and that the U cone contributes little to the sticklebacks' ability to detect the proportion of astaxanthin in a signal. The angular position of a signal colour around the horizontal plane (i.e. θ) was a significant predictor of the proportion of astaxanthin ($R^2 = 0{\cdot}71$, $F_{1,47} = 114{\cdot}32$, $P < 0{\cdot}001$; Fig. 3b), but not carotenoid concentration ($F_{1,47} = 0{\cdot}59$, $P = 0{\cdot}45$). Moreover, incorporating the output of the U cone (by using PC1) actually markedly decreased the proportion of variation in astaxanthin content explained ($R^2 = 0{\cdot}54$), although it was still a highly significant predictor ($F_{2,47} = 55{\cdot}4$, $P < 0{\cdot}001$). PC1 did not predict the concentration of carotenoids ($F_{1,47} < 0{\cdot}01$, $P = 0{\cdot}93$). The angular position of a signal colour around the vertical (UV) plane (i.e. φ) was a significant, but much poorer predictor of astaxanthin content ($R^2 = 0{\cdot}23$, $F_{1,47} = 14{\cdot}35$, $P < 0{\cdot}001$) and did not predict carotenoid concentration ($F_{1,47} = 0{\cdot}89$, $P = 0{\cdot}35$).

Signal brightness, which is likely to be perceived independently of colour, was negatively, although non-significantly, related to carotenoid concentration ($F_{1,47} = 3{\cdot}40$, $P = 0{\cdot}072$), and significantly negatively related to the proportion of astaxanthin ($R^2 = 0{\cdot}28$, $F_{1,47} = 18{\cdot}36$, $P < 0{\cdot}001$).

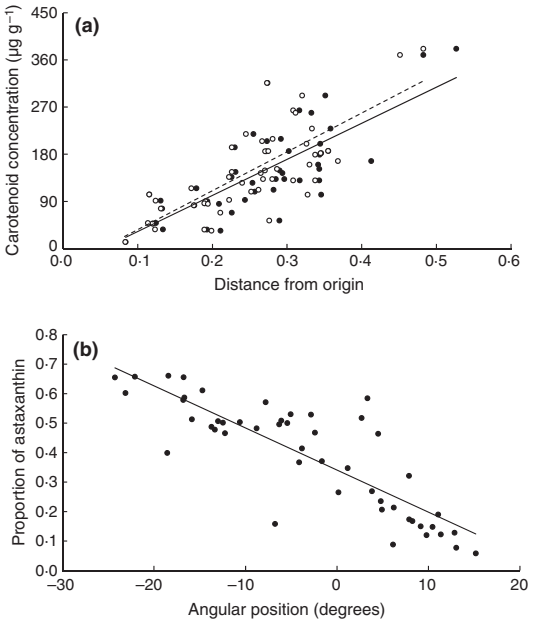

Fig. 3. Relationships between (a) the total concentration of carotenoids in the males' signals and the distance of a colour from the origin of the graph (the stickleback's achromatic point) in tetrahedral colour space (black points, solid line) and triangular (UV-deficient) colour space (white points, dashed line), and (b) the relative proportion of astaxanthin and the angular position of a signal colour around the horizontal (human-visible) plane of the colour space (where lower values indicate a redder signal, higher values a more orange/yellow signal).

Putative opponent processing mechanisms

The orientation of the LSED regression lines (Fig. 2b,c) suggest that particular opponent processing mechanisms would be well-suited to detect variation in both carotenoid concentration and composition. The regression line explaining variation in the proportion of astaxanthin runs approximately parallel to the L-M axis of the tetrahedron, suggesting that an opponent mechanism subtracting the equally weighted outputs of the L and M cones (i.e. an L-M comparison) would be sensitive to a large proportion of the variation in carotenoid composition. Indeed, such a comparison is a significant predictor of the proportion of astaxanthin in the signal ($R^2 = 0{\cdot}54$, $F_{1,47} = 54{\cdot}86$, $P < 0{\cdot}001$; Fig. 4a), but also explains a smaller yet still significant proportion of the variation in carotenoid concentration ($R^2 = 0{\cdot}23$, $F_{1,47} = 14{\cdot}20$, $P < 0{\cdot}001$).

The regression line explaining variation in concentration suggests that comparisons between the L, U and S cones may provide a good estimate of carotenoid concentration. A cone opponent mechanism that subtracts the equally weighted output of the S cone from the summed outputs of the L and U cones (i.e. an $[(L + U)/2] - S$ comparison) would be sensitive to a large proportion of the variation in carotenoid concentration ($R^2 = 0{\cdot}59$, $F_{1,47} = 75{\cdot}54$, $P < 0{\cdot}001$; Fig. 4b). However, a UV-insensitive opponent mechanism comparing

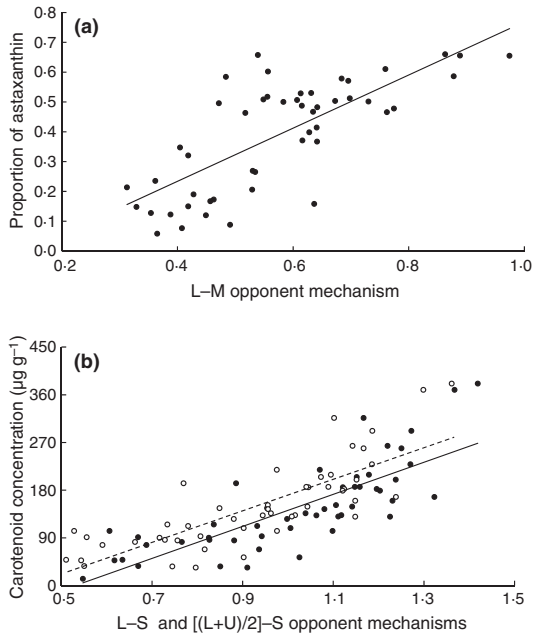

Fig. 4. (a) Relationship between an opponent mechanism comparing the L and M cones [L-M = $(Q_L - Q_M)/(Q_L + Q_M)$] and the proportion of astaxanthin in the signal. (b) Relationships between the output of opponent processing mechanisms comparing the equally weighted outputs of the L and S cones [L-S = $(Q_L - Q_S)/(Q_L + Q_S)$, where greater values indicate a greater relative stimulation of the L cone; black data points] and the summed outputs of the L and U cones with the S cone {$[(L + U)/2] - S = [(Q_L + Q_U)/2 - Q_M]/[(Q_L + Q_U)/2 + Q_M]$; white data points} against the carotenoid concentration of males' nuptial signals. See text for a definition of the symbols used, and Endler & Mielke (2005) for a full description of the calculations. Data on the abscissas have been arcsine-square root transformed.

only the L and S cones also significantly predicts carotenoid content ($R^2 = 0{\cdot}57$, $F_{1,47} = 62{\cdot}05$, $P < 0{\cdot}001$; Fig. 4b) and explains a similar proportion of the variation. Neither mechanism predicted the proportion of astaxanthin ($F_{1,47} = 0{\cdot}61$, $P = 0{\cdot}44$ and $F_{1,47} = 0{\cdot}95$, $P = 0{\cdot}34$ respectively).

Visual system-independent metrics

The visual system-independent metric C_{CAR} significantly predicted the carotenoid concentration ($R^2 = 0{\cdot}35$, $F_{1,47} = 25{\cdot}64$, $P < 0{\cdot}001$), but not the proportion of astaxanthin ($F_{1,47} = 1{\cdot}64$, $P = 0{\cdot}21$). The metric λ_{R50} significantly predicted the proportion of astaxanthin ($R^2 = 0{\cdot}70$, $F_{1,47} = 109{\cdot}38$, $P < 0{\cdot}001$) but not carotenoid concentration ($F_{1,47} = 0{\cdot}87$, $P = 0{\cdot}36$).

Discussion

The nuptial colouration of the male three-spined stickleback is composed predominantly of esters of astaxanthin and lutein/tunaxanthin, concentrations of which varied considerably between individuals but did not correlate within a colour patch (see also Wedekind *et al.* 1998), suggesting that they are physiologically independent of each other. Our data demonstrate that the stickleback's visual system is ideally suited to detect variation in both the total concentration of carotenoids allocated to a male's sexual signal, and the proportion of those carotenoids that consist of astaxanthin. In particular, when the colour data are plotted in tetrahedral colour space (Fig. 2b) it is evident that the concentration of carotenoids increased with increasing distance from the stickleback's achromatic point (which in humans corresponds to the perception of saturation), while the angular position (which corresponds to the human perception of hue) significantly predicted the proportion of astaxanthin in the signal. Note that, while comparisons with human vision are useful for descriptive purposes, we know of no data showing that sticklebacks have an analogous concept to the human perception of 'saturation' and 'hue'. Because this paper is primarily concerned with the ability of the sticklebacks' visual system to detect biologically relevant variation in the concentration and composition of carotenoids in males' sexual signals, we will avoid imposing such assumptions in the subsequent discussion. Instead, we will focus on the functional significance of stickleback's discrimination ability in the context of male sexual signalling.

Variation in carotenoid concentration may indicate individual variation in the ability to assimilate (or in the wild, locate) dietary carotenoids, and so may provide honest information on foraging ability (Lozano 1994) or the extent to which carotenoids are required for somatic functions other than signalling, such as immunostimulation (e.g. Blount *et al.* 2003; Faivre *et al.* 2003) or antioxidant activity (von Schantz *et al.* 1999; Pike *et al.* 2007). Our results suggest that conspecifics would be well-suited to detect variation in the concentration of carotenoids, allowing females to choose high quality mates on the basis of their ability to allocate sufficient carotenoids to sexual signalling (e.g. Pike *et al.* 2007), and potentially allowing males to assess the quality of intrasexual competitors.

However, while there have been several reasons suggested as to why sticklebacks should exhibit large variation in the concentration of carotenoids allocated to their signal, it is less clear why sticklebacks appear so sensitive to the relative proportion of astaxanthin. One explanation is that it may allow males to adapt to intra-population variation in female preference for signal colour. For example, some stickleback females have been reported to prefer orange or yellow over red males, perhaps because of condition-dependent visual constraints or differences in female 'motivation' to choose (Baube, Rowland & Fowler 1995; Bakker, Künzler & Mazzi 1999), and there is evidence from studies on guppies (*Poecilia reticulata*) that males may attempt to match a particular population-specific pigment ratio in their sexual signal to exploit female preference for colour-based signals (Grether, Cummings & Hudon 2005). If this is the case, then it may explain why in sticklebacks astaxanthin is converted to the yellow carotenoid tunaxanthin, rather than other orange–red carotenoids such as canthaxanthin or idoxanthin (astaxanthin metabolites commonly found in the

muscle and skin of other fish; e.g. Christiansen & Wallace 1998; Aas *et al.* 1997). A second explanation is that males may adjust the appearance of their signal in order to maximize its contrast against the background in response to variation in the photic environment or the transmission properties of the water (Endler 1992; Baube, Rowland & Fowler 1995; cf. Grether, Cummings & Hudon 2005), or allow males to optimize their signal expression for species recognition (Rowe *et al.* 2004). Different pigments may also provide different information to conspecifics if, e.g. they are used by the immune system to combat different types of infections (Wedekind *et al.* 1998). Further studies are needed to differentiate between these alternative explanations.

Recent work has shown that both the UV and 'red' components of sticklebacks' signals may be sufficient to elicit a response from females (Rick & Bakker 2008). While our data do not preclude the potential for UV reflectance to be used during mate choice in sticklebacks (Boulcott, Walton & Braithwaite 2005; Rick, Modarressie & Bakker 2006), the strong correlation between reflectance in the long- (red) and short- (UV) wavelength regions of the spectrum suggests that sticklebacks are unable to vary the UV component of the signal independently of redness, making it unlikely to act as a special channel of communication in three-spined sticklebacks (cf. Rick & Bakker 2008), at least in this population. Indeed, given the relatively low intensity of short-wave illumination and low relative abundance of U cones in the stickleback retina (Rowe *et al.* 2004), the output of the U cone (and any opponent mechanisms including a direct comparison with this cone class) would be subject to considerable noise; it may therefore be efficacious for sticklebacks to sum the U and L cone outputs to give a trichromatic eye with high sensitivity to variation in signal expression (van Hateren 1993). Indeed, our data suggest that if the U cone is involved in colour perception in sticklebacks, it would most likely have the effect of reducing chromatic resolution. For example, assuming an absence of UV radiation (i.e. no stimulation of the U cone) – a situation that is likely to have been common in the majority of published lab-based studies of stickleback mating preferences and male–male competition – actually markedly increased the ability of the stickleback's visual system to detect variation in the proportion of astaxanthin. This is backed up by the objective metric, λ_{R50} (Andersson, Prager & Johansson 2007), which makes only the assumption of wavelength discrimination in the red region of the spectrum, and explains a similar proportion of the variation in the proportion of astaxanthin to the U cone-deficient measure. Whether cone summation actually occurs in this species has not been tested, and it remains possible that in some situations sticklebacks preferentially use information from the U cone, perhaps because, like goldfish, they drop the L cone signal at low light intensities (Neumeyer & Arnold 1989). However, numerous studies have shown that signal redness (as detected at least in part by the L cone) is both sufficient and necessary for eliciting a behavioural response in sticklebacks, even in the absence of UV radiation (Baube, Rowland & Fowler 1995). At the very least, our data suggest that sticklebacks should be able to make similarly meaningful assessments of carotenoid allocation in the presence or absence of UV radiation, and that non-visual system-based approaches to quantifying stickleback colouration (e.g. based on photography or reflectance spectra) should provide a meaningful interpretation of colouration in this species.

Our data suggest the possible existence of at least two opponent processing mechanisms in sticklebacks that would be suitable for detecting variation in the concentration and composition of carotenoids allocated to signalling. The first, subtracting equally weighted outputs of the L and M cones (L-M), would encode much of the variation in the proportion of astaxanthin (see also Cronly-Dillon & Sharma 1968; McDonald & Hawryshyn 1995; Rowe *et al.* 2004), but also explains a significant proportion of the variation in carotenoid concentration. The second, subtracting equally weighted outputs from the L and S cones (L-S) (or potentially incorporating information from the U cone in an $[(L + U)/2] - S$ comparison) would be a strong predictor of variation in the concentration of carotenoids (see also Rowe *et al.* 2004). We know of no empirical data supporting these hypothesized mechanisms, although Rowe *et al.* (2004) suggested that an L-S mechanism would be the most effective means for sticklebacks to discriminate between males' nuptial colouration (for the means of quality assessment, for instance), while an L-M mechanism would be the most efficient means for them to detect a male's signal (for means of species recognition). These conclusions are supported by our data, in that an L-S mechanism would be sensitive to variation in carotenoid concentration which, as discussed above, may be indicative of physiological and physical quality, while the an L-M mechanism, which is sensitive to variation in both the carotenoid concentration and the proportion of astaxanthin, may be involved in initial detection of the signal prior to subsequent assessment of individual quality.

Brightness was found to significantly predict variation in the proportion of astaxanthin in the signal (albeit only a fairly small proportion compared with chromatic measures: 28%). This is likely to arise in part because of our method of estimating brightness, which meant that colours that stimulated more cone types would appear brighter (e.g. yellow stimulates both the L and M cones to a greater extent than red; see also Saks, Ots & Hõrak (2003) for similar results in the breast plumage of male greenfinches). However, as it is not known how sticklebacks perceive brightness, whether they could use brightness as a means of determining astaxanthin content of a male's signal is not clear.

In conclusion, we demonstrate that the visual system of sticklebacks is acutely sensitive to variation in both the total concentration of carotenoids in the male's nuptial signal and the relative proportion of its constituents, and suggest that this may allow sticklebacks to accurately assess male quality and thereby inform mate choice and intrasexual competition decisions.

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