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1 The behavioural effects of supplementing diets with synthetic and
2 naturally sourced astaxanthin in an ornamental fish (*Puntius titteya*).

3

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12

13 Abstract

14 Carotenoids are routinely incorporated into ornamental fish diets with
15 the aim of enhancing companion fish colouration which may
16 concomitantly affect fish behaviour. Previously, colour enhancement
17 has typically been achieved using synthetic carotenoids, however,
18 there is now growing public demand for food additives such as
19 carotenoids to be derived from natural sources, which can be acquired
20 from microalgae and cyanobacteria. There has been very little research
21 into whether natural carotenoids alter fish behaviour in a similar way
22 to synthetic carotenoids; the present study aimed to determine whether
23 behavioural changes typically associated with increased carotenoid
24 consumption differed according to carotenoid source in the cherry barb
25 (*Puntius titteya*). Cherry barbs were fed one of four diets (carotenoid-
26 free, 20 ppm synthetic astaxanthin (AX) sourced from Carophyll

27 pink®, 20 or 40 ppm of natural-AX sourced from Panaferd) over a 12
28 week period and then observed for colour changes, mate-choice and
29 aggressive behaviours. The diets containing 20 ppm synthetic-AX and
30 natural-AX enhanced male red colouration of the anal fin and anterior
31 dorsal area, *via* a reduction in hue, in comparison to the carotenoid-
32 free control diet whereas only the 20 ppm natural-AX altered the hue
33 of female colour. In the mate choice trials, males spent more time with
34 females fed the 20ppm synthetic-AX and 40ppm natural-AX
35 compared with the carotenoid-free control and 20 ppm natural AX.
36 Experiments conducted under red-blocking and UV blocking
37 conditions demonstrated an effect of red colouration and ultraviolet
38 reflectance on mate discrimination. Interestingly, males fed both the
39 synthetic and natural AX diets reduced aggressive interactions with a
40 mirror image, even though they displayed enhanced red colouration,
41 which is often used by fish as a signal of increased competitive ability.
42 In conclusion, source of dietary AX affected the behaviour of cherry
43 barbs, to the extent that synthetic AX exerted a stronger effect on mate-
44 choice behaviour under full spectrum lighting in comparison to a
45 similar concentration of natural AX. This therefore demonstrates that
46 the behaviour of companion fish can be influenced by the source of
47 carotenoids within their food.

48 Keywords: Carotenoids, mate-choice behaviour, mirror-image tests,
49 cherry barbs, ornamental fish.

50 1. Introduction

51 There are numerous nervous (Amiri and Shaheen, 2012),
52 endocrine (Leclercq *et al.*, 2010) and dietary (Harpaz and Padowicz,
53 2007) processes which can affect the colouration of teleost fishes, and

54 therefore the transfer of information within colour-based visual signals
 55 (Evans and Norris, 1996; Baron *et al.*, 2008). Carotenoid pigments are
 56 an important dietary requirement; their properties as antioxidant
 57 compounds (Sies and Stahl, 1995) and ability to alter colouration have
 58 been well documented in teleost fish as well as many other taxonomic
 59 groups (McGraw *et al.*, 2002; Blount, 2004). Baron *et al.* (2008)
 60 demonstrated that alterations in colour through carotenoid
 61 consumption can have subsequent effects for colour based behaviours.
 62 Female flame-red dwarf gourami (*Colisa lalia*) preferentially
 63 associated with male fish exhibiting lighter colouration after Lucantin
 64 Pink consumption (Baron *et al.*, 2008). Evans and Norris (1996) found
 65 that male fire-mouth cichlids (*Cichlasoma meeki*) fed with increased
 66 carotenoids were more successful in aggressive interactions than
 67 opponents fed with a reduced amount of carotenoids. This difference
 68 was not seen when the experiments were conducted under green
 69 lighting that prevented fish from discriminating between red colours.
 70 Hence the difference in success of individual males was directly
 71 attributed to the effects of carotenoids on the red colour patches used
 72 for signalling, and not to any other factor such as mass or size.

73 In addition to assessing the effects of carotenoids on the use
 74 of colour signals within the human visual spectrum, it must be noted
 75 that a number of fish species are sensitive to ultraviolet light: UVA
 76 wavelengths specifically, with a peak absorption of 360 nm in teleost
 77 cone cells (Losey *et al.*, 1999). Guppies (*Poecilia reticulata*) and
 78 three-spined sticklebacks (*Gasterosteus aculeatus*) both use ultraviolet
 79 reflectance during mate choice assessment (Kodric-Brown and
 80 Johnson, 2002; Rick and Bakker 2006; Rick and Bakker, 2008a).
 81 Dietary carotenoids can affect ultraviolet reflectance either directly (by

82 interacting with ultraviolet light) or indirectly (by affecting the
83 presence of other pigments) (Kodric-Brown & Johnson, 2002). When
84 assessing the impacts of a carotenoid diet on colour based behaviours,
85 it is therefore important to consider the visual capacity of the species
86 involved (Bennett and Cuthill, 1994) and whether UV-reflectance-
87 based behaviours are used.

88   Generally, reproductive output is limited by female capacity
89 to breed, which therefore drives male competition for access to
90 females (Sargent *et al.*, 1986). As reproductive investment is generally
91 reduced for males in comparison to females, mate choice studies have
92 predominantly used females as focal individuals and assessed female
93 mate choice. However, this does not necessarily mean discrimination
94 between potential mates solely occurs by females; male fish are also
95 selective in their choice of mating partners. For instance, male Pacific
96 blue-eye (*Pseudomugil signifier*) fish discriminate between females
97 based on size and preferentially associate with larger females
98 providing there is no additional cost to them (Wong & Jennions, 2003).
99 Additionally, male two-spotted gobies (*Gobiusculus flavescens*)
100 associate with potential mates based on assessments of female
101 coloured ornaments (Amundsen & Forsgren, 2001). Preliminary
102 behavioural observations of cherry barbs revealed female fish to be
103 shyer than males, with male fish constantly attempting to court
104 females. Subsequently, a mate choice model was established which
105 used male fish as the focal fish which enabled assessment of both male
106 and female mate choice.

107   Carotenoid consumption alters the expression of certain
108 behaviours to correlate with resource-holding potential, however,
109 carotenoid absorption and storage in tissues is dependent upon its

110 chemical form as well as the ability of the fish to convert it into other
111 carotenoids, which differs according to taxonomic grouping. In recent
112 years there has been an increase in consumer demand for the use of
113 products and raw materials which are naturally derived with less
114 dependence on synthetic or highly processed goods. Whether natural
115 or synthetic additives exhibit different effects due to bioavailability is
116 contested. For instance, vitamin C as an additive is synthetically
117 produced with an identical chemical structure to its naturally occurring
118 counterpart (Carr & Vissers, 2013). In human experiments natural and
119 synthetic vitamin C are equally bioavailable, however in animal
120 studies there is greater variation in natural *versus* synthetic
121 bioavailability dependent upon the animal model used (Vissers *et al.*,
122 2011; Carr & Vissers, 2013). Naturally produced supplements are
123 often synthesised in conjunction with other compounds which are
124 thought to influence bioavailability, an example being the interaction
125 between flavonoids and vitamin C affecting uptake (Song *et al.*, 2002;
126 Vissers *et al.*, 2011). Despite variable bioavailability in animal models,
127 the trend for naturally derived additives has moved from human foods
128 into those fed to our companion animals and wherever possible natural
129 colourants, preservatives and flavourings are utilised. For fish,
130 ingredients such as natural colourants, particularly those which help to
131 enhance the colouration of fish and may provide additional health
132 benefits, such as carotenoids have been focussed on (Sinha & Asimi,
133 2007; Yanar *et al.*, 2008). In this study, colour expression and colour-
134 associated behaviours were assessed in cherry barbs fed one of two
135 astaxanthin-based flake diets, Carophyll-pink and Panaferd.
136 Carophyll-pink is a synthetically produced astaxanthin (AX) whereas
137 Panaferd is sourced from a novel natural fermentation method from

138 *Paracoccus carotinifaciens*. The main carotenoid component of
 139 Panaferd is astaxanthin, however, as it is naturally occurring it also
 140 contains several other carotenoids in lower quantities, it is not known
 141 whether the presence of additional carotenoids or other naturally
 142 occurring compounds alter the bioavailability of astaxanthin.
 143 Panaferd, a new ingredient proposed for commercial diets, was tested
 144 at two concentrations (20 and 40 ppm) alongside Carophyll-pink (20
 145 ppm) to determine the effects of consumption of astaxanthin produced
 146 from a natural source. A number of different parameters were
 147 measured to assess the effects of diets on male and female cherry
 148 barbs, *Puntius titteya*. These included changes to mate choice and
 149 competitive ability, as well as changes in colour.

150 2.1 Methods

151 Cherry barbs were sourced from a local pet store and held in
 152 high density stock tanks until experiments began (dissolved oxygen
 153 $94.1\% \pm 0.8\%$; pH 7.39 ± 0.04 ; temperature $28.0^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$; light:dark
 154 period 12:12; all values $\pm$ SEM). Each diet treatment (see below for
 155 diet details) consisted of six replicate tanks, with four males and four
 156 females placed in each tank. In three tanks of each diet treatment,
 157 barriers physically and visually separated sexes ($n=24$ fish per diet
 158 treatment: 12 male, 12 female); of these groups of fish, three males
 159 and three females from each tank were selected for mate-choice trials
 160 ($n=18$ fish per diet treatments: 9 male, 9 female). Physical separation
 161 of the sexes within these tanks was used to prevent fish from exhibiting
 162 preferences during mate choice trials according to prior social
 163 encounters. The remaining three tanks of each diet treatment contained
 164 fish in mixed sex groups. All six tank replicates were included in

165 colour change analyses (n=48 fish per diet treatment: 24 male, 24
166 female).

167 The four diets were supplied by WALTHAM, Mars
168 (<http://www.waltham.com/>), a negative control containing no
169 pigments, a 20 ppm Carophyll-pink positive control and a 20 ppm and
170 40 ppm novel diet containing Panaferd-AX. Nutritional content of the
171 diets is given in Table 1.

172

173 2.1. HPLC analyses

174 Carotenoid content of flake diets was determined using high
175 performance liquid chromatography. A sample (3.0 g) of each diet was
176 used for carotenoid determination. As carotenoids from the Panaferd
177 source are produced by fermentation of bacteria, different extraction
178 solvents were required to remove cell walls, as opposed to those from
179 control and synthetic astaxanthin diet treatments. Once extracted, the
180 samples were ultimately processed through HPLC in the same manner.
181 Flake samples from negative and synthetic diet treatments were shaken
182 with 0.5 ml Protex 6L, 100 mg butylated hydroxytoluene (BHT) and 6
183 ml D.I. water and sonicated at 50°C for 30 min. 40 ml of ethanol was
184 added to the suspension, shaken and 50 ml of dichloromethane added.
185 The mixture was allowed to cool to room temperature in the dark for
186 2 hours. Extracts were then purified by open column chromatography
187 on silica gel. Carotenoids were then eluted from the silica gel with 5
188 ml iso hexane : diethyl ether (1:1) and evaporated under nitrogen.
189 Carotenoids were reconstituted in 1 ml of iso hexane : acetone (82:18).
190 Flake samples of Panaferd diets were sonicated with 2.5 D.I. water at
191 60°C then shaken with 5 ml of tetrahydrofuran (THF) : methanol
192 (20:1) for 5 min. Solutions were then centrifuged at 1300 rpm for 10

min with 10 ml of isohexane. A 5 ml aliquot was then dried under nitrogen and reconstituted in 5 ml of isohexane. The HPLC (Dionex Ultimate 3000) used an autosampler with an injection volume of 33 μ l. The mobile phase used was iso-hexane : acetone : iso-propanol (82:16:2) at 25°C with flow rate at 1.5 ml min⁻¹. The column used was a Luna 3 μ m silica analytical column (length: 100 mm, diameter: 4.6 mm), carotenoid amounts were quantified at 474 nm. Carotenoid contents are expressed as mg kg⁻¹ in Table 1.

2.2. Colour analysis

At the start of the experiment, male and female cherry barbs were lightly anaesthetised using MS-222 (0.08 g l⁻¹) according to Sloman *et al.* (2003) and held in a petri dish containing enough water to cover the body of the fish. The right hand lateral side of each fish was photographed using a Canon EOS 60D dSLR. The following camera settings were used according to the recommendations of Stevens *et al.*, (2007); manual white balance, manual focus, relative aperture f/8, shutter speed 1/40s, ISO 320. The camera was mounted on a tripod at a set distance above the fish, a spotlight was used to provide constant illumination. All fish recovered from anaesthesia without any observable adverse effects. Different anaesthesia methods have previously been shown to affect spectral reflectance patterns (Gray *et al.*, 2011), one method (MS-222) was therefore used across all colour measurements. Following this, fish were fed their respective diets for a period of 12 weeks, fish were fed to satiation to ensure food intake was even within groups and not controlled by the formation of social hierarchies. Nutritional differences between diets were minimal (Table 1), thus it was assumed that diets did not provoke differences in appetite and that carotenoid intake was maintained at intended levels

221 *via* relative concentrations within diets. No underweight or overweight
 222 fish were observed during feeding trials. Fish were then photographed
 223 again according to the methods previously outlined, and behavioural
 224 trials then took place.

225 Images were calibrated to a full colour standard (x-rite
 226 ColorChecker Passport <http://www.xrite.com/home.aspx>:
 227 ColorChecker Passport v1.0.1) and graphical software (Photoshop
 228 CS5) was used to isolate specific areas of an image to allow for colour
 229 analyses in various body areas (Fig. 1). These areas consisted of the
 230 whole body which was then broken down into caudal fin, anal fin and
 231 anterior dorsal areas. Images were then analysed using two different
 232 MATLAB codes.

233 2.2.1. %Red and %Yellow calculations

234 MATLAB analysed the percentage of pixels within an image
 235 that were either 'red' or 'yellow' based on predefined colour
 236 parameters. The red and yellow parameters were adapted from Maan
 237 *et al.* (2010) in which pixels would be identified as red if the hue was
 238 within 0-26 or 232-255 of the 0-255 RGB hue scale, yellow was
 239 defined as hues of 27-45. If pixels fell within these hue ranges they
 240 were then counted as red or yellow providing they met saturation
 241 criteria of 40-97 (Fig. 1). MATLAB analysed colouration within the
 242 HSV (hue, saturation and value) scale and not the RGB (red, green and
 243 blue) scale, which runs from 0-1.0 rather than 0-255. Red and yellow
 244 parameters were adapted to fit within the HSV scale. MATLAB
 245 therefore identified red and yellow pixels based on the following
 246 criteria:

247 Red: Hue = 0-0.0833 or 0.9167-1.0, Saturation = 0.40-0.97

248 Yellow: Hue = 0.0833-0.2499, Saturation = 0.40-0.97

2.2.2. Hue distribution

MATLAB also identified the distribution of hue within an image as an indication of overall colouration. The hue of each pixel was analysed and a histogram generated, the peak of which represents the most prevalent hue. Hue was plotted against normalised pixel count in order to standardise different numbers of pixels per image. This method also works within the HSV scale, therefore the hue of the peak has the same colour parameters set out within the %Red and %Yellow calculations.

2.3. Behavioural assays

Mate choice behaviour was assessed by allowing an individual male visual access to four females in a purpose built mate choice chamber. Each male was allowed to assess four females each from different diet treatments under three different scenarios: 1) under full spectrum lighting, 2) with red reflectance blocked using green lighting and 3) with ultraviolet reflectance blocked using UV filters.

The three mate choice scenarios were run simultaneously. Each of the three lighting scenarios contained four randomly chosen female fish, each from a different diet treatment (n=9 females per diet treatment). Three males from each diet replicate were randomly divided amongst the three lighting conditions and rotated until each male experienced all three conditions successively but in a different order. This was done for males of all four diet treatments. The order in which males completed mate choice scenarios was randomised to negate effects of prior experience. Thus, in total nine male fish per diet treatment (all replicates included) participated in a series of three mate choice trials (n=9). As discussed in the introduction, enhanced red colouration is used as a measure of attractiveness, therefore, in theory

277 female cherry barbs should discriminate between potential mates more
278 than males. However, in preliminary observations, male cherry barbs
279 were found to be bolder than females and female fish did not make
280 appropriate focal subjects. As males were bolder, they acclimated to
281 the mate choice chambers rapidly and began associating with
282 separated females. The experimental set up also allowed female
283 motivation to be analysed by assessing their interaction with males
284 when the male was visible.

285   At the start of the choice trials, male fish were contained
286 within a clear start box at the centre of the mate choice chamber, from
287 which all females were visible, for 10 minutes to allow acclimation to
288 the mate choice chamber. After this acclimation period, males were
289 released from the start box and allowed to explore the mate choice
290 chamber and assess females for 20 minutes while being digitally
291 recorded from above. The resulting video footage was then analysed
292 using JWatcher (<http://www.jwatcher.ucla.edu/>) to determine the
293 proportion of time spent associated with each female. Time spent
294 associated with a female was determined as when the male was within
295 a proximity of 5 cm from the dividing partition separating the sexes.

296   Male fish from each diet treatment were also subjected to
297 mirror-image tests (n= 9 fish per diet treatment). Males were held in
298 isolation in 5 l tanks in which there was a covered mirror at one end.
299 After 20 h within the tank, the mirror was uncovered for 10 min after
300 which the mirror was recovered for a further hour. This was done to
301 allow the fish to acclimate to the action of uncovering the mirror
302 (Sloman, 2010). The mirror was then uncovered and the number of
303 aggressive interactions fish made with the mirror, defined as bites or

304 lateral displays, was recorded for 1 h. The number of aggressive
305 interactions per minute was then calculated.

306 2.4. Statistical analyses

307 All data were tested for normality by assessment of residual
308 plots and using Kolmogorov-Smirnoff and Levene's test for
309 homogeneity of variance. Data reported in percentage were arc-sin
310 transformed prior to analysis. Male and female colouration data were
311 analysed separately using one-way ANOVAs with diet treatment as a
312 fixed factor and tank replicate as a random factor. Tank replicate was
313 used as a random factor to take into account the within and between
314 tank variability. Mate association data were analysed using a four way
315 ANOVA, with female diet, male diet, lighting conditions and tank
316 replicate as fixed factors to examine differences in behaviour between
317 mate-choice trials held under different lighting conditions. Male fish
318 were not individually identifiable between mate-choice trials held
319 under different lighting conditions, thus, a random effect within a
320 mixed model could not be used. Further analysis examined each
321 lighting condition individually to determine differences within lighting
322 conditions. Mirror-image interactions per minute were analysed using
323 a one-way ANOVA with diet as a fixed factor and tank replicate as a
324 random factor. Where significant overall effects were found, Tukey's
325 HSD was used for post-hoc testing to identify differences between
326 treatments, using the 5% significance level.

327 3. Results

328 3.1. Colouration

329 Diet treatment significantly affected the hue of male fish in
330 two isolated areas; the anal fin and the anterior dorsal areas (Table 2:

one-way ANOVA: anal fin $F_{3,15.82}=3.22$, $P=0.05$; anterior dorsal area $F_{3,16.43}=3.67$, $P=0.03$), although Tukey's post hoc testing could not identify specific differences between treatments at the 5% level. There was no difference in the percentage change of red or yellow pixels within male or female fish images as a result of diet treatment (data not shown).

3.2. Behaviour

When mate choice trials were considered across all lighting treatments, the amount of time male fish spent with females was affected by female diet treatment (Fig. 2: four-way ANOVA: Diet: $F_{3,288}=6.059$, $P<0.001$), but not affected by male diet treatment (Fig. 2: four-way ANOVA: $F_{3,288}=1.149$, $P=0.330$). As expected, there was a significant interaction between female diet treatments and lighting conditions (four-way ANOVA: $F_{6,288}=37.776$, $P<0.001$) and so each of the lighting conditions was analysed separately. Female diet affected male association within each of the lighting conditions (Fig. 2: two-way ANOVA: full colour lighting: $F_{3,96}=30.876$, $P<0.001$; Red blocked: $F_{3,96}=55.356$, $P<0.001$; UV blocked: $F_{3,96}=8.602$, $P<0.001$). Under full colour lighting, male fish spent a significantly greater amount of time with females fed the 20ppm synthetic-AX and 40ppm natural-AX (Fig. 2). This differed to mate choice trials which were conducted under red-blocking and UV blocking conditions in which males spent the greatest time with females fed the negative control and 20 ppm natural-AX respectively (Fig. 2).

In mirror image tests, male fish fed the negative control diet were significantly more aggressive in comparison to fish fed any other carotenoid diet treatments (Fig 3: one-way ANOVA: Diet: $F_{3,31}=14.51$, $P<0.003$).

4. Discussion

When carotenoids are incorporated into ornamental fish diets with the aim to enhance colouration and welfare, appropriate research should be carried out to determine how this might affect colour-based behaviours. In the present study, carotenoid consumption significantly changed mate choice and competitive behaviours, both of which are likely to be influenced by colour based signals in cherry barbs.

Colour changes were expected to be more apparent between diet treatments but were observed only within hue changes in isolated areas of the male body, however, there were still substantial effects to colour-associated behaviours due to carotenoid consumption. Male fish were not individually identifiable between lighting treatments, thus, analysis of all lighting conditions together to determine differences between lighting conditions resulted in pseudoreplication. Therefore, the interpretation of behavioural differences between lighting conditions may be limited. However, to remove this pseudoreplication each lighting condition was analysed separately to determine behavioural differences within each lighting condition. It was found that male cherry barbs spent the greatest amount of time with females that were fed the 20 ppm synthetic-AX and the 40 ppm natural-AX diets, when mate choice trials were conducted under full colour spectrum lighting (Fig. 2). There was no difference in male association with females fed the 20 ppm natural-AX diet compared to

383 those fed the carotenoid free negative control, indicating that males
384 preferred females fed either a synthetic astaxanthin or a comparatively
385 high concentration of natural astaxanthin. Therefore, astaxanthin
386 source may affect mate-choice behaviour, whereby 20 ppm of
387 synthetic carotenoids was sufficient to induce a male mate-choice
388 preference similar to that of 40 ppm of naturally sourced astaxanthin.

389   To explore the effects of red colouration further, mate choice
390 trials were repeated under green lighting which has been used
391 previously in similar studies to block red colouration (Evans and
392 Norris, 1996). Results should then be causally related to the effects of
393 carotenoids on red colouration and disassociated from other potential
394 physiological factors which could influence mate choice assessment.
395 However, dependent upon the visual assessment capabilities of male
396 cherry barbs, green lighting may still allow for the assessment of other
397 physiological factors such as UV reflectance. Under green lighting,
398 male fish spent the most time associating with females from the
399 carotenoid-free diet. This suggests that male fish were indeed using
400 differences in red colouration to discriminate between females in the
401 full lighting condition. However, it is not completely clear why under
402 green lighting, male cherry barbs particularly associated with those
403 females fed the carotenoid-free diet. It is possible that if there was less
404 red colouration on these females due to lack of carotenoids in their
405 diet, that their natural colouration would have been the least affected
406 by green lighting and therefore they appeared the most natural of a
407 selection of fish.

408   Other aspects of physiology can be used as social signals. For
409 instance, ultraviolet reflection has been shown to enhance male
410 attractiveness to females in guppies, where females will preferentially

411 associate with a male reflecting ultraviolet light when presented with
412 two carotenoid matched males (Kodric-Brown and Johnson, 2002).
413 Indeed, Rick and Bakker (2008b), went further in selectively
414 excluding certain wavelengths from stickleback discrimination trials.
415 It was found that UV wavelengths carried as much information as a
416 signal as the long, red, wavelengths did and that removal of UV
417 reflectance reduced attractiveness. The importance of UV signalling
418 has only been realised recently and has been suggested to act as a
419 private communication channel (Rick and Bakker, 2008a). The short
420 wavelength of ultraviolet light is scattered easily in water, meaning
421 that ultraviolet signalling is only effective in close proximities
422 allowing information to be conveyed to intended individuals whilst not
423 making the organism more detectable by predators. This was
424 confirmed by Cummings *et al.* (2003) showing ultraviolet reflectance
425 enhanced northern swordtail (*Xiphophorus nigrensis*) attractiveness to
426 mates but not to predators. This study also established that the use of
427 ultraviolet reflectance by northern swordtails was more prevalent in
428 populations with greater predation pressures. The Mexican tetra
429 (*Astyanax mexicanus*) is the natural predator of northern swordtails
430 and is less sensitive to ultraviolet wavelengths enabling swordtails to
431 communicate effectively while staying discreet which establishes an
432 evolutionary basis and selection pressure for the use of ultraviolet
433 signalling.

434   Mate choice trials were therefore also conducted under
435 ultraviolet reflectance blocking conditions so that any differentiation
436 from mate choice results under full colour spectrum lighting could be
437 attributed to a mate choice assessment that incorporates ultraviolet
438 information. Under UV blocking conditions, males spent the least

439 amount of time with females fed the carotenoid-free diet, as they did
 440 under full colour spectrum lighting. However, there were differences
 441 in mate preference for females fed the three carotenoid diets. Under
 442 full spectrum lighting, male fish spent the greatest amount of time with
 443 females fed the 20 ppm synthetic-AX and 40 ppm natural-AX diet;
 444 under UV blocking conditions males spent the greatest amount of time
 445 associating with females fed the 20 ppm natural-AX diet (Fig. 2). As
 446 demonstrated by the flake analysis (Table 1), there was a greater
 447 amount of total carotenoids in the 20 ppm natural-AX diet than the 20
 448 ppm synthetic-AX, however, the concentration of astaxanthin was
 449 similar between these two diets. This change in association suggests
 450 that male cherry barbs may utilise ultraviolet reflection in
 451 discriminating between mates and that naturally produced astaxanthin
 452 may modify ultraviolet reflectance differentially to synthetic
 453 astaxanthin. It is unclear why this effect was seen in 20 ppm natural-
 454 AX but not at a higher concentration of 40 ppm natural-AX; there may
 455 be effects of carotenoid source and concentration on ultraviolet
 456 reflectance and how conspecifics perceive this.

457   Female mate choice allows an individual to pick the most
 458 sexually fit male, often through visual signals including carotenoid-
 459 based red colouration (Maan *et al.*, 2006). Kodric-Brown (1988) found
 460 male guppies with enhanced red/orange colour morphology, due to
 461 increased carotenoid consumption, had a greater mating success rate
 462 due to female preference. However, it was found that male cherry barb
 463 association with females was not influenced by the diet treatment the
 464 male fish was fed, thus, females were not interacting with males in a
 465 way to increase male motivation. This may further indicate that in
 466 cherry barbs female selection of mates is weaker than male selectivity,

467 however, further experiments in which the female is the focal animal
468 would be required to confirm this.

469   Colour morphology can also be used to assess social status and
470 competitive ability of conspecifics. For example, salmonid species are
471 known to darken the colour of their skin and sclera to signal
472 subordination to opponents, which informs opponents of defeat and
473 reduces the subsequent aggression towards subordinates (O'Connor *et*
474 *al.*, 1999). Similarly, carotenoid-based red colouration has been shown
475 to increase aggression of fire mouth cichlids (*Cichlasoma meeki*)
476 (Evans and Norris 1996). Carotenoids are a limited resource, thus
477 individuals with higher carotenoid consumption are likely to have
478 higher resource holding potential and are able to compete effectively
479 enough to gain access to limited resources (Evans and Norris 1996,
480 Briffa and Sneddon, 2007). The red colouration, therefore, acts as a
481 signal to opponents informing them of their competitive ability and is
482 considered an honest signal as it cannot be replicated by other means
483 (Olsen and Owens, 1988). Within the current study, male fish were
484 held in isolation and their behaviour in response to a mirror image was
485 recorded. Males that were fed carotenoids were less aggressive than
486 males fed the carotenoid-free diet (Fig. 3). As fish respond to their
487 mirror image as if it was another individual, it is possible that the
488 carotenoid fed fish perceived their reflection to be an opponent with a
489 high resource holding potential inferred from assessing colour
490 morphology. This may reduce aggression as fish may perceive their
491 chance of winning a contest to be low. Conversely, male fish fed the
492 carotenoid-free diet may have made an assessment of their 'opponents'
493 colour morphology and may have considered their chances of winning
494 a contest to be greater than those fed carotenoids. It is again possible

495 that ultraviolet reflectance is used in agonistic signalling. For a fish to
496 physically attack its mirror image it has to be in close proximity to it,
497 which would also allow for assessment of information garnered
498 through ultraviolet reflectance. Further experimentation could be done
499 to block the ultraviolet reflectance within mirror image trials to
500 ascertain whether this private channel of communication is utilised in
501 agonistic bouts.

502   It was expected that, regardless of whether the novel, natural
503 astaxanthin diet altered colour morphology, there would be an
504 observable colour change between fish fed the carotenoid-free and
505 synthetic-AX controls. However, observed colour changes were only
506 minimal i.e., only occurring in specific body areas. Therefore, it may
507 be possible that more extensive colour changes in cherry barbs are
508 dependent on a different carotenoid, a different type of pigment (i.e.
509 flavonoids, pteridines or pyridines) or there may have been
510 digestibility and absorption factors which prevented more pronounced
511 colour changes i.e. astaxanthin esterification, solubility and diet lipid
512 content or carotenoid conversion (Bories *et al.*, 2007, Guillaume *et al.*,
513 2001, White *et al.*, 2002). There may also have been an effect of using
514 adult fish within this study, for instance, if the fish have consumed
515 carotenoids during growth from juveniles they may have already
516 saturated the amount of pigments within the skin prior to feeding trials.
517 To counteract this further experiments could use fish at earlier life
518 stages or fade the colouration of adults by feeding all fish negative
519 controls prior to being fed trial diets. This study examined colouration
520 from a human perspective, measuring colour within the visible range
521 of humans, and therefore does not account for the fish's own

522 perception of colouration. Thus, changes to colour out-with the red and
523 yellow colour space cannot be ruled out.

524   In conclusion, based on behavioural trials it appears that there
525 were changes in red colouration in fish fed the different carotenoid
526 diets, but that the changes were very subtle. It seems likely that
527 ultraviolet reflectance in conjunction with red colouration is used by
528 cherry barbs in making mate choice decisions. This study has
529 demonstrated that very subtle changes in colour morphology due to
530 consumption of carotenoid diets still impact colour-associated
531 behaviours. There is a need for further research into the effects of a
532 wider range of diet ingredients fed to companion fish, to examine
533 whether they may alter colouration, the impacts these may have on fish
534 behaviours and the underlying mechanisms. Furthermore, different
535 species are likely to alter their colour and/or colour-based behaviours
536 differently which may also impact interactions seen in multi-species
537 assemblages normally found in home aquaria.

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545 **References**

546 Amiri, M. H., Shaheen, H. M., 2012. Chromatophores and color
547 revelation in the blue variant of the Siamese fighting fish (*Betta*
548 *splendens*). *Micron* **43**, 159-169. doi: 10.1016/j.micron.2011.07.002.

- 549 Amundsen, T., Forsgren, E., 2001. Male mate choice selects for
550 female coloration in a fish. *PNAS* **98**, 13155-13160. doi:
551 10.1073/pnas.211439298
- 552 Baron, M., Davies, S., Alexander, L., Snellgrove, D., Sloman, K. A.,
553 2008. The effect of dietary pigments on the coloration and behaviour
554 of flame-red dwarf gourami, *Colisa lalia*. *Animal Behaviour* **75**,
555 1041-1051. doi:10.1016/j.anbehav.2007.08.014
- 556 Bennett, A. T. D., Cuthill, I. C., 1994. Ultraviolet vision in birds:
557 What is its function? *Vision Research* **34**, 1471-1478.
558 DOI: 10.1016/0042-6989(94)90149-X
- 559 Bjerkeng, B., Carotenoids in Aquaculture: Fish and Crustaceans. In:
560 G. Britton, S. Liaaen-Jensen, H. Pfander, Eds.), *Carotenoids*, Vol. 4.
561 Birkhäuser Basel, 2008, pp. 237-254.
- 562 Blount, J. D., 2004. Carotenoids and life-history evolution in
563 animals. *Archives of Biochemistry and Biophysics* **430**, 10-15.
564 doi:10.1016/j.abb.2004.03.039
- 565 Bories, G., Brantom, P., Brufau de Barberà, J., Chesson, A.,
566 Cocconcelli, P. S., Debski, B., Dierick, N., Franklin, A., Gropp, J.,
567 Halle, I., Hogstrand, C., de Knecht, J., Lubomir Leng, Haldorsen, A.
568 K. L., Mantovani, A., Mézes, M., Nebbia, C., Rambeck, W., Rychen,
569 G., von Wright, A., Wester, P., 2007. Safety and efficacy of
570 Panaferd-AX (red carotenoid-rich bacterium (*Paracoccus*
571 *carotinifaciens*) as feed additive for salmon and trout. *The EFSA*
572 *Journal* **546**, 1-30.
- 573 Briffa, M., Sneddon, L. U., 2007. Physiological constraints on
574 contest behaviour. *Functional Ecology* **21**, 627-637. DOI:
575 10.1111/j.1365-2435.2006.01188.x

- 576 Carr, A. C., Vissers, M. C. M., 2013. Synthetic or food-derived
 577 vitamin C – Are they equally bioavailable? *Nutrients* **5**, 4284-4304.
 578 DOI: 10.3390/nu5114284
- 579 Cummings, M. E., Rosenthal, G. G., Ryan, M. J., 2003. A private
 580 ultraviolet channel in visual communication. *Proceedings of the*
 581 *Royal Society B* **270**, 897-904. DOI: 10.1098/rspb.2003.2334
- 582 Evans, M. R., Norris, K., 1996. The importance of carotenoids in
 583 signaling during aggressive interactions between male firemouth
 584 cichlids (*Cichlasoma meeki*). *Behavioral Ecology* **7**, 1-6.
 585 doi: 10.1093/beheco/7.1.1
- 586 Gray, S. M., Hart, F. L., Tremblay, M. E. M., Lisney, T. J.,
 587 Hawryshyn, C. W., 2011. The effects of handling time, ambient light,
 588 and anaesthetic method, on the standardized measurement of fish
 589 colouration. *Canadian Journal of Fisheries and Aquatic Sciences* **68**,
 590 330-342. Doi: 10.1139/F10-151
- 591 Guillaume, J., Kaushik, S., Bergot, P., Metailler, R., 2001. Nutrition
 592 and feeding of fish and crustaceans. Praxis Publishing, UK.
- 593 Harpaz, S., Padowicz, D., 2007. Color enhancement in the
 594 ornamental dwarf cichlid *Microgeophagus ramirezi* by addition of
 595 plant carotenoids to the fish diet. *Israeli Journal of Aquaculture-*
 596 *Bamidgeh* **59**, 195-200.
- 597 Kodric-Brown, A., 1998. Dietary carotenoids and male mating
 598 success in the guppy: an environmental component to female choice.
 599 *Behavioral Ecology and Sociobiology* **25**, 393-401.
 600 10.1007/BF00300185
- 601 Kodric-Brown, A., Johnson, S. C., 2002. Ultraviolet reflectance
 602 patterns of male guppies enhance their attractiveness to females.
 603 *Animal Behaviour* **63**, 391-396. doi:10.1006/anbe.2001.1917

- 604 Leclercq, E., Taylor, J. F., Migaud, H., 2010. Morphological skin
 605 colour changes in teleosts. *Fish and Fisheries* **11**, 159-193. DOI:
 606 10.1111/j.1467-2979.2009.00346.x
- 607 Losey, G. S., Cronin, T. W., Goldsmith, T. H., Hydes, D., Marshall,
 608 N. J., McFarland, W. N., 1999. The UV visual world of fishes: a
 609 review. *Journal of Fish Biology* **54**, 921-943. DOI: 10.1111/j.1467-
 610 2979.2009.00346.x
- 611 Maan, M. E.m van der Spoel, M., Jimenez, P. Q., van Alphen, J. J.
 612 M., Seehausen, O., (2006). Fitness correlates of male coloration in a
 613 Lake Victoria cichlid fish. *Behavioral ecology*. **17**, 691-699. doi:
 614 10.1093/beheco/ark020
- 615 Maan, M. E., Seehausen, O., Van Alphen. J. J. M., 2010. Female
 616 mating preferences and male coloration covary with water
 617 transparency in a Lake Victoria cichlid fish. *Biological Journal of the*
 618 *Linnean Society* **99**, 398-406. DOI: 10.1111/j.1095-
 619 8312.2009.01368.x
- 620 McGraw, K. J., Hill, G. E., Stradi, R., Parker, R. S., 2002. The effect
 621 of dietary carotenoid access on sexual dichromatism and plumage
 622 pigment composition in the American goldfinch. *Comparative*
 623 *Biochemistry and Physiology Part B: Biochemistry and Molecular*
 624 *Biology* **131**, 261-269. DOI: 10.1016/S1096-4959(01)00500-0
- 625 Metcalfe, N. B., 1986. Intraspecific varitation in competitive ability
 626 and food intake in salmonids: consequences for energy budgets and
 627 growth rates. *Journal of Fish Biology* **28**, 525-531. DOI:
 628 10.1111/j.1095-8649.1986.tb05190.x
- 629 O'Connor, K. I., Metcalfe, N. B., Taylor, A. C., 1999. Does
 630 darkening signal submission in territorial contests between juvenile

- 631 Atlantic salmon, *Salmo salar*? *Animal Behaviour* **58**, 1269-1276.
 632 doi:10.1006/anbe.1999.1260
- 633 Olsen, V. A., Owens, I. P. F., 1998. Costly sexual signals: are
 634 carotenoids rare, risky or required? *Trends in Ecology and Evolution*
 635 **13**, 510-514. doi:10.1016/S0169-5347(98)01484-0
- 636 Rick, I. P., Bakker, T. C. M., 2008a. UV wavelengths make female
 637 three-spined sticklebacks (*Gasterosteus aculeatus*) more attractive
 638 for males. *Behavioral Ecology and Sociobiology* **62**, 439-445. DOI:
 639 10.1007/s00265-007-0471-6
- 640 Rick, I. P., Bakker, T. C. M., 2008b. Color signaling in conspicuous
 641 red sticklebacks: do ultraviolet signals surpass others? *BMC*
 642 *Evolutionary Biology* **8**, 189-198. doi:10.1186/1471-2148-8-189
- 643 Rick, I. P., Modarressie, R., Bakker, T. C. M., 2006. UV wavelengths
 644 affect female mate choice in three-spined sticklebacks. *Animal*
 645 *Behaviour* **71**, 307-313. doi:10.1016/j.anbehav.2005.03.039
- 646 Sargent, R. C., Gross, M. R., Van Den Berghe, E. P., 1986. Male
 647 mate choice in fishes. *Animal Behaviour* **34**, 545-550.
 648 doi:10.1016/S0003-3472(86)80123-3
- 649 Sies, H., Stahl, W., 1995. Vitamins E and C, beta-carotene, and other
 650 carotenoids as antioxidants. *The American Journal of Clinical*
 651 *Nutrition* **62**, 1315S-1321S.
- 652 Sinha, A., Asimi, O. A., 2007. China rose (*Hibiscus rosasinensis*)
 653 petals: a potent natural carotenoid source for goldfish (*Carassius*
 654 *auratus* L.). *Aquaculture Research*. **38**, 1123-1128. DOI:
 655 10.1111/j.1365-2109.2007.01767.x
- 656 Sloman, K. A., Baker, D. W., Ho, C. G., McDonald, D. G., Wood, C.
 657 M., 2003. The effects of trace metal exposure on agonistic encounters

658 in juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquatic Toxicology*
 659 **63**, 187-196. doi:10.1016/S0166-445X(02)00176-5
 660 Sloman, K.A., 2010. Exposure of ova to cortisol pre-fertilisation
 661 affects subsequent behaviour and physiology of brown trout.
 662 *Hormones and Behavior* **58**, 433-439.
 663 DOI: 10.1016/j.yhbeh.2010.05.010
 664 Song, J., Kwon, O., Chen, S., Daruwala, R., Eck, P., Park, J. B.,
 665 Levine, M., 2002. Flavanoid inhibition of sodium-dependent vitamin
 666 C transporter (SVCT1) and glucose transporter isoform 2 (GLUT2),
 667 intestinal transporters for vitamin C and glucose. *The Journal of*
 668 *Biological Chemistry* **277**, 15252-15260. DOI:
 669 10.1074/jbc.M110496200
 670 Stevens, M., Parraga, C. A., Cuthill, I. C., Partridge, J. C.,
 671 Troscianko, T. S., 2007. Using digital photography to study animal
 672 coloration. *Biological Journal of the Linnean Society*. 90, 211-237.
 673 DOI: 10.1111/j.1095-8312.2007.00725.x
 674 Vissers, M. C. M., Bozonet, S. M., Pearson, J. F., Braithwaite, L. J.,
 675 2010. Dietary ascorbate intake affects steady state tissue
 676 concentrations in vitamin C-deficient mice: tissue deficiency after
 677 suboptimal intake and superior bioavailability from a food source
 678 (kiwifruit). *American Society for Nutrition*. **93**, 292-301. DOI:
 679 10.3945/ajcn.110.004853
 680 White, D. A., Page, G. I., Swaile, J., Moody, A. J., Davies, S. J.,
 681 2002. Effect of esterification on the absorption of astaxanthin in
 682 rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture*
 683 *Research* **33**, 343-350. DOI: 10.1046/j.1365-2109.2002.00680.x

684 Wong, B. B. M., Jennions, M. D., 2003. Costs influence male mate
685 choice in a freshwater fish. *Proceedings of the Royal Society B:*
686 *Biological Sciences* **270**, S36-S38. DOI 10.1098/rsbl.2003.0003
687 Yanar, M., Erçen, Z., Hunt, A. Ö., Büyükçapar, H. M., 2008. The use
688 of alfalfa, *Medicago sativa* as a natural carotenoid source in diets of
689 goldfish, *Carassius auratus*. *Aquaculture*. **284**, 196-200. DOI
690 10.1016/j.aquaculture.2008.07.050

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711 Table 1: Nutritional content and carotenoid concentrations in negative
 712 control, 20 ppm synthetic astaxanthin (AX), 20 ppm and 40 ppm
 713 natural AX diets. Concentrations expressed as mg kg⁻¹. Analyses
 714 conducted on 3 g samples of each diet. Limit of quantification (LOQ)
 715 was 0.03 mg kg⁻¹.

Proximate composition (%)	Negative control	20 ppm synthetic-AX	20 ppm natural-AX	40 ppm natural-AX
Protein	32.5	32.6	31.7	32.8
Total lipid	9.8	9.9	10.2	9.9
Moisture	6.8	7.6	7.7	6.9
Ash	11.3	11.0	10.7	11.2
Carotenoid				
Astaxanthin	<LOQ	19.98	22.1	44.07
Canthaxanthin	<LOQ	<LOQ	3.45	6.84
Astacene	<LOQ	0.58	-	-
Lutein	<LOQ	<LOQ	<LOQ	<LOQ
Beta-carotene	-	-	<LOQ	<LOQ
Echinone	-	-	<LOQ	<LOQ
Cis-echinone	-	-	<LOQ	<LOQ
3-hydroxyechinone	-	-	0.22	0.45
Adonirubin	-	-	11.21	22.97
Asteroidenone	-	-	0.25	0.38
Adonixanthin	-	-	1.81	3.55
Total carotenoids	<LOQ	20.56	39.04	78.26

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723 Table 2: Analyses (one-way ANOVA) of the effects of diet treatment on the change in hue distribution over a 12 week period in various body areas in male
 724 and female cherry barbs (n= 24 fish per diet treatment).

	Diet		Negative control	20 ppm synthetic-AX	20 ppm natural-AX	40 ppm natural-AX
Male fish	F_{3,67}	P	Mean (lower, upper bound 95% confidence interval)			
Whole body	3,15.65=2.18	0.13	-0.008(-0.011, -0.004)	-0.012(-0.016, -0.009)	-0.011(-0.015, -0.007)	-0.007(-0.011, -0.004)
Caudal fin	3,15.43=2.47	0.10	-0.012(-0.015, -0.008)	-0.016(-0.02, -0.013)	-0.015(-0.019, -0.011)	-0.009(-0.13, -0.005)
Anal fin	3,15.82=3.22	0.05	-0.007(-0.011, -0.004)	-0.013(-0.011, -0.004)	-0.01(-0.014, -0.007)	-0.008(-0.012, -0.004)
Anterior dorsal area	3,16.43=3.67	0.03	-0.006(-0.01, -0.002)	-0.01(-0.014, -0.006)	-0.011(-0.016, -0.007)	-0.006(-0.011, -0.002)
Female fish	F_{3,65}	P				
Whole body	3,17.03=1.67	0.21	-0.002(-0.009, 0.005)	0.00(-0.009, 0.008)	0.004(-0.003, 0.011)	-0.004(-0.011, 0.002)
Caudal fin	3,19.99=1.79	0.18	0.005(-0.004, 0.014)	0.00(-0.011, 0.012)	0.01(0.001, 0.02)	0.006(-0.004, 0.015)
Anal fin	3,17.74=2.16	0.13	-0.004(-0.009, 0.001)	-0.001(-0.008, 0.006)	0.00(-0.005, 0.005)	-0.006(-0.012, -0.001)
Anterior dorsal area	3,15.74=1.94	0.17	0.001(-0.004, 0.006)	0.00(-0.007, 0.006)	0.008(0.003, 0.013)	-0.004(-0.009, 0.001)

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727 Figure 1: Male and female cherry barb colouration was analysed across
 728 the A) whole body, B) caudal fin, C) anal fin and D) anterior dorsal
 729 area. Representation of male cherry barb whole body image E) before
 730 and F) after %R and %Y calculations. Pixels within an image that fit
 731 predefined red and yellow criteria are coloured accordingly and a
 732 percentage is automatically calculated. Pixels which do not meet red
 733 or yellow criteria are coloured black. The white background of the
 734 image is recognised as not being part of the fish and is discounted from
 735 percentage calculations.

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739 Figure 2: Mean ($\pm$ S.E.M.) percentage of time male fish spent

740 associated with females from different diet treatments (negative

741 control, 20 ppm synthetic-AX, 20 ppm and 40 ppm natural-AX diets)

742 under different lighting conditions (full colour spectrum lighting, red

743 light blocked and UV light blocked) (n= 9 fish per diet treatment).

744 Letters indicate homogenous groups between diet treatments within

745 lighting treatments at the 5% significance level (Tukey's HSD).

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749 Figure 3: The mean ($\pm$ S.E.M.) number of aggressive interactions
 750 performed per minute to a mirror image by male fish from different
 751 diet treatments (n= 9 fish). Letters indicate homogenous groups at the
 752 5% significance level (Tukey's HSD).