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Maternal effects of carotenoid supplementation in an ornamented cichlid fish

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Summary

1. Carotenoid-ornamented animals may transfer dietary carotenoid pigments to offspring as well as to their ornament and other competing physiological demands. If carotenoid supplementation increases offspring growth or survival, females should preferentially allocate supplemental carotenoids to offspring rather than ornamentation.

2. We tested whether ornamented convict cichlid (*Amatitlania siquia*) mothers increase carotenoid allocation to eggs with access to dietary carotenoid supplementation, and whether beneficial maternal effects of carotenoid supplementation were transferred to offspring.

3. Maternal effects of carotenoid supplementation were found; offspring from mothers that ate carotenoids benefited in terms of growth and survival parameters compared with offspring from non-supplemented mothers. Contrary to predictions, maternal coloration was not sacrificed for fecundity: mothers maintained their coloration and integument carotenoids even though increased carotenoids in the maternal diet benefited the offspring. Furthermore, carotenoid supplementation did not increase the carotenoids deposited in eggs.

4. This work is the first to show that indirect maternal effects of carotenoid supplementation increased the survival and growth of offspring. Indirect benefits may reduce the cost of carotenoid allocation to offspring, but the mechanism for how carotenoids support offspring development is still unknown.

Key-words: *Amatitlania siquia*, carotenoids, maternal effects, dimorphism, offspring fitness, diet

Introduction

Conspicuous colours of many birds, fishes and lizards are produced by pigmentation of the skin and feathers. Animals can synthesize some pigments, such as pterins and melanins, but other pigments, such as carotenoids, must be obtained from the diet. The carotenoid trade-off hypothesis predicts that the expression of carotenoid ornaments is constrained by (i) carotenoid availability and (ii) competing physiological demands. In addition to pigmentation, biochemical demands for carotenoids include antioxidant protection (Bertrand *et al.* 2006; Costantini & Moller 2008; Garratt & Brooks 2012), immune function (Pap *et al.* 2009; Toomey, Butler & McGraw 2010; McGraw, Nolan & Crino 2011) and reproduction (Surai *et al.* 2001; Newbrey & Reed 2009). Carotenoids are also used in egg production, so carotenoid ornamentation can honestly signal the foraging ability, molecular health or offspring quality of a potential mate (Lozano 1994).

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Females that allocate carotenoids to ornaments at the expense of eggs should incur fitness costs (Fitzpatrick, Berglund & Rosenqvist 1995; Chenoweth, Doughty & Kokko 2006). Because of this, ornamented mothers should allocate carotenoids to eggs at the expense of body pigmentation (Morales, Velando & Torres 2009). To decrease the cost of distributing carotenoids among eggs, ornamentation and other physiological demands, ornamented females can use alternative strategies to enhance ornament expression. Alternative strategies may include translucent skin over pigmented gonads (Baird 1988; Svensson *et al.* 2005), involvement of endogenous pigments (Weiss *et al.* 2011) or structural colour (Brown, McGraw & Clotfelter 2013). These strategies allow females to increase their carotenoid investment in offspring by placing more of the pigment into egg yolks.

Yolk carotenoids and their derivatives may benefit parental fitness in terms of increased offspring growth rate (Lakeh *et al.* 2010), reduced offspring oxidative stress (Blount *et al.* 2002) and enhanced offspring immunity (Bendich & Olson 1989; Blount *et al.* 2003; McGraw &

Ardia 2003). The following study tests whether female reverse sexually dimorphic fish allocate dietary carotenoids to yolk at the expense of pigmentation, and whether maternally derived yolk carotenoids benefit offspring. Our study system is the convict cichlid (*Amatitlania siquia,* formerly *A. nigrofasciata;* Schmitter-Soto 2007). Several forms of sexual dimorphism occur in this species, including body size, fin morphology and brightly coloured ventral patches over the gonads of females only (Noonan 1983). Interaction of skin carotenoids and light-reflective guanine crystals may interact to control the colour and brightness of the ornament (Brown, McGraw & Clotfelter 2013).

Mate choice decisions are based on body size for both males and females (Wisenden 1993; Robart 2012) – ventral patches do not appear to be the product of intersexual selection (Beeching *et al*. 1998). Convict cichlid parents are generally socially monogamous (Wisenden 1995; Beeching & Hopp 1999), although males occasionally desert the female and offspring (Wisenden 1994). Females, and sometimes males under laboratory conditions, can successfully raise offspring without a mate (Keenleyside & Mackereth 1992; Lavery & Reebs 1994; Itzkowitz, Santangelo & Richter 2001), but both sexes provide food for offspring and defend the nest site (Wisenden, Lanfranconi-Izawa & Keenleyside 1995). In general, female convicts are smaller but more aggressive than their mates (Lamprecht & Rebhan 1997), spend more time with the offspring (Lavery & Colgan 1991; Lavery & Reebs 1994) and display more parental behaviours (Lavery & Reebs 1994).

The two hypotheses of the present study are as follows: (i) carotenoid-supplemented females should show increased ventral patch yellow colour, decreased oxidative damage and increased egg carotenoids compared with non-supplemented females. Furthermore, (ii) broods from carotenoid-supplemented mothers may be larger, grow faster and show enhanced survival compared with offspring from non-supplemented mothers.

To address these questions, we fed female fish a carotenoid-supplemented or a non-supplemented diet regime and measured their ornament colour and carotenoid content. After females laid eggs, carotenoids in a sample of eggs were also measured. Size, growth and survival of broods that hatched from these eggs were measured to determine whether and how maternal carotenoids benefit offspring. Finally, we measured oxidative stress in both mothers and offspring to determine whether maternal carotenoids reduced oxidative stress in mothers and fry.

Materials and methods

DIETS AND ANIMALS

All procedures were approved by the Amherst College Institutional Animal Care and Use Committee (IACUC). Sexually mature, laboratory-reared offspring of wild-caught convict cichlids (Río Cabuyo, Lomas Barbudal Biological Reserve, Guanacaste, Costa Rica) were used in this work ($n = 52$). They were fed to satiation twice daily for 14 weeks. The base for convict cichlids diets used in this study was a modified H440 diet, which consists of an agar-cellulose-detrin base (Halver 1989). The level of carotenoid supplementation was within the range of biologically relevant levels (Olson 2006) and previously shown sufficient to produce an increase in yellow ventral patch reflectance in convict cichlid females (Brown, McGraw & Clotfelter 2013). There were two diet groups: 'non-supplemented' (<1·0 μg g^{-1} carotenoids: 29·5% β-carotene, 70·5% unknown carotenoids by HPLC) and 'supplemented' with carotenoids from marigold [GNC, Inc. Pittsburgh, PA, USA; diet: 5·82 μg g^{-1} carotenoids: 84·9% lutein, 10·7% β-carotene, 4·3% zeaxanthin, 0·1% unknown carotenoids by high-performance liquid chromatography (HPLC)]. Non-supplemented diets contained no added carotenoids. A preliminary study showed that convict cichlids have no preference for carotenoid-supplemented or non-supplemented H440 diets (A. C. Brown & E. D. Clotfelter, unpublished data).

HOUSING AND CARE

During weeks 0–8, female fish were individually housed in 2-L clear plastic tanks with an airstone in each tank providing constant oxygenation. Cardboard partitions were placed around the tanks to visually isolate the females. Once daily, 50% of the tank water was removed and replaced with DI water and aquarium salts (Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA) to maintain conductivity between 100 and 200 μS cm^{-1}. Algae growth was controlled by scraping the inside walls of each tank with an algae-removing magnet (Mag-Float®, Dania Beach, FL, USA) as necessary, followed by a water change, in order to limit sources of non-experimental carotenoids.

On week 8, each female fish was placed in a filtered 38-L breeding tank with a clay pot refugia. At the same time, two potential mates from a pool of unrelated laboratory-bred males were also placed into the breeding tank. Prior to introduction to the breeding tanks, males were maintained on commercial cichlid food (Hikari, Inc., Hayward, CA, USA). While in the breeding tanks, males ate female experimental diets to prevent female access to non-experimental carotenoids.

Potential mates were up to 10% smaller than or equal to the standard length of the female. When a male appeared injured or was observed engaging in mouth-wrestling with the female, the male was removed and replaced. These procedures were used to decrease female mortality. When a male was observed defending the female and the refugia from the other male, the male and female were considered paired. This usually occurred with 2 days of co-habitation. Males that were not selected as mates were immediately removed. Pairs remained in the breeding tanks until they produced eggs or until week 14, when the diet treatment of mothers ended.

MATERNAL BODY AND REFLECTANCE MEASURES

At 0, 8 and 14 weeks, fish were weighed (±0·01 g), measured for standard length with digital callipers (±0·01 mm) and subjected to colour reflectance measurements (described below). The residuals of a mass–standard length regression were used as the measure of body condition (Roh, Mirza & Brown 2004; Bentley & Schindler 2013).

Reflectance of the ornamental patch on the left flank of each fish was recorded with a fibre optic spectrometer with a pulsed xenon light source (USB 4000; Ocean Optics, Dunedin, FL, USA). The probe was placed at a 45° angle to reduce scatter (Lahti 2006; Clotfelter, Ardia & McGraw 2007; Brown, McGraw & Clotfelter 2013). Data were binned into 5-nm increments and trimmed to a range of 300–700 nm, as convict cichlids' visible spectrum falls within 400–700 nm (Jackson 2003). Reflectance was standardized, or centred, by subtracting the mean reflectance

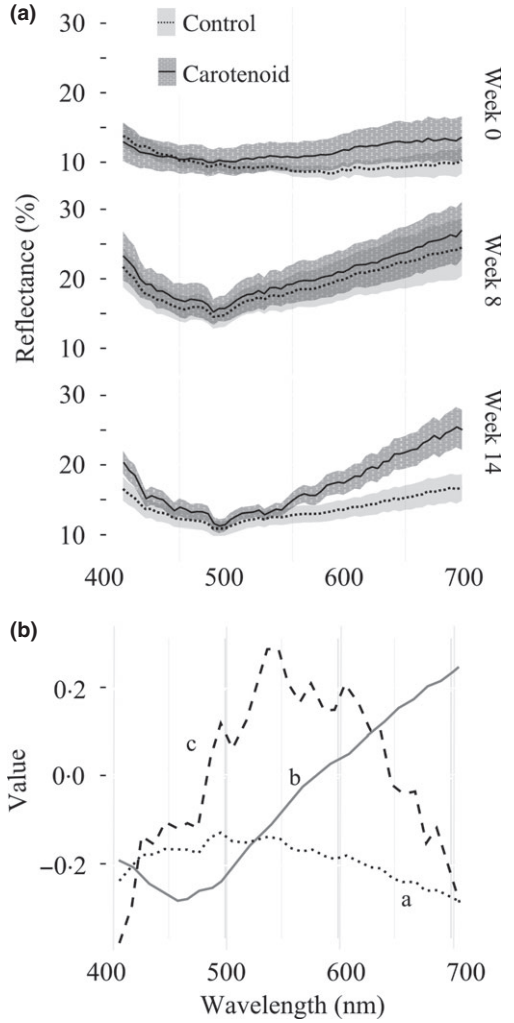

Fig. 1. Mean reflectance for carotenoid and control diet female *Amatitlania siquia* ventral patches on weeks 0, 8 and 14 (top). Shading in the upper figure represents standard error. Principal component loadings (bottom) of these reflectance data show a: PC1; b: PC2; and c: PC3.

across all wavelengths before principal component analysis (PCA), as described by Cuthill *et al.* (1999).

Principal component analysis (PCA) is often used to measure animal coloration because it makes no assumptions about the visual system of the viewer and can compress many highly correlated reflectance values into a few independent variables (Hill & McGraw 2006). PCA generates a series of orthogonal eigenvectors (the 'loadings') to account for the maximum variation in a data set. Principal component values, or eigenvalues, explain the residual of each fish from the eigenvector (Cuthill *et al.* 1999; Jolliffe 2002). As many PC values as variables will be generated by PCA; here, only components that explain >1% of the variance were retained.

The first principal component (PC1) explained 72·53% of maternal ornament reflectance, while PC2 and PC3 explained 19·64% and 4·89%, respectively (Fig. 1). The first component refers to the total size of the area under the spectral curve (or 'brightness'), and the second and third components show the shape of the reflectance curve ('hue' and 'chroma') (Cuthill *et al.* 1999; Hill & McGraw 2006). For our data, PC2 shows yellow, orange and red hues (Cuthill *et al.* 1999; Hill & McGraw 2006), while PC3 shows the contribution of the blue and green values. Additional details of eigenvector interpretation are available in the study by Brown, McGraw and Clotfelter (2013) or Cuthill *et al.* (1999).

MATERNAL ORGAN COLLECTION AND ANALYSIS

After laying eggs, females were euthanized with 10 g L^{-1} tricaine methanesulfonate (MS-222) followed by cervical dislocation with surgical scissors. Microhematocrit tubes collected blood from the caudal vein of each fish. Filled microhematocrit tubes were centrifuged at 13 000× ***g*** to determine blood hematocrit. Blood hematocrit, or packed cell volume, provides a measurement of the proportion of red blood cells (erythrocytes) in whole blood as well as an estimate of blood oxygen carrying capacity (Wells *et al.* 1986; Houston 1997).

The ventral patch was excised from the left side of each female by clipping around the perimeter of the patch with surgical scissors. Integument samples were stored at −80 °C for subsequent analysis by high-performance liquid chromatography (HPLC). Livers were removed, weighed and stored at −80 °C for analysis of oxidative damage by acrolein assay.

CHROMATOGRAPHY

Tissues were frozen in liquid nitrogen and ground to powder using a mortar and pestle. Extractions were performed using hexane, and samples were dried and suspended in acetone and Tris–HCl buffer. Cholesterol esterase (C-9281; Sigma, St. Louis, MO, USA) hydrolysed the carotenoids in a 37 °C water bath for 45 min. Carotenoids were extracted using sodium sulphate decahydrate and hexane, which was dried down under nitrogen gas and reconstituted in mobile phase. Analyses were performed on a Waters 600 HPLC using a Luna 3-μm analytical silica column (00F-4162-E0; Phenomenex, Torrance, CA, USA). The flow rate was 1·2 mL min^{-1}, and the mobile phase was an isocratic mixture of 82 : 18 hexane : acetone. The injection loop size was 20 μL. A Waters 600E wavelength absorbance detector was used to produce a chromatogram at 474 nm. Total amounts of carotenoids were calculated using peak area integration values and the standard curves for each identifiable carotenoid type (Millennium 32; Waters Corp. Milford, MA, USA).

ACROLEIN ASSAY

Oxidative damage was measured using an ELISA assay to measure acrolein, a stable product of lipid oxidation. Across biological systems, acrolein binds covalently to lysine and can be quantified in protein samples as a measure of unmediated oxidative damage (Uchida *et al.* 1998). We homogenized each sample in 1 mL mg^{-1} iced phosphate-buffered saline. Following centrifugation at 4500 ***g*** for 10 min, a modified Bradford protein assay kit (Bio-Rad, Hercules, CA, USA) determined the supernatant protein concentrations. Samples were then adjusted to a concentration of 1 mg protein 1 per mL for the acrolein assay. For the acrolein assay, anti-acrolein monoclonal antibody (mAb5F6) determined mM acrolein mg^{-1} protein in each sample following the protocol of Kurtz *et al.* (2006). Serially diluted standards of acrolein-modified bovine serum albumin (BSA) provided a standard curve. Microplates were read at 474 nm on a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

OFFSPRING HOUSING

Female fish produced 74–334 eggs each (mean = 202·4 ± 89·35), and live offspring hatched from 26 of 27 clutches (carotenoid maternal diet n = 12, non-supplemented maternal diet n = 14). Eggs were removed from breeding tanks and placed in incubation chambers as soon as spawning had ceased. Each chamber was an 8-cm-diameter PVC plastic cylinder with a mesh bottom, suspended within a filtered 38-L tank to expose offspring to uniform

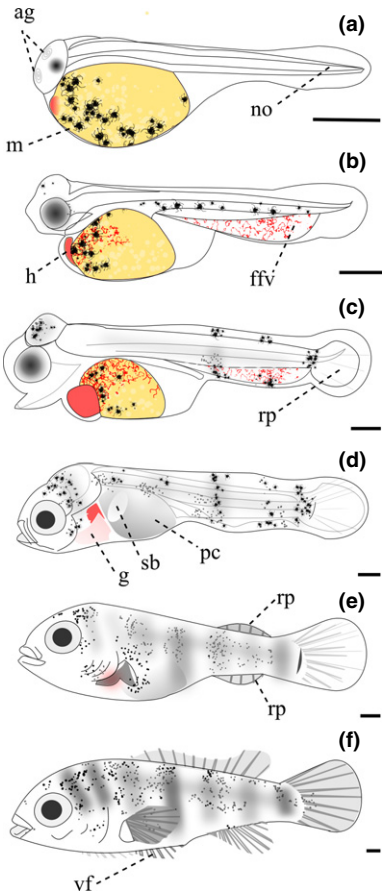

Fig. 2. Development of *Amatitlania siquia* larvae during the experimental observation period of 0–28 days. (a) post-hatching stage (2 days); (b) yolk sac stage (3·5 days); (c) opened mouth stage (4·5 days); (d) free swimming stage (8 days); (e) unpaired fins stage (14 days); (f) pre-juvenile stage (25 days). ag, adhesive glands; ffv, fin fold vessels; g, gills; h, heart; m, melanocytes; no, notochord; pc, peritoneal cavity lining; rp, rays primordia; sb, swim bladder; vf, ventral fin. Scale bars = 500 μm.

rearing conditions. Each brood was placed in a separate incubation chamber within the incubation tank. Tanks were maintained at 23 °C (±1 °C), and water changes were implemented at least once per week or as needed. Control diet was the only food provided to offspring regardless of maternal diet. Feedings occurred once per day until fry began to self-feed (around 7–8 days), at which time feeding frequency increased to 3× daily, *ad libitum* (Fig. 2). Uneaten food was removed with a bulb syringe.

FRY MEASUREMENTS

Eggs and fry that were removed for biochemical analyses were included in brood size measures, but not survival and growth analysis. Samples of ≈ 20 unhatched eggs were collected as the brood was transferred to the incubation tanks. These eggs were removed from the nesting substrate with a razor blade, counted and frozen at −80 °C until later determination of carotenoid content by HPLC using the methods detailed above.

After hatching, ≈ 20 yolk-dependent fry (<12 h post-hatch; Fig. 2) were harvested from each brood for acrolein analysis using methods detailed above. These fry were euthanized in a solution of 40% ethanol, rinsed gently in deionized water, weighed, counted and stored at −80 °C until analysis.

Photographs were taken of each brood (i) on being transferred to the incubation tanks (as eggs), (ii) on hatching day and (iii) every 7 days for 28 days. At stages 1 and 2, fry samples for carotenoid analysis and acrolein assay, respectively, were collected prior to photography. Each brood was transferred to a sterile 100-mm petri dish in 200 mL incubation water. The petri dish was placed within a circle drawn onto a white background. A digital camera (P90; Nikon, Melville, NY, USA) was secured to a small tripod so that the camera lens was 18 cm above (90°) the petri dish. A metric ruler was included within each frame for scale. Afterwards, each brood was returned to its incubation chamber.

IMAGEJ software (Rasband 1997) was used to count eggs and measure the length of each fry within a clutch. Average egg volume was calculated using equations optimized by Narushin (2005) for analysis of avian eggs, where B is the maximum breadth (mm), and L is the maximum length (mm):

$$V = (0{\cdot}65057 - 0{\cdot}0018 * B)(\mathrm{LB}^2) \qquad \text{eqn 1}$$

After hatching, average (AGR) was calculated from the average length for each brood (Stobart *et al.* 1986; Vijayagopal, Gopakumar & Vijayan 2008). AGR was calculated as the average gain in size over the course of the monitoring period as follows, where y is the average length at time t:

$$\mathrm{AGR} = \frac{\ln(y_1) - \ln(y_2)}{t_2 - t_1} \qquad \text{eqn 2}$$

Instantaneous survival rates for each brood were calculated by dividing the fry number at t_{+1} by fry number at t.

STATISTICS

Analyses were conducted using R statistical software v. 2.14.2 (R Development Core Team 2010). To test the effect of dietary carotenoids on ventral ornament colour or maternal body condition, we used repeated-measures ANOVA. Diet was the main effect, with week (0, 8 or 14) as a fixed effect and fish (subject) nested within diet treatment as the error term. The dependent variable was PC1, PC2, PC3 or body condition. ANOVA was also used to test the effect of breeding status on colour, with breeding status as the main effect with diet and an interaction term. The dependent variable was PC1, PC2 or PC3. The effect of diet treatment on integument carotenoids, egg carotenoids, egg volume, egg number, liver acrolein, offspring acrolein or blood hematocrit was determined by 2-way ANOVA.

Offspring analyses were performed using repeated-measures ANOVA. Average total length per brood, AGR or survival was the dependent variable. Maternal diet was the main effect, with day post-hatch as a fixed effect and brood (subject) nested within the maternal diet treatment as the error term.

Multiple linear regression was used to test for relationships between egg volume or egg number and fry lengths or survival within the diet groups. Linear regression was used to test for a relationship between maternal mass and egg number, liver carotenoids and liver acrolein, or maternal acrolein and offspring acrolein. All residuals were normally distributed except acrolein levels, which were log-transformed to achieve normality. P-values were considered significant when $P < 0{\cdot}05$.

Results

EFFECTS OF CAROTENOIDS ON MOTHERS

We predicted that females would allocate carotenoids to their offspring at the expense of their own coloration and

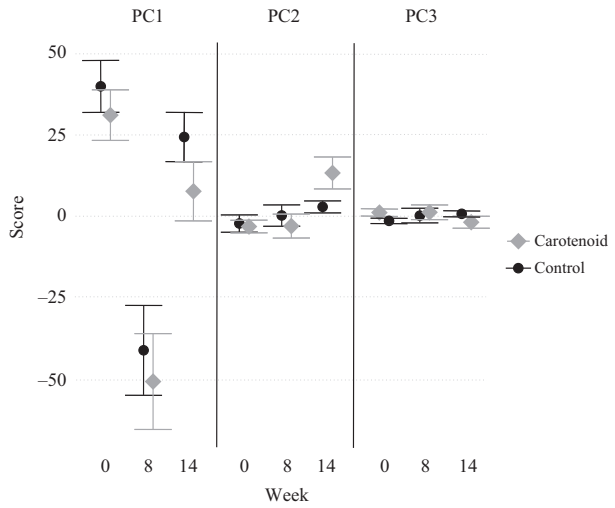

Fig. 3. Female *Amatitlania siquia* colour reflectance on weeks 0, 8 and 14 during the course of carotenoid-supplemented or unsupplemented diet trial. Bars show the standard error.

health and that carotenoids in eggs would benefit offspring. Maternal integument total carotenoids ranged between 0·08 and 1·43 μg g^{-1} and consisted of esterified β-carotene and two esterified xanthophylls: astaxanthin and tunaxanthin. Of 52 female fish that completed the 14-week experiment, 27 fish produced eggs. Carotenoid supplementation did not affect body condition in mothers (week 8: $F_{1,25} = 3{\cdot}05$, $P = 0{\cdot}09$, week 14: $F_{1,25} = 2{\cdot}00$, $P = 0{\cdot}17$).

Diet did not affect the proportions of carotenoids (carotenes/xanthophylls) found in the integument ($F_{1,25} = 0{\cdot}15$, $P = 0{\cdot}70$) or total carotenoids in maternal integument ($F_{1,25} = 1{\cdot}34$, $P = 0{\cdot}25$). Supplemented mothers showed significantly decreased blood hematocrit compared with controls ($F_{1,25} = 14{\cdot}32$, $P < 0{\cdot}001$). Diet did not affect acrolein levels in the liver ($F_{1,24} = 0{\cdot}01$, $P = 0{\cdot}91$).

All female fish became significantly less bright between weeks 0 and 8 and brighter again by week 14 ($F_{1,47} = 5{\cdot}311$, $P = 0{\cdot}02$; PC1, Fig. 3). Fish that produced eggs were significantly more yellow (PC2) at week 8 than fish who did not produce eggs ($F_{1,47} = 25{\cdot}62$, $P < 0{\cdot}001$; Fig. 4). Changes in brightness and coloration were not affected by carotenoid supplementation ($F_{1,47} \leq 0{\cdot}11$, $P > 0{\cdot}74$ for all) or related to carotenoids found in the integument ($R^2 = 0{\cdot}01$, $P > 0{\cdot}26$, for all). Yellow coloration was not affected by diet supplementation when the analysis was restricted to fish that spawned ($F_{1,25} = 3{\cdot}37$, $P = 0{\cdot}07$), although supplemented fish became more yellow (PC2) on average.

EFFECTS OF MATERNAL CAROTENOIDS ON OFFSPRING

The mean carotenoid concentration of eggs was 2·04 (±0·97) μg g^{-1}. HPLC detected esterified carotenes, tunaxanthin and astaxanthin, in addition to trace amounts of astacene, lutien and zeaxanthin, and several unidentified carotenoids. Maternal mass at week 8 predicted egg number ($R^2 = 0{\cdot}13$, $P = 0{\cdot}02$, $n = 26$).

Growth and survival analysis in the present study could not continue beyond 28 days because most of the offspring from non-supplemented mothers died, resulting in sample sizes too small to compare. Maternal carotenoid supplementation significantly increased offspring size ($F_{1,72} = 28{\cdot}15$, $P < 0{\cdot}001$), but this effect did not appear until the fry were older than 14 days (Fig. 5a). Offspring from carotenoid-supplemented females survived longer than offspring from control females ($F_{1,21} = 13{\cdot}13$, $P = 0{\cdot}003$; Fig. 5b). Maternal diet did not increase AGR ($F_{1,101} = 1{\cdot}15$, $P = 0{\cdot}28$).

Maternal diet also did not affect oxidative damage (acrolein) in larvae ($F_{1,25} = 0{\cdot}24$, $P = 0{\cdot}63$; mean = 2·05 ± 12·49 μg g^{-1}), egg number ($F_{1,21} = 0{\cdot}13$, $P = 0{\cdot}72$), egg volume ($F_{1,21} = 0{\cdot}74$, $P = 0{\cdot}40$) or egg carotenoid content ($F_{1,19} = 0{\cdot}43$, $P = 0{\cdot}52$). Egg carotenoids did not affect offspring length, survival ($R^2 < 0{\cdot}01$, $P > 0{\cdot}50$, $n = 22$ for both) or oxidative damage in larvae ($R^2 = 0{\cdot}03$, $P = 0{\cdot}16$, $n = 22$). Mean egg volume did not affect growth ($R^2 < 0{\cdot}01$, $P = 0{\cdot}82$, $n = 10$) or survival ($R^2 < 0{\cdot}01$,

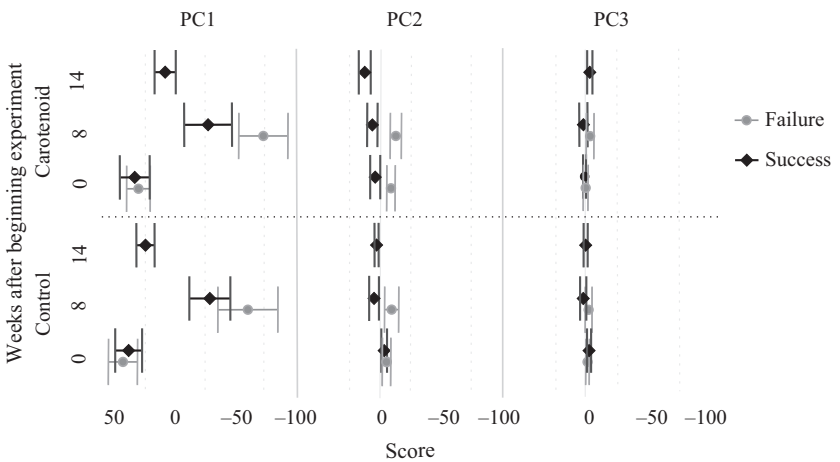

Fig. 4. Comparison of female *Amatitlania siquia* that produced offspring vs. females that did not. Fish that failed to reproduce were excluded at week 14. Bars represent standard error.

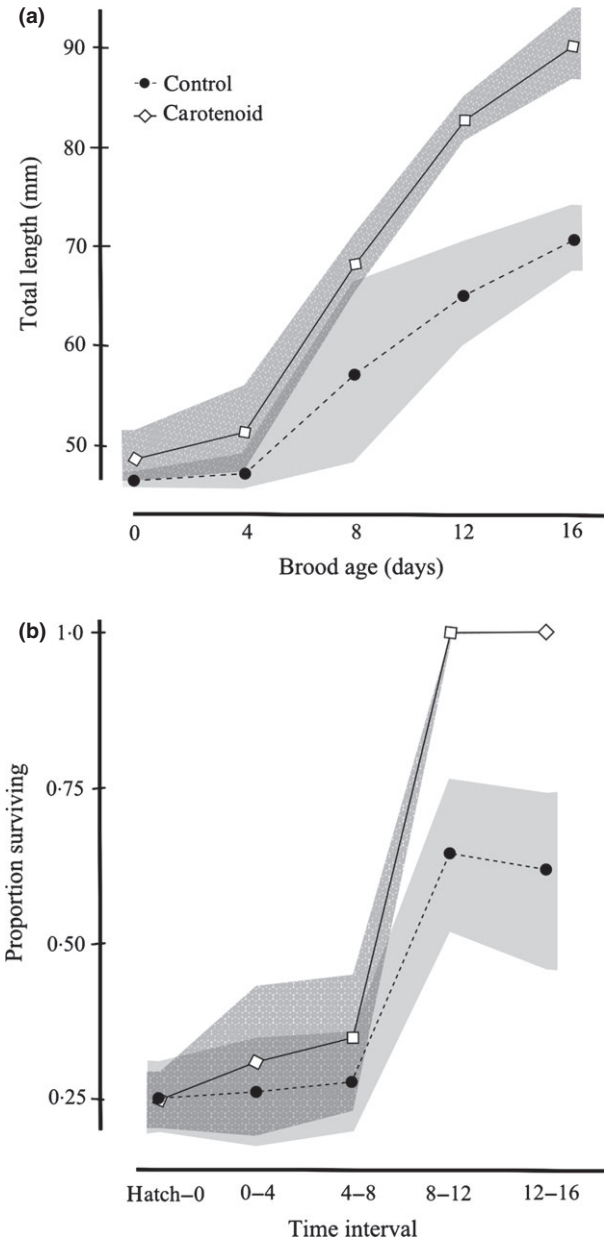

Fig. 5. (a) Total lengths of *Amatitlania siquia* offspring from carotenoid-supplemented and unsupplemented mothers. Shading represents the standard error. (b) The proportion of surviving offspring from each maternal diet group from one measurement time to the next (7 days). Shading represents standard error.

$P > 0{\cdot}50$, $n = 22$). Maternal acrolein did not reflect offspring acrolein ($R^2 = 0{\cdot}04$, $P = 0{\cdot}15$, $n = 22$).

Discussion

If dietary carotenoids are limited and beneficial to offspring development, females that allocate carotenoids to somatic demands at the expense of eggs may suffer reduced fitness (Morales, Velando & Torres 2009). We found that offspring from mothers on the carotenoid-supplemented diet grew larger and survived longer than offspring from non-supplemented mothers, but this was not linked to carotenoids deposited into eggs. Likewise, mothers maintained carotenoid stores in integument, yellow ornament coloration and antioxidant status even without supplemented carotenoids. It seems that carotenoids in the bodies of female fish were not depleted by egg production, although carotenoids in the maternal diet may enhance offspring fitness indirectly.

The result that ventral ornament colour and integument carotenoids were maintained across both diets is consistent with previous work proposing a role for iridophores and pterin pigments in reducing the cost of carotenoid ornamentation (Weiss, Foerster & Hudon 2012; Brown, McGraw & Clotfelter 2013). The present study also found that fish that produced eggs were more yellow prior to introduction to the breeding tank on week 8 than fish that would not produce eggs (Fig. 3). Although the function of the yellow ventral patch was not what this experiment was designed to test, this result offers some support to the hypothesis that the patch is a visual signal that may contain information about female reproductive status.

Maternal carotenoids may have a role early in offspring development in some species (Winston, Lemaire & Lee 2004), but we observed benefits of maternal carotenoids to offspring around 14 days. In convict cichlid larvae, exogenous feeding begins on day 8, and the yolk sac disappears by day 9–10 (Meijide & Guerrero 2000). Starvation, developmental errors and heightened predation risk may converge during this time to result in the stage of highest mortality in larval fishes (Sifa & Mathias 1987). The mechanism for the survival benefits we report here, however, is still unknown.

After 14 days, maternal dietary carotenoids increased the length of offspring. This was accompanied by an increased growth rate (AGR) on average, although this effect was not significant. A delay in growth-enhancing benefits of maternally derived carotenoids has also been reported in gray partridge chicks, *Perdix perdix* (Cucco *et al.* 2008). The delay in growth and survival benefits that we report further support the hypothesis that maternal diet affects offspring indirectly, as benefits were first detectable after the yolk was fully consumed (Fig. 2).

Maternal carotenoid supplementation to eggs affects offspring growth rate in many birds, fish and shrimp (Rema & Gouveia 2005; Wang, Chien & Pan 2006; Cucco *et al.* 2008; Lakeh *et al.* 2010; Jha, Sarma & Qureshi 2012; Niu *et al.* 2012). But we found no link between maternal diet and egg volume. Egg size often predicts offspring success in birds (Krist 2011), but the relationship between maternal fitness and increased egg size is less clear in fishes, as fish often encounter a trade-off between egg number and egg size (Mann & Mills 1985; Morrongiello *et al.* 2012; Semmens & Swearer 2012). Our results show that egg volume did not influence growth or survival in convict cichlids in the laboratory environment. Antioxidant carotenoids may allow for increased offspring growth by relieving oxidative stress in some species (Catoni, Peters & Schaefer 2008), but neither decreased egg carotenoids nor

increased fry acrolein were found in offspring from non-supplemented fish in the present study. Likewise, there was no link between maternal acrolein and offspring acrolein, which might have observed if mothers donate limited antioxidants to eggs.

Although our experimental design could not compare broods into adulthood, the pre-juvenile stage of fish development – which typically begins around 28 days in Neotropical cichlids – is characterized by the presence of adult organs and limbs, including the fins (Meijide & Guerrero 2000). Growth and survival measurement could not continue beyond 28 days in this study because most of the control offspring had died, adding more support to the hypothesis that carotenoids in the maternal diet are important for proper development of offspring. Both carotenoids and retinoids support immunity (Amar *et al*. 2001; Clotfelter, Ardia & McGraw 2007; Mora, Iwata & von Andrian 2008; Mullin 2011), and retinoic acid, a carotenoid derivative, is involved in the development of larval structures, such as the neural crest (Bohnsack & Kahana 2013), the heart (Huang *et al*. 2011) and intestines (Nadauld *et al*. 2005).

Previously, our research group found that carotenoid-supplemented fish placed more carotenoids into gonads than control fish (Brown, McGraw & Clotfelter 2013), but dietary carotenoids did not increase carotenoids in eggs in the present study. The reason for this inconsistency may be that carotenoids are stored in the ovaries and converted to retinoids as eggs mature (Levi *et al*. 2012). Retinoids are nutrients in the vitamin A family which are involved in a variety of cellular processes, including vision (Wald 1968), embryonic development (Johnson & Scadding 1991), gene regulation (Durston *et al*. 1997; Ross *et al*. 2000), immunity (Mora, Iwata & von Andrian 2008; Mullin 2011), redox homeostasis (Alvarez *et al*. 1995) and embryonic development (Durston *et al*. 1997; Antipatis, Grant & Ashworth 2000; Ross *et al*. 2000).

The decrease in hematocrit of fish that consumed carotenoids does not agree with similar studies. There is some evidence that carotenoids may become detrimentally pro-oxidant at pharmacologic dosages (El-Agamey *et al*. 2004; Costantini, Metcalfe & Monaghan 2010), which could potentially alter haematological parameters (Messarah *et al*. 2011), but acrolein data presented here did not show changes in oxidative state, and the carotenoid levels used in this study are not pharmacologic. Further study will determine the biological significance of decreased hematocrit as a result of carotenoid supplementation in convict cichlids.

The present study offers some clues to understanding the interaction between maternal dietary carotenoids and offspring fitness and is the first to characterize it in a female-ornamented species. We found that females did not allocate pigment to offspring rather than their own somatic demands. Also, mothers that consumed carotenoids did not increase allocation to their eggs, yet their fry grew larger and survived better than fry from non-supplemented mothers. We cannot rule out the involvement of carotenoid-derived retinoids in increasing offspring survival and size; retinoids in eggs would have been missed by the carotenoid-specific HPLC techniques used in the present study. However, this is not the only possible mechanism for producing offspring benefits without increased egg carotenoids. How dietary carotenoids might produce indirect trans-generational effects is an important topic for future research.

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References

Alvarez, R., de Andrés, J., Yubero, P., Viñas, O., Mampel, T., Iglesias, R. *et al*. (1995) A novel regulatory pathway of brown fat thermogenesis. Retinoic acid is a transcriptional activator of the mitochondrial uncoupling protein gene. *The Journal of Biological Chemistry*, **270**, 5666–5673.

Amar, E.C., Kiron, V., Satoh, S. & Watanabe, T. (2001) Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, **32**, 162–173.

Antipatis, C., Grant, G. & Ashworth, C.J. (2000) Moderate maternal vitamin A deficiency affects perinatal organ growth and development in rats. *The British Journal of Nutrition*, **84**, 125–132.

Baird, T.A. (1988) Abdominal windows in straighttailed razorfish, *Xyrichtys martinicensis*: an unusual female sex character in a polygynous fish. *Copeia*, **1988**, 496–499.

Beeching, S. & Hopp, A. (1999) Male mate preference and size-assortative pairing in the convict cichlid. *Journal of Fish Biology*, **55**, 1001–1008.

Beeching, S.C., Gross, S.H., Bretz, H.S. & Hariatis, E. (1998) Sexual dichromatism in convict cichlids: the ethological significance of female ventral coloration. *Animal Behaviour*, **56**, 1021–1026.

Bendich, A. & Olson, J.A. (1989) Biological actions of carotenoids. *FASEB Journal*, **3**, 1927–1932.

Bentley, K.T. & Schindler, D.E. (2013) Body condition correlates with instantaneous growth in stream-dwelling rainbow trout and arctic grayling. *Transactions of the American Fisheries Society*, **142**, 747–755.

Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J. & Sorci, G. (2006) Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. *Oecologia*, **147**, 576–584.

Blount, J.D., Surai, P.F., Houston, D.C. & Moller, A.P. (2002) Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Functional Ecology*, **16**, 445–453.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R. & Surai, P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science*, **300**, 125–127.

Bohnsack, B.L. & Kahana, A. (2013) Thyroid hormone and retinoic acid interact to regulate zebrafish craniofacial neural crest development. *Developmental Biology*, **373**, 300–309.

Brown, A.C., McGraw, K.J. & Clotfelter, E.D. (2013) Dietary carotenoids increase yellow nonpigment coloration of female convict cichlids (*Amantitlania nigrofasciata*). *Physiological and Biochemical Zoology*, **86**, 312–322.

Catoni, C., Peters, A. & Schaefer, H.M. (2008) Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. *Animal Behaviour*, **76**, 1107–1119.

Chenoweth, S., Doughty, P. & Kokko, H. (2006) Can non-directional male mating preferences facilitate honest female ornamentation? *Ecology Letters*, **9**, 179–184.

Clotfelter, E.D., Ardia, D.R. & McGraw, K.J. (2007) Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. *Behavioral Ecology*, **18**, 1139–1145.

Costantini, D., Metcalfe, N.B. & Monaghan, P. (2010) Ecological processes in a hormetic framework. *Ecology Letters*, **13**, 1435–1447.

Costantini, D. & Moller, A.P. (2008) Carotenoids are minor antioxidants for birds. *Functional Ecology*, **22**, 367–370.

Cucco, M., Guasco, B., Malacarne, G., Ottonelli, R. & Tanvez, A. (2008) Yolk testosterone levels and dietary carotenoids influence growth and immunity of grey partridge chicks. *General and Comparative Endocrinology*, **156**, 418–425.

Cuthill, I.C., Bennett, A.T.D., Partridge, J.C. & Maier, E.J. (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. *American Naturalist*, **153**, 183–200.

Durston, A.J., van der Wees, J., Pijnappel, W.W.M., Schilthuis, J.G. & Godsave, S.F. (1997) Retinoid signalling and axial patterning during early vertebrate embryogenesis. *Cellular and Molecular Life Sciences*, **53**, 339–349.

El-Agamey, A., Lowe, G.M., McGarvey, D.J., Mortensen, A., Phillip, D.M., Truscott, T.G. *et al.* (2004) Carotenoid radical chemistry and antioxidant/pro-oxidant properties. *Archives of Biochemistry and Biophysics*, **430**, 37–48.

Fitzpatrick, S., Berglund, A. & Rosenqvist, G. (1995) Ornaments or offspring: costs to reproductive success restrict sexual selection processes. *Biological Journal of the Linnean Society*, **55**, 251–260.

Garratt, M. & Brooks, R.C. (2012) Oxidative stress and condition-dependent sexual signals: more than just seeing red. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 3121–3130.

Halver, J.E. (1989) *Fish Nutrition*. Academic Press, San Diego.

Hill, G.E. & McGraw, K.J. (2006) *Bird Coloration*. Harvard University Press, Cambridge, MA.

Houston, A.H. (1997) Review: are the classical hematological variables acceptable indicators of fish health? *Transactions of the American Fisheries Society*, **126**, 879–894.

Huang, S., Ma, J., Liu, X., Zhang, Y. & Luo, L. (2011) Retinoic acid signaling sequentially controls visceral and heart laterality in zebrafish. *The Journal of Biological Chemistry*, **286**, 28533–28543.

Itzkowitz, M., Santangelo, N. & Richter, M. (2001) Parental division of labour and the shift from minimal to maximal role specializations: an examination using a biparental fish. *Animal Behaviour*, **61**, 1237–1245.

Jackson, J.K. (2003) *Science and Categories: Representations of Mating Behaviour in Convict Cichlids (Archocentrus nigrofasciatum)*. ProQuest, UMI Dissertations Publishing, Ann Arbor, MI, USA.

Jha, G.N., Sarma, D. & Qureshi, T.A. (2012) Effect of spirulina (*Spirulina platensis*) and marigold (*Tagetes erecta*) fortified diets on growth, body composition and total carotenoid content of *Barilius bendelisis*. *Indian Journal of Animal Sciences*, **82**, 336–340.

Johnson, K.J. & Scadding, S.R. (1991) Effects of vitamin A and other retinoids on the differentiation and morphogenesis of the integument and limbs of vertebrates. *Canadian Journal of Zoology*, **69**, 263–273.

Jolliffe, I.T. (2002) *Principal Component Analysis*. Springer, New York.

Keenleyside, M.H.A. & Mackereth, R.W. (1992) Effects of loss of male parent on brood survival in a biparental cichlid fish. *Environmental Biology of Fishes*, **34**, 207–212.

Krist, M. (2011) Egg size and offspring quality: a meta-analysis in birds. *Biological Reviews*, **86**, 692–716.

Kurtz, J., Wegner, K.M., Kalbe, M., Reusch, T.B.H., Schaschl, H., Hasselquist, D. *et al.* (2006) MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach. *Proceedings of the Royal Society Biological Sciences Series B*, **273**, 1407–1414.

Lahti, D.C. (2006) Persistence of egg recognition in the absence of cuckoo brood parasitism: Pattern and mechanism. *Evolution*, **60**, 157–168.

Lakeh, A.A.B., Ahmadi, M.R., Safi, S., Ytrestoyl, T. & Bjerkeng, B. (2010) Growth performance, mortality and carotenoid pigmentation of fry offspring as affected by dietary supplementation of astaxanthin to female rainbow trout (*Oncorhynchus mykiss*) broodstock. *Journal of Applied Ichthyology*, **26**, 35–39.

Lamprecht, J. & Rebhan, T. (1997) Factors influencing pairbond stability in convict cichlids (*Cichlasoma nigrofasciatum*). *Behavioural Processes*, **39**, 161–176.

Lavery, R.J. & Colgan, P.W. (1991) Brood age and parental defence in the convict cichlid, *Cichlasoma nigrofasciatum* (Pisces: Cichlidae). *Animal Behaviour*, **41**, 945–951.

Lavery, R.J. & Reebs, S.G. (1994) Effect of mate removal on current and subsequent parental care in the convict cichlid (Pisces, Cichlidae). *Ethology*, **97**, 265–277.

Levi, L., Ziv, T., Admon, A., Levavi-Sivan, B. & Lubzens, E. (2012) Insight into molecular pathways of retinal metabolism, associated with vitellogenesis in zebrafish. *American Journal of Physiology-Endocrinology and Metabolism*, **302**, E626–E644.

Lozano, G.A. (1994) Carotenoids, parasites, and sexual selection. *Oikos*, **70**, 309–311.

Mann, R.H.K. & Mills, C.A. (1985) Variations in the sizes of gonads, eggs and larvae of the dace, *Leuciscus leuciscus*. *Environmental Biology of Fishes*, **13**, 277–287.

McGraw, K.J. & Ardia, D.R. (2003) Carotenoids, immunocompetence, and the information content of sexual colors: An experimental test. *American Naturalist*, **162**, 704–712.

McGraw, K.J., Nolan, P.M. & Crino, O.L. (2011) Carotenoids bolster immunity during moult in a wild songbird with sexually selected plumage coloration. *Biological Journal of the Linnean Society*, **102**, 560–572.

Meijide, F.J. & Guerrero, G.A. (2000) Embryonic and larval development of a substrate-brooding cichlid *Cichlasoma dimerus* (Heckel, 1840) under laboratory conditions. *Journal of Zoology*, **252**, 481–493.

Messarah, M., Saoudi, M., Boumendjel, A., Boulakoud, M.S. & Feki, A.E. (2011) Oxidative stress induced by thyroid dysfunction in rat erythrocytes and heart. *Environmental Toxicology and Pharmacology*, **31**, 33–41.

Mora, J.R., Iwata, M. & von Andrian, U.H. (2008) Vitamin effects on the immune system: vitamins A and D take centre stage. *Nature reviews. Immunology*, **8**, 685–698.

Morales, J., Velando, A. & Torres, R. (2009) Fecundity compromises attractiveness when pigments are scarce. *Behavioral Ecology*, **20**, 117–123.

Morrongiello, J.R., Bond, N.R., Crook, D.A. & Wong, B.B.M. (2012) Spatial variation in egg size and egg number reflects trade-offs and bet-hedging in a freshwater fish. *Journal of Animal Ecology*, **81**, 806–817.

Mullin, G.E. (2011) Vitamin A and immunity. *Nutrition in Clinical Practice*, **26**, 495–496.

Nadauld, L.D., Shelton, D.N., Chidester, S., Yost, H.J. & Jones, D.A. (2005) The zebrafish retinol dehydrogenase, rdh1 l, is essential for intestinal development and is regulated by the tumor suppressor adenomatous polyposis coli. *The Journal of Biological Chemistry*, **280**, 30490–30495.

Narushin, V.G. (2005) Egg geometry calculation using the measurements of length and breadth. *Poultry science*, **84**, 482–484.

Newbrey, J.L. & Reed, W.L. (2009) Growth of yellow-headed blackbird *Xanthocephalus xanthocephalus* nestlings in relation to maternal body condition, egg mass, and yolk carotenoids concentrations. *Journal of Avian Biology*, **40**, 419–429.

Niu, J., Li, C.-H., Liu, Y.-J., Tian, L.-X., Chen, X., Huang, Z. *et al.* (2012) Dietary values of astaxanthin and canthaxanthin in *Penaeus monodon* in the presence and absence of cholesterol supplementation: effect on growth, nutrient digestibility and tissue carotenoid composition. *British Journal of Nutrition*, **108**, 80–91.

Noonan, K.C. (1983) Female mate choice in the cichlid fish *Cichlasoma nigrofasciatum*. *Animal Behaviour*, **31**, 1005–1010.

Olson, V. (2006) Estimating nutrient intake in comparative studies of animals: an example using dietary carotenoid content in birds. *Oikos*, **112**, 620–628.

Pap, P.L., Vagasi, C.I., Czirjak, G.A., Titilincu, A., Pintea, A. & Barta, Z. (2009) Carotenoids modulate the effect of coccidian infection on the condition and immune response in moulting house sparrows. *Journal of Experimental Biology*, **212**, 3228–3235.

R Development Core Team (2010) *R: A Language and Environment For Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rasband, W.S. (1997–2012) ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/.

Rema, P. & Gouveia, L. (2005) Effect of various sources of carotenoids on survival and growth of goldfish (*Carassius auratus*) larvae and juveniles. *Journal of Animal and Veterinary Advances*, **4**, 654–658.

Robart, A.R. (2012) Effect of mate size on maternal reproductive effort in the convict cichlid *Amatitlania siquia*. *Current Zoology*, **58**, 66–72.

Roh, E., Mirza, R.S. & Brown, G.E. (2004) Quality or quantity? The role of donor condition in the production of chemical alarm cues in juvenile convict cichlids. *Behaviour*, **141**, 1235–1248.

Ross, S.A., McCaffery, P.J., Drager, U.C. & Luca, L.M.D. (2000) Retinoids in embryonal development. *Physiological Reviews*, **80**, 1021–1054.

Schmitter-Soto, J.J. (2007) A systematic revision of the genus *Archocentrus* (Perciformes: Cichlidae), with the description of two new genera and six new species. *Zootaxa*, **1603**, 1–78.

Semmens, D. & Swearer, S.E. (2012) Trade-offs obscure the relationship between egg size and larval traits in the diadromous fish *Galaxias maculatus*. *Marine Ecology-Progress Series*, **461**, 165–174.

Sifa, L. & Mathias, J. (1987) The critical period of high mortality of larvae fish: a discussion based on current research. *Chinese Journal of Oceanology and Limnology*, **5**, 80–96.

Stobart, R., Bassett, J., Cartwright, T. & Blackwell, R. (1986) An analysis of body weights and maturing patterns in western range ewes. *Journal of Animal Science*, **63**, 729–740.

Surai, P., Bortolotti, G., Fidgett, A., Blount, J. & Speake, B. (2001) Effects of piscivory on the fatty acid profiles and antioxidants of avian yolk: studies on eggs of the gannet, skua, pelican and cormorant. *Journal of Zoology*, **255**, 305–312.

Svensson, P.A., Forsgren, E., Amundsen, T. & Skold, H.N. (2005) Chromatic interaction between egg pigmentation and skin chromatophores in the nuptial coloration of female two-spotted gobies. *Journal of Experimental Biology*, **208**, 4391–4397.

Toomey, M.B., Butler, M.W. & McGraw, K.J. (2010) Immune-system activation depletes retinal carotenoids in house finches (*Carpodacus mexicanus*). *Journal of Experimental Biology*, **213**, 1709–1716.

Uchida, K., Kanematsu, M., Sakai, K., Matsuda, T., Hattori, N., Mizuno, Y. *et al.* (1998) Protein-bound acrolein: potential markers for oxidative stress. *Proceedings of the National Academy of Sciences USA*, **95**, 4882–4887.

Vijayagopal, P., Gopakumar, G.N. & Vijayan, K.K. (2008) Empirical feed formulations for the marine ornamental fish, striped damsel, *Dascyllus aruanus* (Linne 1758) and their physical, chemical and nutritional evaluation. *Aquaculture Research*, **39**, 1658–1665.

Wald, G. (1968) The molecular basis of visual excitation. *Nature*, **219**, 800–807.

Wang, Y.-J., Chien, Y.-H. & Pan, C.-H. (2006) Effects of dietary supplementation of carotenoids on survival, growth, pigmentation, and antioxidant capacity of characins, *Hyphessobrycon callistus*. *Aquaculture*, **261**, 641–648.

Weiss, S.L., Foerster, K. & Hudon, J. (2012) Pteridine, not carotenoid, pigments underlie the female-specific orange ornament of striped plateau lizards (*Sceloporus virgatus*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **161**, 117–123.

Weiss, S.L., Kennedy, E.A., Safran, R.J. & McGraw, K.J. (2011) Pterin-based ornamental coloration predicts yolk antioxidant levels in female striped plateau lizards (*Sceloporus virgatus*). *Journal of Animal Ecology*, **80**, 519–527.

Wells, R.M.G., McIntyre, R.H., Morgan, A.K. & Davie, P.S. (1986) Physiological stress responses in big gamefish after capture: observations on plasma chemistry and blood factors. *Comparative Biochemistry and Physiology A*, **84**, 565–571.

Winston, G.W., Lemaire, D.G.E. & Lee, R.F. (2004) Antioxidants and total oxyradical scavenging capacity during grass shrimp, *Palaemonetes pugio*, embryogenesis. *Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology*, **139**, 281–288.

Wisenden, B.D. (1993) Female convict cichlids adjust gonadal investment in current reproduction in response to relative risk of brood predation. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **71**, 252–256.

Wisenden, B.D. (1994) Factors affecting mate desertion by males in free-ranging convict cichlids (*Cichlasoma nigrofasciatum*). *Behavioral Ecology*, **5**, 439–447.

Wisenden, B.D. (1995) Reproductive-behavior of free-ranging convict cichlids, *Cichlasoma nigrofasciatum*. *Environmental Biology of Fishes*, **43**, 121–134.

Wisenden, B.D., Lanfranconi-Izawa, T.L. & Keenleyside, M.H.A. (1995) Fin digging and leaf lifting by the convict cichlid, *Cichlasoma nigrofasciatum* - examples of parental food provisioning. *Animal Behaviour*, **49**, 623–631.

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