Dietary carotenoids and bacterial infection in wild and domestic convict cichlids (*Amatitlania* spp.)

Alexandria C. Brown · Matthew D. Cahn · Sara Choi · Ethan D. Clotfelter

Abstract Honest signaling of carotenoid-based ornaments may be reinforced by dietary limitation and by competing physiological demands for carotenoids. This study measured dietary carotenoids in a natural population of the convict cichlid *Amatitlania siquia*, a species in which females possess carotenoid-based ventral coloration. The zoonotic pathogen *Edwardsiella* spp. was detected in wild *A. siquia*, but carotenoids in the stomachs of wild fish did not vary significantly with parasite infection. We followed this with a laboratory experiment on domestic convict cichlids to test whether increased dietary carotenoid (ß-carotene) would decrease oxidative stress and facilitate clearance of experimental infections of *Edwardsiella tarda*. Fish maintained on a medium carotenoid diet (similar to the diets of wild fish) recovered from *E. tarda* infections more rapidly than fish on control diets, though fish on high carotenoid diets did not experience a similar benefit. There was an interaction between carotenoid diet and *E. tarda* such that uninfected fish on the medium carotenoid diet were significantly more colorful along their ventral surface compared to fish on the control and high carotenoid diets. Neither diet nor *E. tarda* infection affected oxidative damage, antioxidant capacity, or carotenoid content of the skin. From our field and laboratory data we conclude that carotenoid intake by wild convict cichlids occurs at a rate sufficient to affect bacterial infection and ventral coloration, but more data are needed from wild populations to verify the functional significance of these interactions.

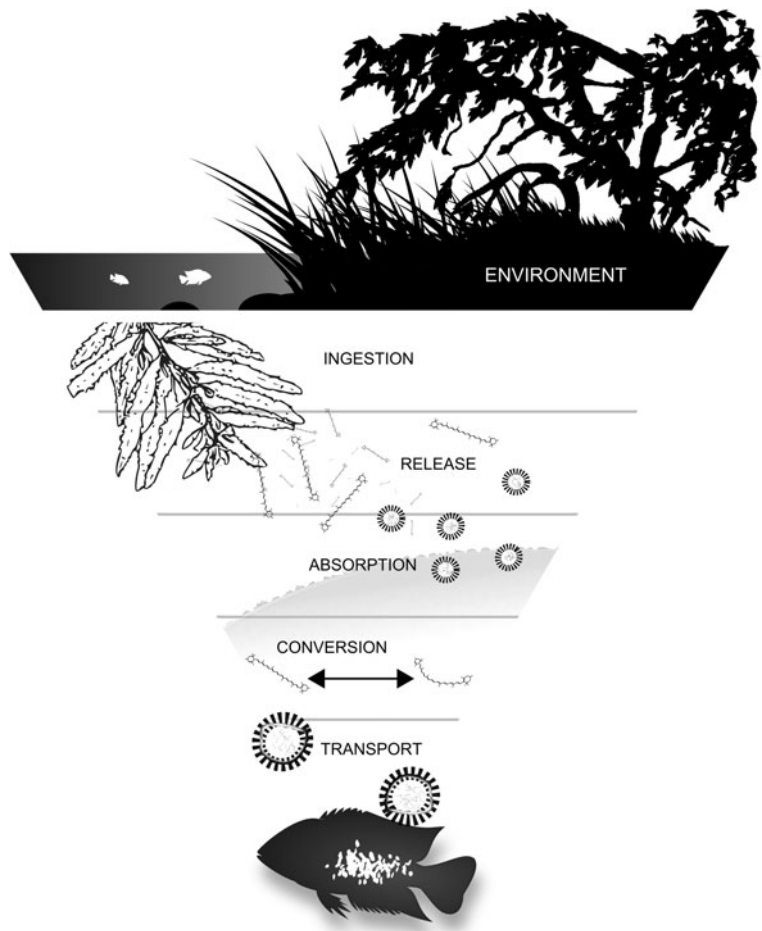**Keywords** Parasite · Diet · Trade-off · Oxidative stress · Immunity

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Introduction

Vertebrates must obtain carotenoid pigments from their diet. The carotenoid trade-off hypothesis predicts that the honesty of carotenoid-based ornaments is reinforced by competing physiological demands for carotenoids, including immunity (Lozano 1994; Blount et al. 2003; McGraw et al. 2006) and antioxidant balance (Cohen et al. 2008; Hõrak et al. 2010). Dietary limitation is often cited as the major factor determining carotenoid availability for ornamented animals, though this assumption is rarely tested (Grether et al. 2001; Blount et al. 2012). There are likely many levels at which carotenoid limitation could be reinforced, including absorption, transportation, conversion and utilization (Fig. 1; Hill and Johnson 2012).

The honesty of carotenoid-based ornamentation may be particularly important in the context of parasite resistance and sexual selection (Hamilton and Zuk 1982). Parasites may cause reduced expression of carotenoids as the demand for the immune-related functions of the pigments increases. This hypothesis is supported primarily through observations of parasite load and pigmentation in wild animals, but few studies test it directly with controlled experimental infections. Our study focuses on convict cichlids, reverse sexually dichromatic fishes in the genus *Amatitlania*. Females (but not males) bear yellow- or orange-flecked ventral patches along their flanks, the function of which is not fully understood. Previous work has shown that both carotenoid pigments and structural components contribute to ventral patch coloration in convict cichlids (Brown et al. 2013). Among fishes, experimental and correlative studies have generally supported the underlying assumption that carotenoid-based coloration is an indicator of

parasite load (e.g. *Pundamilia nyererei*, Maan et al. 2006; bluefin killifish *Lucania goodie*, Johnson and Fuller 2015; three-spined stickleback *Gasterosteus aculeatus*, Milinski and Bakker 1990, but see Sparkes et al. 2013). In all of these species, males are the more ornamented sex. Convict cichlids are somewhat unusual among fishes in that they are reversed sexually-dichromatic.

Beeching et al. (1998) reported that female convict cichlids directed more aggression toward brightly-colored female intruders, whereas males showed no preference for colorful or neutral model females. In a recent field study, Anderson et al. (2015) found that female coloration decreased as the reproductive cycle progressed, and as the number of behavioral interactions with potential predators and heterospecific competitors increased. Therefore, if yellow-orange ventral coloration is an indicator of parasite and pathogen resistance in female convict cichlids, it is unclear who the intended

recipient of that signal is. The first part of the current paper describes a brief field study, in which we measured carotenoids in the stomach contents of wild convict cichlids (*A. siquia*), as well as pathogenic *Edwardsiella* bacteria in their skin mucus. Edwardsiellosis, the disease caused by *Edwardsiella* spp., is a disease reported in many species of fishes, reptiles, birds and mammals (Rashid et al. 1997; Mohanty and Sahoo 2007).

Following the field study, we performed a laboratory experiment to test how three biologically relevant levels of the carotenoid ß-carotene affected *E. tarda* clearance rate and oxidative stress parameters. Dietary carotenoids have been shown to increase pathogen clearance rate of *Vibrio harveyi* in the crustacean *Gammarus pulex* (Babin et al. 2010) and *Isospora* spp. in a bird, *Turdus merula* (Baeta et al. 2008). An important source of oxidative stress is reactive oxygen species (ROS) produced by the innate immune system (Finkel and Holbrook 2000). ROS are molecules that can oxidize intracellular components, such as lipids, proteins, and DNA (Dowling and Simmons 2009). Organisms can mitigate ROS with endogenous antioxidants such as superoxide anion dismutase, or exogenous antioxidants such as carotenoids (Sies et al. 1992). Furthermore, carotenoid-based ornamentation has been shown to decrease under conditions of increased oxidative stress (Bertrand et al. 2006). In our study, we predict that increased oxidative stress as a result of immune activation should cause a reduction in coloration when dietary carotenoids are limited, and conversely, fish supplemented with dietary carotenoids should show decreased oxidative stress.

This work will test the assumption that dietary availability of carotenoids is limiting for reverse sexually-dichromatic female convict cichlids. Relatively few studies have measured carotenoid intake in free-living fish. A notable exception is a study by Grether et al. (1999), in which the authors quantified differences in carotenoid availability in three river drainages in Trinidad, which they related to gut contents and carotenoid-based ornamentation in the resident guppies *Poecilia reticulata*. If our results show that carotenoids are not limited by their availability in the environment, or by an environment × parasite interaction, this would indicate another avenue for potential limitation, such as transportation, storage, and assimilation of carotenoid pigments (Fig. 1; Sefc et al. 2014). The mechanism by which carotenoid-based ornament honesty is reinforced

is vital to our understanding of how these ornaments evolve and whether the carotenoid trade-off hypothesis is relevant to cichlid fishes.

Materials and methods

Study animals

The subjects of the field study were *Amatitlania siquia*, which were distinguished from *A. nigrofasciata* by Schmitter-Soto (2007). Recent molecular work by McMahan et al. (2014) contradicts one of the new species proposed by Schmitter-Soto (2007); therefore, it is likely that the proposed species *A. siquia* is actually synonymous with *A. nigrofasciata*. The limited availability of *A. siquia* stock in captivity, and the ethical considerations of experimentally infecting wild-caught fish, made it necessary for us to perform the laboratory experiment on the closely-related, and commercially available, *A. nigrofasciata*. *A. nigrofasciata* and *A. siquia* interbreed freely in the laboratory, produce viable offspring, and share life history characteristics.

Field collection

We sampled seven sites along the Río Cabuyo in Lomas Barbudal Biological Reserve, Guanacaste Province, Costa Rica (10° 30' N, 85° 23' W). Each sampling site consisted of a slow-moving pool ≈50 m apart, distinct from other pools by riffles or cascades. Upstream immigration (above the sampling areas) was blocked by a waterfall. Wisenden and Keenleyside (1995) provide additional details about the study area. Lomas Barbudal is primarily tropical dry forest, with distinct wet and dry seasons. We collected the field data for this study in the dry season from 12 to 23 January, 2013. During this period, water and air temperatures ranged from 26 to 30 °C throughout the day. Each pool was sampled twice, at intervals of 2 days. Individual differences in body masses ensured that the same fish were not sampled more than once.

Fish were observed grazing on algae in the morning and late afternoon. During these times, fish were captured in clear, acrylic dip nets (Aquatic Ecosystems, Inc.) by snorkeling upstream with face mask and flippers, and transported to holding containers on shore. GPS location was recorded for each fish. Sex was determined by examination of orange or yellow patch

coloration on the skin (Beeching et al. 1998; Brown et al. 2013). Fish were weighed using a spring balance (±0.1 g; Pesola, Switzerland). Both flanks of each fish were swabbed for *Edwardsiella* spp. with a sterile cotton swab, which was immediately plated on *Edwardsiella* isolating media (EIM) in sterile slant tubes. EIM takes advantage of *Edwardsiella* resistance to the antibiotic colistin and was made according to methods outlined in (Shotts and Waltman 1990). Innoculated EIM was incubated at room temperature for 48 h before analysis. Infection was confirmed by the presence of smooth dark green colonies. Fish were considered infected with *Edwardsiella* if either stomach contents (see below) or mucus contained live bacterium.

Stomach contents were obtained using gastric lavage (Hartleb and Moring 1995), which in preliminary laboratory tests resulted in a high recovery of stomach contents (98 % by mass) and no mortality. In the field, fish were held in a dampened, gloved hand, with a wet paper towel over the eyes. The lavage probe was inserted into the mouth to the stomach, and then an attached rubber bulb was pumped by hand until water regurgitated from the mouth ran clear. Fish were immediately returned to the water within 1 m of the capture area.

Carotenoids in stomach contents

Stomach contents were swabbed and plated on EIM prior to filtration through a paper filter (Whatman). Filter papers and solids from the gut were preserved in isopropyl alcohol until extraction and carotenoid analysis. Stomach contents were rinsed from paper filters with ethanol, dried under nitrogen gas, and weighed. Carotenoids were extracted using hexane, dried under nitrogen, and re-suspended in acetone and Tris-HCl buffer for de-esterification. Enzymatic de-esterification used cholesterol esterase (Sigma C-9281) with bile salts to hydrolyze the carotenoids, as described in Jacobs et al. (1982). Carotenoids were then extracted again using sodium sulfate decahydrate and petroleum ether (Vecchi et al. 1987). These were evaporated under nitrogen gas and reconstituted in 82:18 hexane:acetone mobile phase.

A Waters 600 high performance liquid chromatograph (HPLC) with a 20 μl injection loop and a Luna 3 μm analytical silica column (Phenomenex 00F-4162-E0) separated carotenoid types. The mobile phase consisted of an isocratic mixture of 82:18

hexane:acetone set to a flow rate of 1.2 ml/min. A Waters 600E wavelength absorbance detector set at 474 nm produced a chromatogram. Total amounts of carotenoids were calculated using peak area integration values and the standard curves for each identifiable carotenoid.

Laboratory fish handling and measurements

Domestic female convict cichlids (*A. nigrofasciata*) were obtained from a commercial distributor and quarantined for 6 weeks. Following quarantine, fish were swabbed to test for *E. tarda* as described above; none tested positive. They were then anesthetized with 1 mg ml^{-1} tricaine methanesulfonate (MS-222) buffered to pH 7 with sodium bicarbonate. Standard length (± 0.01 mm) was measured using digital calipers; mean standard length of these fish was 2.66 cm (range = 1.92– 3.43 cm).

Fish were randomly assigned to diet and exposure groups. The resulting sample sizes were: control diet =12 (7 exposed to *Edwardsiella tarda* – see below, five exposed to control media), 19 medium-carotenoid diet (six *E. tarda*, 13 control), and 17 high-carotenoid diet (nine *E. tarda*, eight control). Unequal treatment groups resulted from housing limitations and exclusion of sexually immature females. Each female then was housed individually in a 7-L tank aerated with an airstone at 26 ± 2 °C with a 12:12 light:dark photoperiod. At the end of the experiment, anaesthetized fish were sacrificed by decapitation following body and color measurements. Liver and skin from the pigmented patch area were collected with surgical scissors and forceps and stored at −80 °C.

Laboratory diets

Diets were based on nutrient-free H440 agar-casein-dextrin formulations as described previously (Halver 1989; Brown et al. 2013, 2014). Ascorbic acid and α-tocopherol (Sigma-Aldrich) were added to the base mixture, as these compounds may act synergistically with carotenoids to remove free radicals (Martinez et al. 2008). To this base, ß-carotene was added to create medium and high carotenoid diets. The high carotenoid diet contained 210 μg g^{-1} ß-carotene, while the medium diet contained 20.0 μg g^{-1} ß-carotene. The carotenoid concentrations of our diets were similar to carotenoid levels used in fish feed in a similar manipulation

(Kolluru et al. 2006). A previous study on another Neotropical cichlid *Amphilophus citrinellus* showed that ß-carotene in present in the integument and increases when ß-carotene is added to the diet (Lin et al. 2010). No carotenoids were added to the control diet; previous analysis via HPLC confirmed that the carotenoid content of the control diet is <1 µg g^{-1} (Brown et al. 2013). Diets were stored at −80 °C and thawed at 4 °C every 3 days to feed fish. Fish were fed to satiation twice daily. Uneaten food was removed after 5 min. Experimental diets were offered for 13 weeks prior to *E. tarda* exposure and until the end of the experiment, which is sufficient time for changes in body carotenoid coloration to occur in fishes (Amar et al. 2001; Lin et al. 2010; Brown et al. 2013).

Reflectance measurements

To measure the color of the yellow-orange ventral patch, an Ocean Optics USB4000 bifurcated fiber optic spectrometer with a pulsating xenon light source recorded the spectral reflectance of each fish's ventral patch between the second and third black vertical stripe (Brown et al. 2013). A black latex cuff on the probe standardized the angle (90°) and distance (3 mm) from the surface of the fish. Spectrometer data was collected using the SpectraSuite software (version 2.0.159) for wavelengths 300–700 nm with boxcar smoothing set to five. For each reading, three spectra were averaged with an integration time of 100 ms. We analyzed reflectance data within 300–700 nm to encompass the range of visual sensitivity of convict cichlids (400–700 nm; Jackson 2003).

Reflectance spectra were reduced with principal components analysis (PCA), which extracts brightness, hue, and chroma from spectral data in studies of carotenoid coloration in animals (Cuthill et al. 1999; Hill and McGraw 2006; Clotfelter et al. 2007). Orthogonal principal components are calculated to account for the greatest amount of variance among the spectra, as discussed in Brown et al. (2013). Only components that accounted for >1 % of the cumulative proportion of variance were retained. The first three components (PC1, PC2, and PC3) accounted for 0.88, 0.07, and 0.03 of the total variance, respectively. Based on the principle component loadings (Fig. 2), we interpret the second component (PC2) as a measure of yellow, orange, and red pigments. The first and third principle components account for achromatic brightness and the relationship between mid-range and very long/short

range values, respectively. For more information on the interpretation of principle component values in the context of animal coloration research, see Cuthill et al. (1999).

Edwardsiella tarda protocols

Virulent *E. tarda* (originally collected from channel catfish *Ictalarus punctalus* at the USGS Leetown Science Center, Kearneysville, WV, USA) were grown in liquid *Edwardsiella* isolating medium at room temperature for 48 h, washed twice with phosphate buffered saline (PBS) and adjusted to 1×10^6 CFU ml^{-1} using a spectrophotometer (Bio-Tek). Species identity was confirmed by production of hydrogen sulfide on triple sugar iron (TSI) test slants (Collins and Lyne 1976). A spectrophotometric standard curve was determined by colony count on solid *Edwardsiella* isolating media plates. Stock concentrations used in the present experiment also were confirmed later by colony count on solid *Edwardsiella* isolating media.

In fishes, *Edwardsiella* enters the bloodstream through the intestines, gills, and epithelium (Mohanty and Sahoo 2007). For the laboratory-based exposure, fish were exposed to 142.8 CFU ml^{-1} *E. tarda* or vehicle only (1 ml PBS) in tank water. We determined in a pilot experiment that this exposure level and duration produces symptoms (excess mucus production, loss of appetite, lethargy; Saleh 2005) but no fatalities in healthy animals. After mixing, tank water from both groups was grown on *Edwardsiella* isolating media and colonies were counted to confirm exposure levels. Exposures lasted for 12 h, after which fish were removed to sterilized tanks with clean water.

Following exposure, *E. tarda* in skin mucus was tested every 3 days post infection (dpi) for 21 days using an inoculating loop brushed over the entire flank of each fish to collect a film of fish mucus. The loop was streaked onto two separate *Edwardsiella* isolating media plates (one per flank) and incubated at 22 °C for 48 h. A fish was considered clear of infection when no *Edwardsiella* colonies were observed for two successive three-day intervals.

Oxidative status

Two assays were used to represent the oxidative status of each animal: acrolein and Trolox equivalent antioxidant capacity (TEAC). The amount of protein-bound

Fig. 2 Principal components loadings (eigenvectors) derived from a principle components analysis of reflectance spectra taken from the ventral surface of female convict cichlids *A. nigrofasciata*. Principal components PC1, PC2 and PC2 accounted for 88 %, 7 % and 3 % of the total variance, respectively. PC2 loads positively across ≈ 450–700 nm, and hereafter is our proxy for yellow-orange coloration
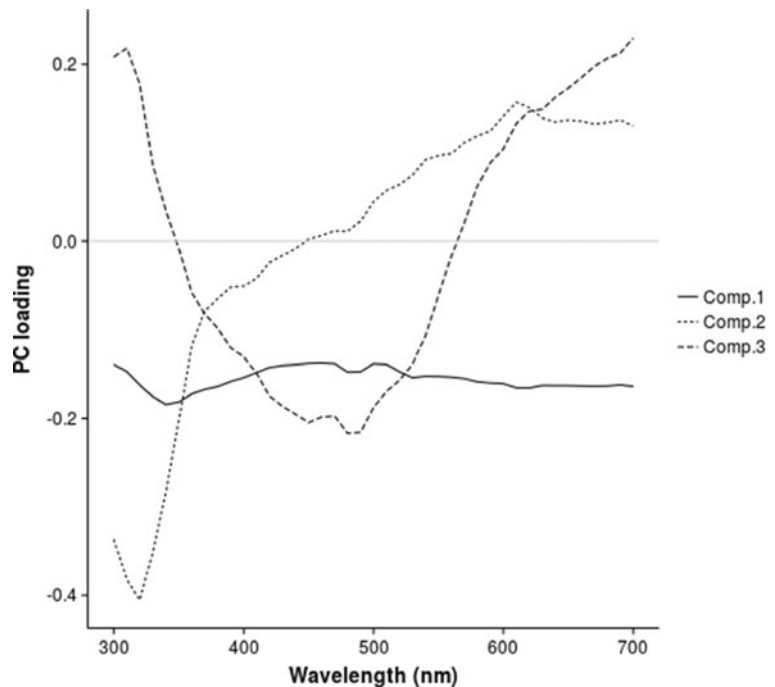
acrolein indicates oxidative damage that already has occurred due to previous, unmitigated oxidative stress (Uchida et al. 1998). The TEAC assay measures the total, short-term antioxidant capacity of all circulating antioxidants, both endogenous and exogenous. Fish livers were homogenized in 0.1 mg ml^{-1} PBS, and then centrifuged at 4500 g for 10 min (4 °C). Supernatant was used for all three assays. A modified Bradford protein assay kit (Sigma-Aldrich) allowed liver supernatant concentration to be adjusted to 0.3 mg ml^{-1} for both acrolein and TEAC.

For the TEAC assay, colorless 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) reacts with potassium persulfate to form a blue ABTS radical. The amount of radical neutralized by an antioxidant-containing sample was then measured using a 734 nm spectrophotometer (Molecular Devices VersaMax). Known concentrations of Trolox, a water soluble analog of vitamin E, served as a standard curve. The capacity of a sample to quench ABTS radical is expressed as Trolox equivalency units.

Acrolein quantification was achieved using the protocol developed by Uchida et al. (1998). In brief, liver supernatant or known concentrations of acrolein-modified bovine serum albumin (BSA) were incubated for 12 h at 37 °C in a round bottom 96-well plate with a medium binding coating (Fisherbrand). Wells were then washed with TBS-tween and blocked with BSA. Acrolein was detected by monoclonal antibody mAb5F6. The secondary antibody was HRP conjugated anti-mouse IgG produced in rabbit. SIGMA Fast OPD (Sigma Aldrich) substrate solution was used to measure acrolein amounts with spectrophotometric detection at 450 nm.

Integument carotenoids

Skin was processed for carotenoid analysis using methods outlined in Brown et al. (2013). Briefly, skin was frozen in liquid nitrogen and ground to powder using a mortar and pestle. Carotenoids were extracted with hexane, and samples were dried, re-suspended in acetone and diluted into Tris-HCl buffer. Carotenoids were hydrolyzed with cholesterol esterase (Sigma C-9281) and extracted using sodium sulfate decahydrate and hexane. Hexane was evaporated under nitrogen gas and reconstituted in 82:18 hexane:acetone mobile phase.

Analyses were performed on a Waters 600 HPLC using a Luna 3 μm analytical silica column (Phenomenex 00F-4162-E0) at a flow rate of 1.2 ml min^{-1}. A Waters 600E wavelength absorbance detector produced a chromatogram at 474 nm. Total amounts of carotenoids were calculated using peak area integration values and

analytical standards (CaroteNature, Switzerland) for each identifiable carotenoid type (Millennium 32, Waters Corp.).

Statistical analysis

All statistics were performed in R 2.14.1 (R Development Core Team 2008). Residuals of data were normally distributed prior to analysis. Tests were considered significant when $P < 0.05$. We used ANOVA with *E. tarda* and fish sex as predictors with no interaction term (as the *E. tarda* group was small) to predict log-transformed gut carotenoid concentrations.

For the laboratory experiment, log transformations were applied to oxidative stress data prior to analysis to resolve influential outliers. Fish were excluded from tests of oxidative stress parameters or HPLC results if there was not enough tissue recovered to allow for analysis. Main and interaction effects of diet and *Edwardsiella* exposure were tested using ANOVA. Tukey's HSD tests were used for pairwise comparisons among levels of the main effects.

Results

Field study

Wild convict cichlids in the Río Cabuyo were observed eating brown algae, small invertebrates, and scavenging on a dead eel. We caught 36 wild fish (24 females, 12 males) and collected their stomach contents via gastric lavage. There were no sex differences in carotenoid contents of the gut or in *Edwardsiella* infection rates (see below), so in this section males and females are pooled together. Fish ranged in mass between 1.5–20.0 g, with a mean of 5.7 g. Each fish had carotenoids in their stomach contents; HPLC identified α- and ß-carotenes, tunaxanthin, lutien, astaxanthin, and cantha-xanthin (Table 1). There were also trace quantities of unidentified carotenoids.

Gut carotenoids ranged from 0.27–298.18 μg g^{-1} of stomach contents with a median of 1.95 μg g^{-1} of stomach contents. There was a marginally significant increase in total carotenoid concentration in the stomach contents of males, the non-ornamented sex ($F_{2,33} = 5.67$, $P = 0.02$). *Edwardsiella* spp. were isolated from the skin and stomach contents of six fish (16.6 %); four of 24 females were infected and two of 12 males were

Table 1 Carotenoids in the stomachs of wild convict cichlids *A. siquia* (μg g^{-1} of stomach contents). Fish indicates the number of fish with each carotenoid type in their stomachs

	Fish	Mean (μg g^{-1})	SD (μg g^{-1})	Maximum (μg g^{-1})
Carotenes	32	17.34	42.56	185.45
Tunaxanthin	5	82.62	94.34	240.00
Lutein	5	11.56	14.75	29.80
Astaxanthin	4	0.42	0.44	0.98
Canthanxanthin	6	38.94	41.64	96.13
Total	36	35.03	68.25	298.18

infected. Fish infected with *Edwardsiella* did not have more carotenoids in their stomach contents than uninfected fish ($F_{2,33} = 2.19$, $P = 0.15$).

Laboratory experiment

In the laboratory experiment, 48 female convict cichlids were maintained on one of the three experimental diets: control ($n = 12$), medium carotenoid ($n = 19$), and high carotenoid ($n = 17$). Of these fish, seven on the control diet, six on the medium carotenoid diet, and nine on the high carotenoid diet were randomly selected to be exposed to *E. tarda*, while the remaining fish were exposed to control (sterile) media.

Edwardsiella exposure

We predicted that dietary carotenoids would help fish eliminate *E. tarda* bacteria from their skin mucus. Fish on carotenoid-supplemented diets cleared *E. tarda* from skin mucus more rapidly than controls ($F_{5,42} = 9.49$, $P < 0.001$). Fish in the control group cleared *E. tarda* after 11.25 ± 7.47 days, while fish in the medium and high groups cleared the pathogen after 7.11 ± 6.50 days and 9.18 ± 6.91 days, respectively. Pairwise comparisons showed that fish on the medium carotenoid diet cleared *E. tarda* significantly faster than the fish on control diets ($P < 0.001$), but not significantly faster than fish on the high carotenoid diet ($P = 0.06$). Neither were fish on the high carotenoid diet able to clear the parasite significantly faster than the fish on the control diet ($P = 0.09$).

Color and carotenoids

We predicted that fish maintained on carotenoid-limited diets or infected with *E. tarda* would experience

decreased coloration (PC2) and carotenoid pigments in the ventral skin patch. After confirming that there were no pre-existing differences in coloration among the treatment groups (diet: $F_{2,41} = 1.24$, $P = 0.30$; infection: $F_{1,41} = 0.13$, $P = 0.72$; diet × interaction: $F_{2,41} = 2.40$, $P = 0.10$), we found that following the 13-week diet treatment and experimental infection with *E. tarda*, there was a significant interaction effect (diet: $F_{2,41} = 0.62$, $P = 0.54$; infection: $F_{1,41} = 1.54$, $P = 0.22$; diet × infection: $F_{2,41} = 6.60$, $P = 0.003$; Fig. 3).

We detected three carotenoid types in the integument of female convict cichlids via HPLC: ß- or α-carotene and the xanthophylls tunaxanthin, and astaxanthin. However, the combined concentration (µg g^{-1}) of carotenoids in the tissue was unaffected by diet ($F_{2,36} = 2.28$, $P = 0.12$), infection ($F_{1,36} = 1.52$, $P = 0.23$), or their interaction ($F_{2,36} = 0.78$, $P = 0.47$). This was also true when we adjusted for fish standard length ($P \geq 0.20$ for all comparisons).

Oxidative status

As would be expected, we found a strong, positive relationship between acrolein – our biomarker of oxidative stress – and the antioxidant activity in the liver, as measured by the TEAC assay ($R^2 = 0.86$, $F_{1,39} = 238.2$, $P < 0.001$). There was no effect of carotenoid diet, *E. tarda* infection, or their interaction on liver acrolein (diet: $F_{2,36} = 0.66$, $P = 0.53$; infection: $F_{2,36} = 1.83$, $P = 0.18$; diet × infection: $F_{2,36} = 1.27$, $P = 0.29$). Similarly, there were no main effects or interaction effects on oxidative balance, as measured by the residuals from a regression of TEAC against acrolein (diet: $F_{2,35} = 1.14$, $P = 0.33$; infection: $F_{2,35} = 1.12$, $P = 0.30$; diet × infection: $F_{2,35} = 1.36$, $P = 0.27$).

Discussion

There was no evidence that fish feeding on high-carotenoid diets suffer less from *Edwardsiella* infections, nor that infected fish sought out carotenoid-rich foods (Kolluru et al. 2009). Males had slightly more carotenoids in their guts than females, but males and females were observed frequently feeding side by side on the same algae so we doubt that this finding is due to biologically-relevant differences. Given the abundance of algae in the Río Cabuyo, and their carotenoid contents (Haugan and Liaaen-Jensen 1994), we conclude that convict cichlids are not limited by their access to

Fig. 3 Female convict cichlids *A. nigrofasciata* were fed one of three carotenoid diets and then infected with *Edwardsiella tarda* or control medium. There was a significant interaction between diet and infection status such that fish on the medium diet showed less yellow-orange ventral coloration when infected ($F_{2,41} = 6.60$, $P = 0.003$). This relationship is reversed in the control and high-carotenoid diet groups, with infected fish showing a slightly higher mean orange color (albeit not significantly)
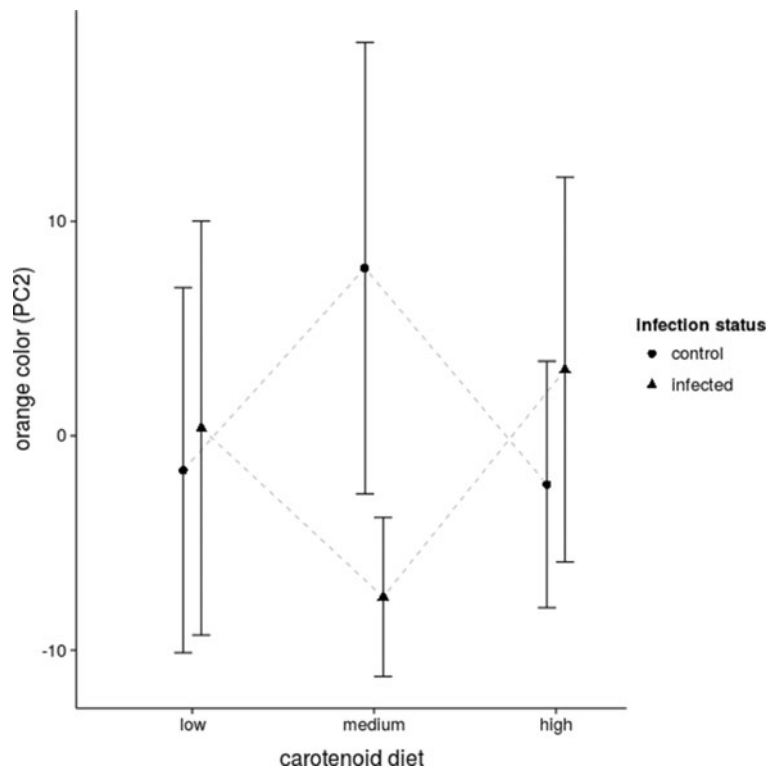
dietary carotenoids in this environment. Carotenoid limitation may occur at other steps in the assimilation pathway, however, such as absorption and transportation (Fig. 1). Alternatively, the *Edwardsiella* endemic to free-living convict cichlid populations may not be virulent enough to exert selection pressure on foraging behavior.

In a similar study, Kolluru et al. (2006) found that levels of the nematode *Gyrodactylus turnbulli* were lowest in guppies (*Poecilia reticulata*) fed a medium carotenoid diet. Those authors speculated that excessive dietary carotenoids could suppress host immunity (perhaps by acting as prooxidants; see below) or benefit the parasites (e.g., Perrot-Minnot et al. 2011). Our findings are also consistent with those of Amar et al. (2000), who found that intermediate doses of dietary ß-carotene caused significant increases in plasma immunoglobulins and serum complement activity in rainbow trout *Oncorhynchus mykiss*. In one of the few studies investigating the specific effect of carotenoids on *Edwardsiella* spp., Kim et al. (2012) fed olive flounder (*Paralichthys olivaceus*) control diets or diets supplemented with 1–3 % astaxanthin, a xanthophyll carotenoid. Then they injected fish with *E. tarda* and found that flounder maintained on the astaxanthin diets had greater non-specific immune responses and suffered reduced cumulative mortality compared to fish on the control diet; they observed no significant differences among the astaxanthin treatment groups. Taken together, these studies suggest a threshold above which dietary carotenoids provide fish with no benefit against parasites, and may in fact be detrimental.

We found no evidence of a main effect of carotenoid diet on the yellow-orange ventral coloration (PC2) in female convict cichlids in the laboratory experiment. This finding conflicts with an earlier study in our laboratory (Brown et al. 2013). In that study, Brown et al. (2013) maintained fish on diets containing ß-carotene as well as the xanthophylls lutein and zeaxanthin. The authors found that fish maintained on a diet similar in total carotenoids to the medium carotenoid in the current study (23 µg g^{-1}) increased in yellow-orange coloration along their ventral surface. They also found that fish fed a less-concentrated diet (26.5 ng g^{-1}) were even more yellow-orange. The discrepancy between the two studies could be due to the combination of carotenoids used by Brown et al. (2013), or because both the medium- and high-carotenoid diets in the current study were more concentrated than the optimal level for absorption and color expression.

The mechanism by which dietary carotenoids facilitate the clearance of *E. tarda* infections is unknown. Carotenoids could support immunity by facilitating or allowing production of endogenous oxidants from the innate immune system, or becoming pro-oxidant themselves (Palozza 1998; Palozza et al. 2003; El-Agamey et al. 2004). Mechanisms for pro-oxidant action of carotenoids occur in the presence of chronic oxidative stress, oxygen exposure, or pharmacologic carotenoid concentrations. *In vitro* studies of ß-carotene have shown that, while it is an effective quencher of some types of ROS, hydrogen peroxide may react with ß-carotene to form carbon-centered carotenoid radicals (Woods et al. 1999; Tanumihardjo 2012). In the current study we found no evidence that our experimental diets affected oxidative damage (acrolein) or antioxidant capacity (TEAC), therefore we cannot conclude that pro-oxidant activity of ß-carotene decreased pathogen clearance rate. Future work should consider the potential benefits of short-term oxidative stress in addition to long-term dangers, as well as quantify vitamin A conversion in the gut to address the possibility that ß-carotene supplementation increases the antioxidant pool with directly enhancing the immune system.

Our field study found considerable individual variation in the carotenoid content of convict cichlid stomach contents, which were unrelated to *Edwardsiella tarda* infection. Furthermore, our field study provided little evidence that dietary carotenoids were limited in the natural environment. Our laboratory study found an interaction between dietary carotenoids and *E. tarda* infection, but not in fish on the carotenoid-limited diet as predicted by the carotenoid tradeoff hypothesis. The results presented here are consistent with a growing body of evidence that individual variation and genetics may be more influential than diet in carotenoid allocation and carotenoid-based signaling (Hill 2011). Individual genetic differences in carotenoid lipoprotein transporters, for example, may explain individual variation in carotenoid allocation dynamics (Lee et al. 1999; Hill and McGraw 2006; McGraw and Parker 2006). Genetic and gene-environment interaction influences on lipid and carotenoid transporter expression should be considered in future studies of carotenoid-based body coloration. Elucidation of the mechanisms controlling individual variability in carotenoid allocation could determine what factors reinforce honesty in carotenoid-based ornamentation and how ornaments evolve.

References

Amar EC, Kiron V, Satoh S, Okamoto N, Watanabe T (2000) Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fish Sci 66:1068–1075

Amar EC, Kiron V, Satoh S, Watanabe T (2001) Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac Res 32:162–173

Anderson C, Wong SC, Fuller A, Zigelski K, Earley RL (2015) Carotenoid-based coloration is associated with predation risk, competition, and breeding status in female convict cichlids (*Amatitlania siquia*) under field conditions. Environ Biol Fish 98:1005–1013

Babin A, Clotilde B, Moret Y (2010) Dietary supplementation with carotenoids improves immunity without increasing its cost in a crustacean. Am Nat 176:234–241

Baeta R, Faivre B, Motreuil S, Gaillard M, Moreau J (2008) Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*). Proc R Soc B 275:427–434

Beeching SC, Gross SH, Bretz HS, Hariatis E (1998) Sexual dichromatism in convict cichlids: the ethological significance of female ventral coloration. Anim Behav 56:1021–1026

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 JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Blount JD, Rowland HM, Drijfhout FP, Endler JA, Inger R, Sloggett JJ, Hurst GDD, Hodgson DJ, Speed MP (2012) How the ladybird got its spots: effects of resource limitation on the honesty of aposematic signals. Funct Ecol 26:334–342

Brown AC, McGraw KJ, Clotfelter ED (2013) Dietary carotenoids increase yellow nonpigment coloration of female convict cichlids (*Amantitlania nigrofasciata*). Physiol Biochem Zool 86:312–322

Brown AC, Leonard H, McGraw KJ, Clotfelter ED (2014) Maternal effects of carotenoid supplementation in an ornamented cichlid fish. Funct Ecol 28:612–620

Clotfelter ED, Ardia DR, McGraw KJ (2007) Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. Behav Ecol 18:1139–1145

Cohen AA, McGraw KJ, Wiersma P, Williams JB, Robinson WD, Robinson TR, Brawn JD, Ricklefs RE (2008) Interspecific associations between circulating antioxidant levels and life-history variation in birds. Am Nat 172:178–193

Collins CH, Lyne PM (1976) Microbiological methods. Butterworths, London

Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. Am Nat 153:183–200

Dowling DK, Simmons LW (2009) Reactive oxygen species as universal constraints in life-history evolution. Proc R Soc B 276:1737–1745

El-Agamey A, Lowe GM, McGarvey DJ, Mortensen A, Phillip DM, Truscott TG, Young AJ (2004) Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Arch Biochem Biophys 430:37–48

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247

Grether GF, Hudon FJ, Millie DF (1999) Carotenoid limitation of sexual coloration along an environmental gradient in guppies. Proc R Soc B 266:1317–1322

Grether GF, Hudon J, Endler JA (2001) Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). Proc R Soc B 268:1245–1253

Halver JE (1989) Fish nutrition. Academic Press, San Diego

Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? Science 218:384–387

Hartleb CF, Moring JR (1995) An improved gastric lavage device for removing stomach contents from live fish. Fish Res 24: 261–265

Haugan JA, Liaaen-Jensen S (1994) Algal Carotenoids 54. Carotenoids of brown algae (Phaeophyceae). Biochem Syst Ecol 22:31–41

Hill GE (2011) Condition-dependent traits as signals of the functionality of vital cellular processes. Ecol Lett 14:625–634

Hill GE, Johnson JD (2012) The vitamin A-redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. Am Nat 180:E127–E150

Hill GE, McGraw KF (2006) Bird coloration. Harvard University Press, Cambridge

Hõrak PE, Sild E, Soomets U, Sepp T, Kilk K (2010) Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. J Exp Biol 213: 2225–2233

Jackson JK (2003) Science and categories: representations of mating behaviour in convict cichlids (*Archocentrus nigrofasciatum*). ProQuest, UMI Dissertations Publishing

Jacobs PB, LeBoeuf RD, McCommas SA, Tauber JD (1982) The cleavage of carotenoid esters by cholesterol esterase. Comp Biochem Physiol B 72:157–160

Johnson AM, Fuller RC (2015) The meaning of melanin, carotenoid, and pterin pigments in the bluefin killifish, *Lucania goodie*. Behav Ecol 26:158–167

Kim S-S, Song J-W, Kim K-W, Lee K-J (2012) Effects of dietary astaxanthin on innate immunity and disease resistance against *Edwardsiella tarda* in olive flounder *Paralichthys olivaceus*. Isr J Aquacult Bamidgeh 64:740

Kolluru GR, Grether GF, South SH, Dunlop E, Cardinali A, Liu L, Carapiet A (2006) The effects of carotenoid and food availability on resistance to a naturally occurring parasite (*Gyrodactylus turnbulli*) in guppies (*Poecilia reticulata*). Biol J Linn Soc 89:301–309

Kolluru GR, Grether GF, Dunlop E, South SH (2009) Food availability and parasite infection influence mating tactics in guppies (*Poecilia reticulata*). Behav Ecol 20:131–137

Lee CM, Boileau AC, Boileau TWM, Williams AW, Swanson KS, Heintz KA, Erdman JW (1999) Review of animal models in carotenoid research. J Nutr 129:2271–2277

Lin SM, Nieves-Puigdoller K, Brown AC, McGraw KJ, Clotfelter EC (2010) Testing the carotenoid trade-off hypothesis in the polychromatic midas cichlid, *Amphilophus citrinellus*. Physiol Biochem Zool 83:333–342

Lozano GA (1994) Carotenoids, parasites, and sexual selection. Oikos 70:309–311

Maan ME, van der Spoel M, Jimenez PQ, van Alphen JJM, Seehausen O (2006) Fitness correlates of male coloration in a Lake Victoria cichlid fish. Behav Ecol 17:691–699

Martinez A, Rodriguez-Girones MA, Barbosa A, Costas M (2008) Donator acceptor map for carotenoids, melatonin and vitamins. J Phys Chem A 112:9037–9042

McGraw KJ, Parker RS (2006) A novel lipoprotein-mediated mechanism controlling sexual attractiveness in a colorful songbird. Physiol Behav 87:103–108

McGraw KJ, Crino OL, Medina-Jerez W, Nolan PM (2006) Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. Ethology 112:1209–1216

McMahan CD, Matamoros WA, Barraza E, Kutz J, Chakrabarty P (2014) Taxonomic status of the Lago Coatepeque endemic convict cichlid *Amatitlania coatepeque* (Teleostei: Cichlidae). Copeia 2014:633–638

Milinski M, Bakker TCM (1990) Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. Nature 344:330–333

Mohanty BR, Sahoo PK (2007) Edwardsiellosis in fish: a brief review. J Biosci 32:1331–1344

Palozza P (1998) Prooxidant actions of carotenoids in biologic systems. Nutr Rev 56:257–265

Palozza PS, Serini S, Di Nicuolo F, Piccioni E, Calviello G (2003) Prooxidant effects of beta-carotene in cultured cells. Mol Asp Med 24:353–362

Perrot-Minnot MJ, Gaillard M, Dodet R, Cezilly F (2011) Interspecific differences in carotenoid content and sensitivity to UVB radiation in three acanthocephalan parasites exploiting a common intermediate host. Int J Parasitol 41:173–181

R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna

Rashid MM, Nakai T, Muroga K, Miyazaki T (1997) Pathogenesis of experimental Edwardsiellosis in Japanese flounder *Paralichthys olivaceus*. Fish Sci 63:384–387

Saleh WD (2005) Isolation and identification of *Edwardsiella tarda* from infected tilapia fish *Oreochromis niloticus*. Bull Facul Agric Cairo Univ 56:839–848

Schmitter-Soto JJ (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

Sefc KM, Brown AC, Clotfelter ED (2014) Carotenoid-based coloration in cichlid fishes. Comp Biochem Physiol A Mol Integr Physiol 173:42–51

Shotts EB, Waltman WD (1990) A medium for the selective isolation of *Edwardsiella ictaluri*. J Wildl Dis 26:214–218

Sies H, Stahl W, Sundquist AR (1992) Antioxidant functions of vitamins. Ann N Y Acad Sci 669:7–20

Sparkes TC, Rush V, Kopp DA, Foster SA (2013) Reproductive success in a natural population of male three-spined stickleback *Gasterosteus aculeatus*: effects of nuptial colour, parasites and body size. J Fish Biol 82:1720–1727

Tanumihardjo SA (2012) Carotenoids and human health. Springer, New York

Uchida K, Kanematsu M, Sakai K, Matsuda T, Hattori N, Mizuno Y, Suzuki D, Miyata T, Noguchi N, Niki E, Osawa T (1998) Protein-bound acrolein: potential markers for oxidative stress. Proc Natl Acad Sci U S A 95:4882–4887

Vecchi M, Glinz E, Meduna V, Schiedt K (1987) HPLC separation and determination of astacene, semiastacene, astaxanthin, and other keto-carotenoids. J High Resolut Chromatogr 10:348–351

Wisenden BD, Keenleyside MHA (1995) Brood size and the economy of brood defence: testing Lack's hypothesis in a biparental cichlid fish. Environ Biol Fish 43:145–151

Woods JA, Bilton RF, Young AJ (1999) β-carotene enhances hydrogen peroxide-induced DNA damage in human hepatocellular hepg2 cells. FEBS Lett 449:255–258