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Carotenoid availability and tradeoffs in female convict cichlids, a reverse sexually-dichromatic fish

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Abstract Carotenoid pigments have myriad functions in fish, including coloration and immunity. The "carotenoid trade-off hypothesis" posits that dietary limitation of carotenoids imposes constraints on animals to allocate to one function at the expense of another. This hypothesis rarely has been tested in fish. We quantified tissue carotenoids in breeding and non-breeding female convict cichlids in Lake Xiloá, Nicaragua. This species is reverse sexually dichromatic such that females possess carotenoid-based coloration that males lack. We also collected algae samples near nest sites to assess carotenoid availability, recorded water depth, and examined cichlids' behavioral interactions with pair mates, conspecifics, heterospecific competitors, and predators. Each of these, we predicted, would mediate potential carotenoid trade-offs. We found that non-breeding females had significantly higher levels of carotenoids in their integument, liver, and gonads compared to breeding fish. We found that algae and total carotenoids declined with depth across our study transects at 9, 11, 13, and 15 m, but the concentration of carotenoids (ng carotenoid g^{-1} algae, or algal quality) did not vary with depth. Furthermore, relationships among carotenoid concentrations of the three tissue types did not vary with depth, and female color status (orange or not) was not affected by behavioral interactions with other community members, reproductive status, or water depth. Our results support previous studies showing that carotenoid pigmentation may serve a signal function that facilitates the establishment of non-breeding females within the breeding population. Our study also uncovered no evidence indicating that carotenoids are limiting in the diet of breeding female convict cichlids.

Keywords Algae · Cichlidae · Gonad · HPLC · Integument · Liver · Water depth

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Introduction

Carotenoids are pigments responsible for many examples of yellow, orange, and red coloration in animals. Carotenoids can stimulate the immune system and bolster protection against pathogens (McGraw and Ardia 2003; Baeta et al. 2008; McGraw et al. 2011; Simons et al. 2012), and their putative role as antioxidants in non-human animals has been the subject of considerable debate (Constantini and Møller 2008; Perez-Rodriguez et al. 2010; Vinkler and Albrecht 2010; Koch et al. 2018). Vertebrates cannot synthesize their own carotenoids, and must obtain them from their diet. This has prompted speculation that carotenoids are limiting (Olson and Owens 1998), an argument that has gained only limited empirical support (Hill 2014; Simons et al. 2014; Brown et al. 2016). An important consequence of this presumed limitation is that carotenoid-based signals convey salient information about the foraging ability, and relative health, of the signaler (Svensson and Wong 2011; Simons et al. 2012). Furthermore, this presumed limitation, together with the myriad physiological functions of carotenoids, has led to the oft-tested "carotenoid trade-off hypothesis." According to this hypothesis, animals are forced to distribute limited dietary carotenoids among competing needs, thus an animal might have to sacrifice ornamental coloration to maintain immunity or antioxidant protection (Lozano 1994; von Schantz et al. 1999). Koch and Hill (2018) recently reviewed the carotenoid trade-off hypothesis and evaluated its underlying assumptions: that carotenoids are limited and beneficial, and that high-quality individuals are less subject to carotenoid trade-offs. Those authors found little evidence – at least in studies on birds – of carotenoid limitation in the environment, and instead suggest that animals might be limited in their ability to extract and assimilate those carotenoids, such as by physiological processes including the availability of transporter lipoproteins or transformation of precursor molecules (Hill 2011, 2014; Hill and Johnson 2012; Koch and Hill 2018). Some evidence suggests that carotenoid depletion (rather than supplementation) studies may be a more effective approach to identifying potential carotenoid trade-offs (Weaver et al. 2018).

Carotenoids are key photosynthetic pigments in plants, and thus are ubiquitous in both marine and freshwater algae (Takaichi 2011). Fishes obtain their necessary carotenoids via direct consumption of algae or by consuming heterotrophic prey (de Carvalho and Caramujo 2017). Variation in local algae abundance (assessed via algae crops or chlorophyll concentrations) is known to be a predictor of integument pigmentation in fishes (Cavraro et al. 2018). However, whether variation in availability represents a physiological limitation is less clear. Grether et al. (1999) found significant variation in algae crops among river drainages in Trinidad, which correlated with canopy cover. Variation in algae crops translated into differences in carotenoid availability and, ultimately, the concentration of carotenoids in the integument of male guppies (*Poecilia reticulata*). They found evidence that carotenoid limitation was most pronounced in streams with the lowest availability of algae (Grether et al. 1999). Kolluru et al. (2006) maintained wild-caught guppies on carotenoid-enriched diets and found that intermediate levels of carotenoids provided the best protection against the ectoparasite *Gyrodactylus*. In another study, Brown et al. (2016) maintained convict cichlids (*Amatitlania nigrofasciata*) in the laboratory on diets supplemented with ß-carotene at two environmentally-relevant levels, and found that fish on the medium-carotenoid diet had greater color expression and reduced infection by pathogenic *Edwardsiella* bacteria compared to fish on the high-carotenoid diet (Brown et al. 2016). These studies suggest that carotenoids are not limiting, and may in fact occur at levels higher than necessary for optimal immune defense.

There have been few explicit tests of carotenoid trade-offs in fishes (Sefc et al. 2014). In a captive study, Clotfelter et al. (2007) reported that carotenoid-enriched diets boosted the T cell response to a mitogen in domestic fighting fish *Betta splendens*, but more so in blue fish that were limited in their ability to deposit carotenoid directly to the integument. Lin et al. (2010) found no evidence that supplemental carotenoids enhanced innate immunity (lysozyme activity) in two color morphs of the Midas cichlid (*Amphilophus citrinellus*), which were caught from Lake Nicaragua and maintained in the laboratory. Theis et al. (2017) found evidence of a trade-off between the number and area of egg spots (carotenoid-pigmented areas of the tail) and immune defenses (lymphocyte ratio) in the cichlid *Astatotilapia burtoni* in Lake Tanganyika. However, Parolini et al. (2018) found that integument coloration and enzymatic and non-enzymatic antioxidant defenses were positively correlated in brown trout (*Salmo trutta*), suggesting that colorful fish do not necessarily face a trade-off between competing functions.

The goal of the current study was to evaluate several components of the carotenoid-trade-off hypothesis in a free-living population of convict cichlids (*A. nigrofasciata*) in Lake Xiloá, Nicaragua. Under the taxonomic arrangement of Schmitter-Soto (2007), convict cichlids from Nicaragua were ascribed to the species *A. siquia*. However, recent genetic analysis suggests that this species is polytypic due to imperfect taxonomy and more likely belongs to *A. nigrofasciata* (Bagley et al. 2016). Therefore, we follow the taxonomy in Bussing (1998) and Allgayer (2001), which is consistent with the genetic data. Convict cichlids are reverse-sexually dichromatic. Female possess yellow-orange coloration along the ventrolateral surface and fins, which is due in part to carotenoid pigmentation (Brown et al. 2013). These pigmented patches are important in both intraspecific and interspecific interactions, particularly in the context of offspring defense, a behavior for which this species is well-known (Wisenden 1995; Beeching et al. 1998; Itzkowitz et al. 2001; Anderson et al. 2016). Furthermore, pigmentation in females of this species appears to vary as a function of reproductive status at both coarse (non-breeding vs. breeding) and fine (breeding females with or without free-swimming offspring) scales (Anderson et al. 2015). The probability of female convict cichlids possessing orange color also declines with increased predation risk and competition with heterospecifics, suggesting that behavioral interactions might mediate patterns of carotenoid allocation to the integument versus other essential physiological functions (Anderson et al. 2015).

We hypothesized that carotenoid availability in the algae attached to rocks near nesting sites would vary as a function of water depth. We also hypothesized that females would face a trade-off between allocation of carotenoids to integument versus other tissues such as liver and gonads, where they might also be useful (Brown et al. 2014). We predicted that these trade-offs, if present, would be more pronounced at greater depths where carotenoids are more limiting and where encounters with predators are more frequent (Anderson et al. 2015). We also predicted that carotenoid trade-offs would differ between mated and unmated females, the former of whom must allocate carotenoids to eggs and engage in vigorous defense of eggs and offspring (Brown et al. 2014; Anderson et al. 2015). Lastly, we predicted that extensive interactions with predators or heterospecific competitors (i.e., community-level stressors) would impose constraints on allocation of carotenoids to the integument such that females that constantly defend their nests and offspring would be less likely to express ornamental coloration.

Materials and methods

Field data collection

This study was conducted at Lake Xiloá in southwestern Nicaragua (12°12′47.12”N, 86°19′2.35”W, Fig. 1) from 20 January – 4 February, 2013. Transects were established and ran approximately east-west along the southeast shore of the lake. Four transects were laid via SCUBA at depths of 9, 11, 13, and 15 m using an Agora 100 m field tape measure; the diver swam parallel to the shore using a depth gauge to maintain proper depth for a given transect. Along the three deepest transects, the habitat consisted of small to large cobblestone with varying amounts of sand and algal cover on the rocks. Along the 9 m transect, the habitat was primarily sand with cobblestones interspersed. The lengths of the 9, 11, 13 and 15 m transects were 56, 60, 71, and 73 m, respectively; transect lengths were limited by the amount of suitable cichlid habitat between two flanking sandy regions. Each transect was marked with a fluorescent orange flag at intervals of 10–12 m.

Pairs of breeding convict cichlids (*Amatitlania nigrofasciata*) with nests located <1 m from each transect line were identified at random and marked with numbered flags; 15 such pairs were identified at each of the four depths. Each pair was observed by a diver for 10 min on 4–5 separate occasions between 24 January and 30 January 2013. During each observation the following information was recorded with dive computers and a depth gauge: (a) time and date and (b) water depth. In addition, we quantified the following information via observation: (c) whether the focal female possessed orange coloration on the ventrolateral surface (binary state: presence or absence), (d) reproductive status [no offspring or just eggs (these categories were combined because eggs are often difficult to see without excavating the nest), offspring (wrigglers: non-motile larvae or fry: free-swimming offspring)]; and (e) number of aggressive interactions performed by both the resident male and female. The recipient of each aggressive

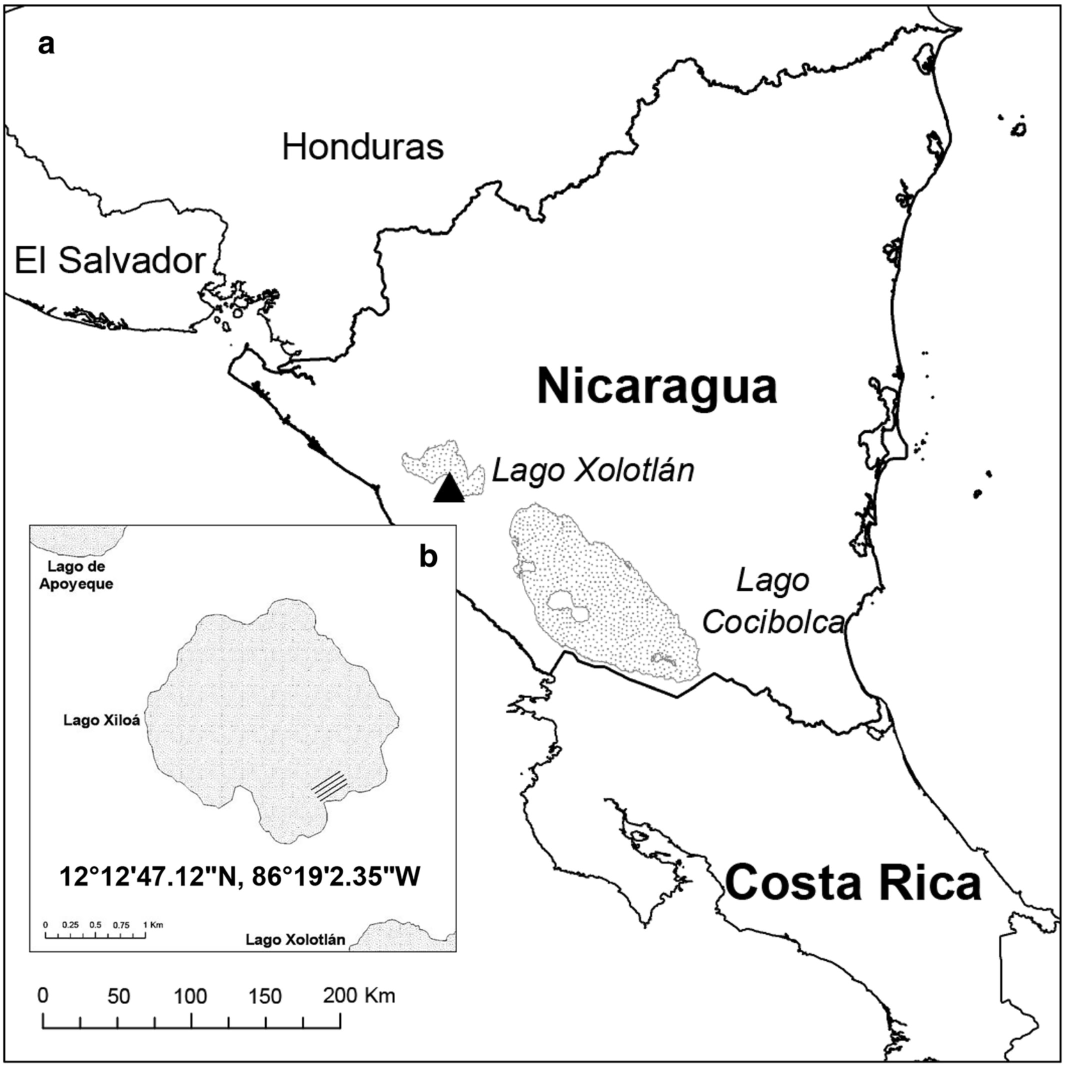

Fig. 1 **a** Lake Xiloá is located on a peninsula in Lake Xolotlán in southwestern Nicaragua, indicated by black triangle. The inset **b** shows the approximate location of our 9, 11, 13 and 15 m transects along the south shore of Lake Xiloá. Transects not drawn to scale

interaction was also recorded, which included (i) heterospecifics (adults and juveniles of *Neetroplus nematopus*, *Hypsophrys nicaraguense*, *Amphilophus citrinellus*, *A. rostratus*, *A. sagittae, A. xiloensis*), (ii) predators (*Gobiomorus dormitor*), (iii) conspecifics, and (iv) pair mate. Aggression typically entailed approaches (slow movement towards the antagonist) with fins erect coupled with frontal display (flaring of opercula) or lateral display (rigid body, fins erect, flank directed at antagonist), attack-chase sequences (rapid and often for a distance), or bites. Approaches towards the predator *G. dormitor* involved either direct attack or frontal display (smaller predators) or movement towards the tail in a curved pattern, often coupled with a bite (larger predators).

Divers conducted 2–3 dives per day for behavioral observations. These dives were performed at 07:30, 12:30, and 13:30 h, and were between 60 and 80 min in duration. To maintain safe, repetitive diving profiles, the 15 m observations were conducted only during the morning and early afternoon dives, and pairs of fish were observed randomly during these dives. For the shallower transects, pair observations were randomized across the three dives each day. Dives typically consisted of 3–4 observations per depth. Observation time included a 2-min acclimation period, during which time the diver would record basic information [(a) through (d) in the previous paragraph] followed by a 10 min behavior quantification period. The diver was always positioned at least 1 m downslope (looking up at the nest) from the nest being observed. Data were collected on 62 mated females at 9 m ($n = 15$), 11 m ($n = 17$), 13 m ($n = 15$), and 15 m ($n = 15$). Two of the nests at 11 m were found to have two attending females. These females were included in the tissue carotenoids analysis ($n = 62$ mated females), but excluded from the behavioral analysis ($n = 60$ mated females) because behavioral interactions could not be attributed to an individual female. 48 non-breeding female convict cichlids also were collected from shallow (< 3 m) vegetated areas in the vicinity of the aforementioned transects (just closer to shore).

On five occasions between 30 January and 4 February 2013, night dives were conducted to excavate the focal nests and retrieve the adult cichlids for processing on land; adults sleep with their brood in the nest at night. Fish were caught and transferred to a holding container underwater, brought to the surface, and maintained for <24 h in an aerated 19-L bucket (all but 10 fish were processed within 9 h). Fish were euthanized in a lethal dose of sodium-bicarbonate buffered MS-222 (Finquel®, Argent Labs; pharmaceutical grade), weighed using an Ohaus field balance to the nearest 0.01 g and decapitated. A small piece of the caudal fin was excised and placed in a 0.5 ml microcentrifuge tube filled with NaCl-saturated DMSO for later genetic analysis. Sex was confirmed by observation of the gonad. The gonad, liver, and ventrolateral integument (~ 3 mm × 3 mm piece from the left side between the fifth and sixth vertical black bars) were removed, placed in 0.5 mL microcentrifuge tubes, and immediately frozen.

On 5 February 2013, rocks were collected from all excavated nests and algae was removed by scraping with a razor blade; the resulting material was weighed, placed into 0.5 mL microcentrifuge tubes, and immediately frozen. Samples were stored at the field station in Lake Xiloá either at room temperature (heads), 4 °C (fin clips), or − 20 °C (gonad, liver, integument, and algae). All samples were transported back to the University of Alabama on dry ice and stored at either 4 °C or − 80 °C for further analysis.

Quantifying carotenoids

A similar high-performance liquid chromatography (HPLC) protocol as in previous studies (Brown et al. 2013, 2014, 2016) was used for carotenoid analysis. Algal samples and fish tissues were frozen in liquid nitrogen and ground to powder using a mortar and pestle. Carotenoids were extracted with hexane, after which they were dried, re-suspended in acetone and diluted with Tris–HCl buffer. Carotenoids were hydrolyzed with cholesterol esterase (C-9281; Sigma, St. Louis, MO, USA) in a 37 °C water bath for 45 min and extracted with sodium sulfate decahydrate and hexane. Hexane was evaporated under nitrogen gas and the sample was reconstituted in a 82:18 hexane:acetone mobile phase. Analyses were performed on a Waters 600 series HPLC system with a model 2478 detector, using a Luna® 3-μm analytical silica column (00F-4162-E0; Phenomenex, Torrance, CA, USA) at a flow rate of 1.2 mL min^{-1} and with an injection loop size of 20 μL. A Waters 600E wavelength absorbance detector was used to produce a chromatogram at 474 nm. Total amounts of carotenoid were calculated using peak area integration values and the standard curves for each identifiable carotenoid type (Millennium 32; Waters Corp. Milford, MA, USA). Total carotenoids in female cichlid gonads was strongly positively correlated with the carotenoid concentration (ng · g^{-1}) of those same tissues ($r = 0.72$, $N = 40$, $P < 0.001$). Due to this strong correlation, and because masses were not available for all samples, only the total carotenoids (ng · sample^{-1}) for each organ/tissue type is reported.

Statistical analysis

One-way analyses of variance (ANOVA) determined how algal mass (g), algal carotenoid content (total ng), and algal quality (ng carotenoid · g^{-1} algae) changed as a function of depth (9, 11, 13, and 15 m). Tukey-Kramer Honest Significant Difference (HSD) was applied to examine differences between depths (e.g., 9 m vs. 11 m; 13 m vs. 15 m). To examine whether tissue carotenoid concentrations of breeding females changed with depth, and to examine whether carotenoid concentrations covaried between tissues, analyses of covariance (ANCOVA) were conducted. Here, depth was categorized as shallow (9 m) or deep (≥ 11 m). In models with integument carotenoid concentrations as the dependent variable, depth was a categorical fixed effect and either liver or gonad carotenoid concentrations were fixed covariates. Models were conducted with and without interactions between depth and tissue (liver or gonad) carotenoid concentrations, and model fit was evaluated with Akaike's Information Criterion (with small sample size correction; AICc). In models with gonad carotenoid concentration as the dependent variable, depth was a categorical fixed effect and liver carotenoid concentration was the fixed covariate. Models were conducted with and without interactions between depth and liver carotenoid concentrations, and model fit was evaluated with AICc. Pearson's correlations also evaluated relationships between tissue carotenoid concentrations, independent of depth, in both breeding and non-breeding females, as well as between algal carotenoids (ng) and tissue carotenoids in breeding females. Algae, liver, gonad, and integument carotenoid concentrations, as well as algal quality (ng carotenoid · g^{-1} algae) were natural log (ln)-transformed to achieve normality; algal mass (g) was not transformed. Fisher's Exact tests determined whether the categorical state of possessing orange (yes/no) varied predictably with depth and reproductive status (no offspring vs. offspring) in the breeding females. Logistic analyses examined whether rates of interaction between breeding females and their pair-mates, other conspecifics, heterospecifics or predators influenced: (i) the probability that females would possess orange color; (ii) female reproductive status (no offspring vs. offspring).

Results

Carotenoid availability and water depth

The total mass of algae collected from the rocks varied significantly with depth. Algae was significantly more abundant at the shallowest depth (9 m) than at any other depth. None of the other depths differed significantly in algal mass (Table 1). Similarly, the total amount of carotenoid availability decreased significantly with depth. Carotenoid abundance was significantly greater at 9 m than at 13 or 15 m, while carotenoid abundance at 11 m was intermediate and not significantly different than any other depth (Table 1). However, the concentration of carotenoids in algae (ng g^{-1}) did not vary with depth (Table 1).

Carotenoids in breeding and non-breeding females

Breeding females had significantly lower liver ($F_{1,65} = 14.93$, $P = 0.0003$), gonad ($F_{1,34} = 30.01$, $P < 0.0001$), and integument ($F_{1,80} = 13.49$, $P = 0.0004$) carotenoid content (ng · sample^{-1}) than non-breeding females (Fig. 2). Among breeding females, there was no significant effect of depth on absolute levels of tissue carotenoids (Table 2). In models with depth as a covariate, there also were no relationships among (ln-transformed) liver, gonad, and integument carotenoids in breeding females, and no significant change in these relationships with depth (interaction terms; Table 2). Pairwise correlations essentially confirmed these results, with positive but non-significant relationships among (ln-transformed) liver, gonad, and integument carotenoids in breeding females (liver-gonad: $r_{13} = 0.55$, $P = 0.051$; liver-integument: $r_{23} = 0.07$, $P = 0.75$; gonad-integument: $r_{16} = 0.40$, $P = 0.12$). Tissue carotenoids in breeding females also were unrelated to algal carotenoid availability (ng g^{-1}) ($F_{1,\ 15\text{–}20} < 3.4$, $P > 0.09$) and these relationships did not change with depth (depth × algal carotenoid availability; $F_{3,\ 15\text{–}20} < 1.1$, $P > 0.39$).

All non-breeding females were collected in shallow (< 3 m), vegetated areas of the lake where they are most abundant. Rarely are non-breeding females encountered in areas where breeding, nesting females are present. Thus, depth-related changes in tissue carotenoids were not analyzed for non-breeding females. Liver and gonad carotenoid content were negatively correlated in non-breeding females but not significantly so ($r_{13} = -0.26$, $P = 0.39$). Liver and integument carotenoid content were positively correlated in non-breeding females but, again, not significantly so ($r_{36} = 0.17$, $P = 0.33$). There was, however, a significant positive correlation between integument and gonad carotenoid content in non-breeding females ($r_{16} = 0.51$, $P = 0.04$).

Female coloration, reproductive status and behavior

Neither depth (9, 11, 13, 15 m; Fisher's Exact: $P = 0.84$) nor reproductive status (no offspring/eggs vs. offspring; Fisher's Exact: $P = 0.28$) was associated with the likelihood that breeding females had visible orange coloration. Rates of interaction between breeding females and their mate, other conspecifics, heterospecifics, or predators also were not associated with whether breeding females possessed visible orange coloration (logistic analyses: Likelihood Ratio [L-R] $\chi^2 < 0.39$, df = 1, $P > 0.53$), a trend that was consistent across depths (behavioral interaction × depth: L-R $\chi^2 < 1.45$, df = 1, $P > 0.23$). In fact, interaction rates in any of the aforementioned contexts did not vary across depths ($F_{3,56} < 2.14$, $P > 0.11$).

Table 1 Summary statistics for algal mass, algal carotenoid abundance, and algal concentration (per g algal mass) at each transect depth

	Algal mass (g)				Total algal carotenoid abundance (ng)				Algal quality (ng carotenoid/g algae)			
Depth (m)	9	11	13	15	9	11	13	15	9	11	13	15
Mean ± SEM	0.60 ± 0.12	0.22 ± 0.04	0.12 ± 0.05	0.05 ± 0.01	52.0 ± 16.2	13.4 ± 4.2	5.1 ± 1.9	3.0 ± 1.3	82.2 ± 28.0	49.1 ± 10.2	130.7 ± 41.4	54.4 ± 21.6
Range	0.13–1.56	0–0.48	0–0.78	0–0.14	4.1–157.9	0.06–39.1	0.19–20.9	0.06–13.7	3.4–270.7	1.6–97.7	9.7–432	0.9–227.9
Median	0.38	0.19	0.02	0.04	41.5	10.39	2.20	1.33	47.80	54.58	90.72	30.59
N	15	15	15	15	10	12	11	10	10	12	11	10
ANOVA stats	$F_{3,56} = 12.64$, $P < 0.0001$				$F_{3,39} = 6.33$, $P = 0.0013$				$F_{3,39} = 1.43$, $P = 0.25$			
Tukey's HSD	a	b	b	b	a	ab	b	b	a	a	a	a

For Tukey-Kramer Honest Significant Difference (HSD), depths with the same letter are not significantly different. *SEM* = standard error of the mean, *N* = sample size, *F* = *F*-value from one-way ANOVA, *P* = *P*-value from one-way ANOVA. Degrees of freedom for the *F*-test are shown as a subscript. Algal quality (ng g^{-1}) is not equivalent to dividing total algal carotenoid abundance (ng) by algal mass (g) because carotenoids concentrations were not successfully obtained from all algae samples

Rates of interaction between breeding females and their mate or with predators were not associated with the likelihood that females would have offspring (logistic analyses; L-R $\chi^2 < 2.37$, df = 1, $P > 0.12$). However, there was a significant positive association between the rate at which breeding females interacted with heterospecifics and the probability that the females would have offspring (logistic analysis; L-R $\chi^2 = 20.57$, df = 1, $P < 0.0001$). For each unit increase in interaction rate with heterospecifics, females were 2.6-times more likely to have offspring than to not have offspring. The rate of interaction between breeding females and conspecifics also was associated with reproductive status but in a negative way (logistic analysis; L-R $\chi^2 = 12.0$, df = 1, $P = 0.0005$). For each unit increase in interaction rate with conspecifics, females were 3.6-times less likely to have offspring than to not have offspring.

Discussion

Non-breeding female convict cichlids caught in the shallows of Lake Xiloá, Nicaragua had significantly more carotenoids in their livers, integument, and gonads than did breeding females caught at greater depths. The rocky substrate near cichlid nests at depths of 11–15 m had less algae, and therefore fewer total carotenoids, than rocks at depths of 9 m. However, this reduced carotenoid availability had no apparent effect on carotenoid allocation patterns in breeding females, as the relationships among liver, gonad, and integument carotenoids were not statistically significant and were unaffected by water depth. In non-breeding females, however, there was a significant positive correlation between integument and gonad carotenoids.

In this study, there was no evidence to indicate that reduced algal abundance limited tissue carotenoids in convict cichlids breeding at greater depths. This result stands in contrast to Grether et al. (1999, 2001), who found geographic variation in carotenoid-based coloration of male Trinidadian guppies (*Poecilia reticulata*) due to variation in the standing crop of carotenoids among river drainages, which was linked to differences in tree canopy cover. Similarly, Cavraro et al. (2018) found that Mediterranean killifish *Aphanius fasciatus* showed more carotenoid-based coloration in streams with greater chlorophyll content in the sediment. Other studies, however, have found no support for

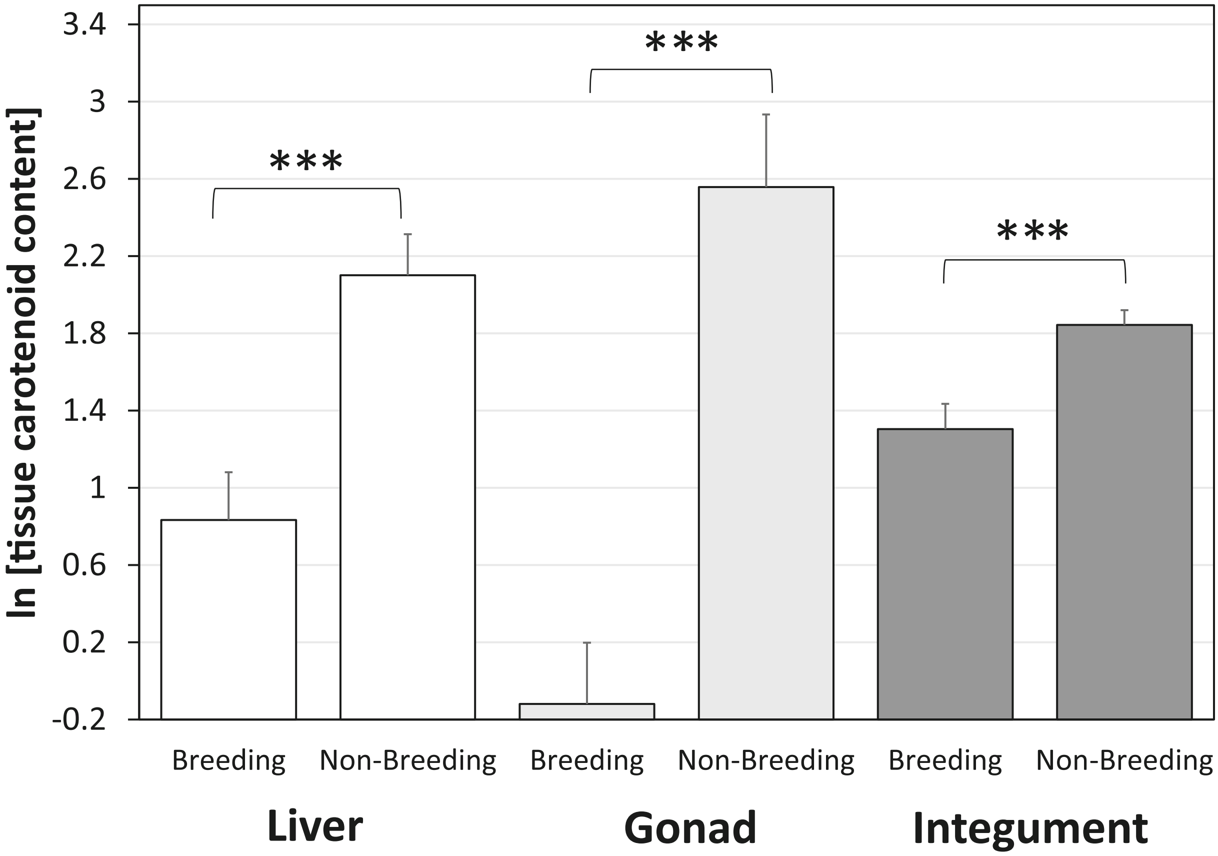

Fig. 2 Differences in tissue carotenoid content (ng/sample) between breeding and non-breeding females. Asterisks indicate significant differences ($P < 0.001$). Histogram bars ± error bars show ln-transformed average ± SEM

the link between dietary carotenoid availability and fish coloration. McNeil et al. (2016) revealed that male carotenoid-based coloration in the eurytopic African cichlid *Pseudocrenilabrus multicolor victoriae* varied with dissolved oxygen content of the water, but not in any measurable way with fish diet. Deutsch (1997) found no evidence that male Malawi cichlids varied in coloration as a function of

Table 2 Relationships among integument, gonad, and liver carotenoids in paired convict cichlid females. The models also account for any effects of depth (binary variable: shallow = 9 m; deep ≥11 m) and for interactions between tissue carotenoids and water depth

	Integument carotenoids		Integument carotenoids		Integument carotenoids		Integument carotenoids		Gonad carotenoids		Gonad carotenoids	
	F_{df}	P	F_{df}	P	F_{df}	P	F_{df}	P	F_{df}	P	F_{df}	P
Overall	$0.15_{3,19}$	0.930	$0.08_{2,20}$	0.926	$0.86_{3,12}$	0.488	$1.28_{2,13}$	0.310	$1.52_{3,9}$	0.274	$2.49_{2,10}$	0.133
Depth	$0.03_{1,19}$	0.863	$0.06_{1,20}$	0.815	$0.00_{1,12}$	0.967	$0.04_{1,13}$	0.844	$0.26_{1,9}$	0.620	$0.43_{1,10}$	0.528
Liver carot.	$0.03_{1,19}$	0.861	$0.12_{1,20}$	0.734					$1.87_{1,9}$	0.205	$2.00_{1,10}$	0.188
Gonad carot.					$1.53_{1,12}$	0.239	$2.12_{1,13}$	0.169				
Depth × Liver carot.	$0.30_{1,19}$	0.593							$0.06_{1,9}$	0.814		
Depth × Gonad carot.					$0.18_{1,12}$	0.682						
AICc	59.14		56.19[a]		54.67		50.54[a]		54.83		49.35[a]	

Carot. = carotenoids, [a] indicates the best-fit model, which was the reduced model (excluding interactions with depth) in all cases water depth, though the author did not quantify algae availability or carotenoid concentrations.

Non-breeding convict cichlid females feeding in shallow waters had significantly more carotenoids in their integument and gonads than breeding females. Furthermore, gonad and integument carotenoids were positively correlated in non-breeding females. Allocation of carotenoids to the gonads can help to amplify the signal function of the ventral orange color (Svensson et al. 2005). Thus, the orange ventrolateral flanks of non-breeding female convict cichlids may function initially in agonistic, intrasexual selection (Beeching et al. 1998), and secondarily as a source of carotenoids for future eggs (Ahmadi et al. 2006). Once non-breeding females have attracted a mate and established a breeding site, they must defend it against conspecifics, heterospecific competitors, and predators; during this period, the orange coloration of females declines (Anderson et al. 2015). Nesting males and females respond more aggressively to fish models with orange patches, particularly when caring for wrigglers/fry (Anderson et al. 2016; see also Lehtonen 2019), suggesting that orange coloration indicates pre-breeding fish that might be potential nest predators or usurpers. Even if intruding females do not successfully take over the nest, attract the mate, or eat the resident female's offspring, our results indicate that extensive interactions with conspecifics might reduce reproductive success; more frequent interactions with conspecifics was associated with a lower probability of having hatched offspring. An alternative explanation is that females in the preparatory phases of reproduction, including nest establishment and possible egg care, might engage conspecifics more often in vigorous attempts to defend the territory.

The decline in female orange coloration during the breeding cycle likely occurs because females reallocate carotenoids from integument to gonads in preparation for egg laying. Egg carotenoids are known to improve offspring survival and growth in many species (reviewed in Janhunen et al. 2011; but see Svensson et al. 2005), including convict cichlids (Brown et al. 2014). We found no significant correlations among liver, integument, and gonad carotenoids among breeding convict cichlids. Previous studies have yielded similarly mixed results. In two-spotted gobies (*Gobiusculus flavescens*) females that exhibit more orange also produce eggs with more carotenoids (Svensson et al. 2005) but there are no correlations between integument carotenoids and egg carotenoids in female Chinook salmon *Oncorhynchus tshawytscha* (Garner et al. 2010). In guppies, dietary intake of carotenoids is more predictive of egg carotenoid content than integument content (Grether et al. 2008). In our study, it is possible that strong relationships among carotenoid concentrations in the three tissue types might have been masked by combining all types of carotenoid into a single measure (total carotenoid). Carotenes and xanthophylls are known to occur in different concentrations in integument, liver, and gonads (e.g., Lin et al. 2010), so future work should fractionate the sample to determine how concentrations of specific carotenoids covary among tissue types.

A major difference between this study and our previous work on wild Lake Xiloá convict cichlids was the relationship between coloration and (i) reproductive status, and (ii) behavioral interactions that the animals had within their community. Anderson et al. (2015) showed that females were less likely to be orange when caring for hatched offspring (e.g., wrigglers, free-swimming fry) and also when interacting more frequently with predators and heterospecific competitors. Our study, however, showed no such relationships. In the current study, 79.7% (47 out of 59) of the females were defending hatched offspring, while in Anderson et al.'s (2015) study, only 48.9% (113 out of 231) were defending hatched offspring. Given that most females in the current study had offspring, and because these females tend to possess less orange coloration, it is possible that we simply did not have enough variation in breeding status to detect any relationship with coloration. There also were considerable differences between the two studies in how the focal females interacted with predators and heterospecifics. In the current study, females interacted with predators 8–18 times less frequently than in Anderson et al. (2015) (average # interactions · day^{-1} ± SEM for females with: no offspring/eggs - 0.12 ± 0.09; offspring - 0.22 ± 0.05 [*this study*] vs. no offspring/eggs – 0.94 ± 0.28; offspring: 4.05 ± 0.35, (Anderson et al. 2015)). Females also interacted about 1.7-times more often with heterospecifics in the current study (5.7 ± 0.46) compared to Anderson et al. (2015) (3.3 ± 0.27). Such dramatic differences are likely responsible for the different findings, and could be due to seasonal or yearly differences in predator/heterospecific abundance (e.g., current study done in January–February 2013; Anderson et al. study done in March 2010 and September–February 2011–2012).

Our findings suggest that carotenoids are not limiting in the diets of breeding female convict cichlids. If they were, we would predict that absolute concentrations of carotenoid in the liver, gonad, and integument and, importantly, relationships between carotenoid concentrations in the tissue types, would change as a function of depth. These relationships might be positive, for example, in shallower water where carotenoids are more abundant, and negative in deeper waters where fish are forced to allocate scarce carotenoids to one function or the other. This did not appear to be the case for the convict cichlids in Lake Xiloá. Brown et al. (2016) analyzed the stomach contents of riverine convict cichlids in Costa Rica and found no relationship between dietary carotenoids and infection by the bacterium *Edwardsiella tarda*. They also confirmed that levels of dietary carotenoids in free-living fish were sufficient (and even better) than higher levels at clearing *E. tarda* infection in the laboratory. From this they concluded that dietary intake of carotenoids was sufficient to support normal immune function. In much the same way, we have shown that algal carotenoids are sufficient across most depths where convict cichlids breed in Lake Xiloá, Nicaragua.

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Authors' contributions EDC and RLE designed the study, organized the field and laboratory research, secured funding for the project, conducted data collection in the field and laboratory, and collaborated to generate the manuscript. CTA, MKM and BBN assisted with field and laboratory data collection and with organizing the manuscript. ACB participated in organizing the field research and conducted carotenoid assays in the laboratory.

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Data availability Raw data will be made available as supplementary material.

Compliance with ethical standards

Conflicts of interest/Competing interests The authors have no conflicts of interest or competing interests.

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Consent to participate All authors fully consented to being involved with the reported research.

Consent for publication All authors approved of the final version of this manuscript.

Code availability This study did not employ any custom code or software applications outside of the statistical packages described in the Methods section.

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