Reference: *Biol. Bull.* 238: 119–130. (April 2020)

DOI: 10.1086/708625

Red Coloration in an Anchialine Shrimp: Carotenoids, Genetic Variation, and Candidate Genes

RYAN J. WEAVER[1,2,*], BRYSON K. GONZALEZ[3], SCOTT R. SANTOS[2], AND JUSTIN C. HAVIRD[1]

[1]*Department of Integrative Biology, University of Texas at Austin, Austin, Texas, 78712;* [2]*Department of Biological Sciences and Molette Biology Laboratory for Environmental and Climate Change Studies, Auburn University, Auburn, Alabama 36849; and* [3]*Hawaii Baptist Academy, Honolulu, Hawaii 96817*

Abstract. Red coloration is a widely distributed phenotype among animals, yet the pigmentary and genetic bases for this phenotype have been described in relatively few taxa. Here we show that the Hawaiian endemic anchialine shrimp *Halocaridina rubra* is red because of the accumulation of astaxanthin. Laboratory colonies of phylogenetically distinct lineages of *H. rubra* have colony-specific amounts of astaxanthin that are developmentally, and likely genetically, fixed. Carotenoid supplementation and restriction experiments failed to change astaxanthin content from the within-colony baseline levels, suggesting that dietary limitation is not a major factor driving coloration differences. A possible candidate gene product predicted to be responsible for the production of astaxanthin in *H. rubra* and other crustaceans is closely related to the bifunctional cytochrome P450 family 3 enzyme *CrtS* found in fungi. However, homologs to the enzyme thought to catalyze ketolation reactions in birds and turtles, *CYP2J19*, were not found. This work is one of the first steps in linking phenotypic variation in red coloration of *H. rubra* to genotypic variation. Future work should focus on (1) pinpointing the genes that function in the bioconversion of dietary carotenoids to astaxanthin, (2) examining what genomic variants might drive variation in coloration among discrete lineages, and (3) testing more explicitly for condition-dependent carotenoid coloration in crustaceans.

Received 24 October 2019; Accepted 11 February 2020; Published online 15 April 2020.

* To whom correspondence should be addressed. Email: ryan.weaver@utexas.edu.

Abbreviations: EP site, Windward Oahu lineage; EWA site, South Oahu lineage; HM site, South Maui lineage; HPLC, high-performance liquid chromatography; HSD, honest significant difference; IH site, East Hawaii lineage; KBP, mixed lineages; KONA site, West Hawaii lineage; MCMC, Markov chain Monte Carlo; OWAI site, West Oahu lineage; pp, posterior probability; WC site, East Maui lineage.

Online enhancements: supplemental figures.

Introduction

Variation in coloration within animal species is hypothesized to result from selective pressures of the environment (Hasson, 1997; Hadfield and Owens, 2006; Sommaruga, 2010), physiological constraints (Számadó, 2011; Weaver *et al.*, 2017), and/or genetic differences (Roulin, 2004; Matrková and Remeš, 2012; Wybouw *et al.*, 2019). Carotenoid-based coloration has been particularly well studied because it is thought that variation in the intensity of this coloration within a species depends on some aspect of an individual's "quality" and/or "condition" (Hill, 1991). For example, male house finches range from vibrant red to drab orange, and redder males tend to be of higher phenotypic quality than drab males across some proxy measures, such as parasite load (Thompson *et al.*, 1997; Brawner *et al.*, 2000) and disease clearance (Hill and Farmer, 2005). However, more generally, evidence of condition-dependent expression of carotenoid coloration is sporadic in birds (Griffith *et al.*, 2006; Simons *et al.*, 2012; Weaver *et al.*, 2018b). Birds and certain selected other vertebrates have been the dominant taxa investigated in the potential roles of carotenoid coloration as honest signals of quality (Candolin, 1999; Pérez-Rodríguez *et al.*, 2010; Simons *et al.*, 2012), yet many invertebrates also use carotenoids for coloration. Results from recent studies of crustaceans suggest that carotenoids act as antioxidants that provide relevant protection against environmental stressors, such as exposure to ultraviolet (UV) radiation, heavy metals, and other xenobiotics that induce oxidative stress (Davenport *et al.*, 2004; Babin *et al.*, 2010; Caramujo *et al.*, 2012; Snoeijs and Häubner, 2014; Wade *et al.*, 2017; Weaver *et al.*, 2018c). This contrasts with

vertebrate studies where growing evidence suggests that carotenoids do not serve key antioxidant roles (Isaksson *et al.*, 2007; Pérez-Rodríguez, 2009; Koch and Hill, 2018; Koch *et al.*, 2018) but instead have been co-opted as sexual signals.

With rare exceptions (Moran and Jarvik, 2010), animals cannot synthesize carotenoids *de novo*; they must be obtained from the diet (Goodwin, 1984; Parker, 1996). In many cases, dietary carotenoids are yellow xanthophylls that animals absorb through the gut, transport through the circulatory system, and deposit to the integument to produce a yellow coloration (Parker, 1996; Weaver *et al.*, 2018b). To produce red coloration, some animals metabolically convert dietary yellow carotenoids to red ketocarotenoids (*i.e.*, carotenoid bioconversion, McGraw, 2006; Weaver *et al.*, 2018b). Crustaceans are among the most colorful aquatic animals, and previous work has shown that red coloration in crustaceans is most often due to accumulation of the red ketocarotenoid astaxanthin (Goodwin and Srisukh, 1949; Matsuno, 2001; Su *et al.*, 2018; Weaver *et al.*, 2018a).

Carotenoid bioconversion genes identified in plants, bacteria, and fungi encode either diiron non-heme monooxygenases or cytochrome P450 enzymes that convert b-carotene to xanthophylls (*e.g.*, zeaxanthin or lutein) *via* 3,3′-hydroxylation or those that convert xanthophylls to ketocarotenoids (*e.g.*, astaxanthin) *via* 4,4′-ketolation. Such genes have been identified mainly in bacteria (*CrtZ*; Makino *et al.*, 2008), plants (*CrtR*; Inoue, 2004), and fungi (*CrtS*; Alvarez *et al.*, 2006; Martín *et al.*, 2008). *CrtS* encodes a P450 belonging to the 3A family (*i.e.*, CYP3A) that putatively acts bifunctionally as both a hydroxylase and a ketolase to convert yellow xanthophyll carotenoids to the red ketocarotenoid astaxanthin in the fungus *Xanthophyllomyces dendrorhous* (Martín *et al.*, 2008). In one of the earliest reports of linking a carotenoid phenotype to a genotype, Mojib *et al.* (2014) identified candidate b-carotene hydroxylase proteins in a copepod and a tunicate on the basis of homology with *CrtS* of *X. dendrorhous*. Later, in 2016 *CYP2J19* was identified as the gene responsible for red carotenoid coloration in birds (Lopes *et al.*, 2016; Mundy *et al.*, 2016; Twyman *et al.*, 2018a) and turtles (Twyman *et al.*, 2016). *CYP2J19* encodes a cytochrome P450 carotenoid ketolase that adds ketone functional groups to the 4,4′ carbons of b-ionone rings of the substrate carotenoid, producing red carotenoids. More recently, *CYP384A1* was identified as the putative gene responsible for red carotenoid coloration in a spider mite (*Tetranychus* sp.). *CYP384A1* encodes a CYP3-like enzyme; and mutations in this gene are suspected to be a putative cause for yellow coloration of mites in *Tetranychus* sp., which are typically red (Wybouw *et al.*, 2019). The genetic basis for red carotenoid coloration from the bioconversion of yellow precursor carotenoids in any other animal taxa, including crustaceans, has yet to be identified.

The red shrimp *Halocaridina rubra* is a small (1-cm) atyid shrimp found exclusively in anchialine habitats of the Hawaiian Islands (Holthuis, 1963, 1973; Bailey-Brock and Brock, 1993). The anchialine ecosystem is characterized by networks of landlocked surface and/or cave ponds and pools connected to subterranean aquifers that experience tidal influences but that do not have surface connections with the ocean. This topography results in a highly variable environment; strong but unpredictable gradients in salinity, UV radiation, temperature, and dissolved oxygen are common (Holthuis, 1973; Bailey-Brock and Brock, 1993; Havird *et al.*, 2014). Across the islands, eight distinct genetic lineages of *H. rubra* have been identified on the basis of mitochondrial cytochrome oxidase subunit I (*COI*), and large subunit ribosomal (*16S-rDNA*) gene sequences, suggesting that they represent a cryptic species complex (Craft *et al.*, 2008). Populations representing these lineages also show drastic variation in coloration that ranges from vibrant red to nearly translucent (Vaught *et al.*, 2014; Fig. 1). Other anchialine shrimp species from Japan with genetically structured populations display similar red coloration (Weese *et al.*, 2013). The morphological basis for coloration in *H. rubra* and related shrimp species has been thoroughly described (Noël and Chassard-Bouchaud, 2004; Flores and Chien, 2011; Vaught *et al.*, 2014). Red coloration results from sequestering red pigments in specialized cells called chromatosomes, which are distributed throughout the exoskeleton and epidermis. The extent and intensity of red coloration are dynamically controlled by the expansion and contraction of chromatosomes, which change shape in response to physiological or environmental cues. The density and surface area of chromatosomes vary both within and among *H. rubra* laboratory colonies—established from the genetic lineages described above—and, as a result, the intensity of red coloration also varies (Vaught *et al.*, 2014). Despite the plastic nature of red coloration in crustaceans, Vaught *et al.* (2014) showed that chromatosomal properties that give rise to red coloration in *H. rubra* varied significantly among laboratory colonies that were maintained under common conditions, suggesting that variation in coloration may be genetically based. However, it is unclear whether carotenoid content also contributes to differences in red coloration in this species.

The goals of this study were to determine that *H. rubra* coloration is carotenoid based, identify those carotenoids, and test the hypothesis that lineage-specific differences in coloration are due to differences in carotenoid abundance. We also experimentally manipulated access to dietary carotenoids to test whether genetic *versus* environmental factors may be driving colony-level differences in coloration. Last, we mined the transcriptomes and genomes of *H. rubra*, five other atyid shrimps, and other crustaceans for homologs of known carotenoid bioconversion genes to determine whether *CYP2J19*, *CrtS*, or other candidate genes may act to bioconvert dietary carotenoids to ketocarotenoids in these taxa.

Materials and Methods

Shrimp husbandry

Specimens of *Halocaridina rubra* Holthuis, 1963 used in this study were collected in 2006 from seven sites across three islands of the Hawaiian archipelago. Each of these sites con-

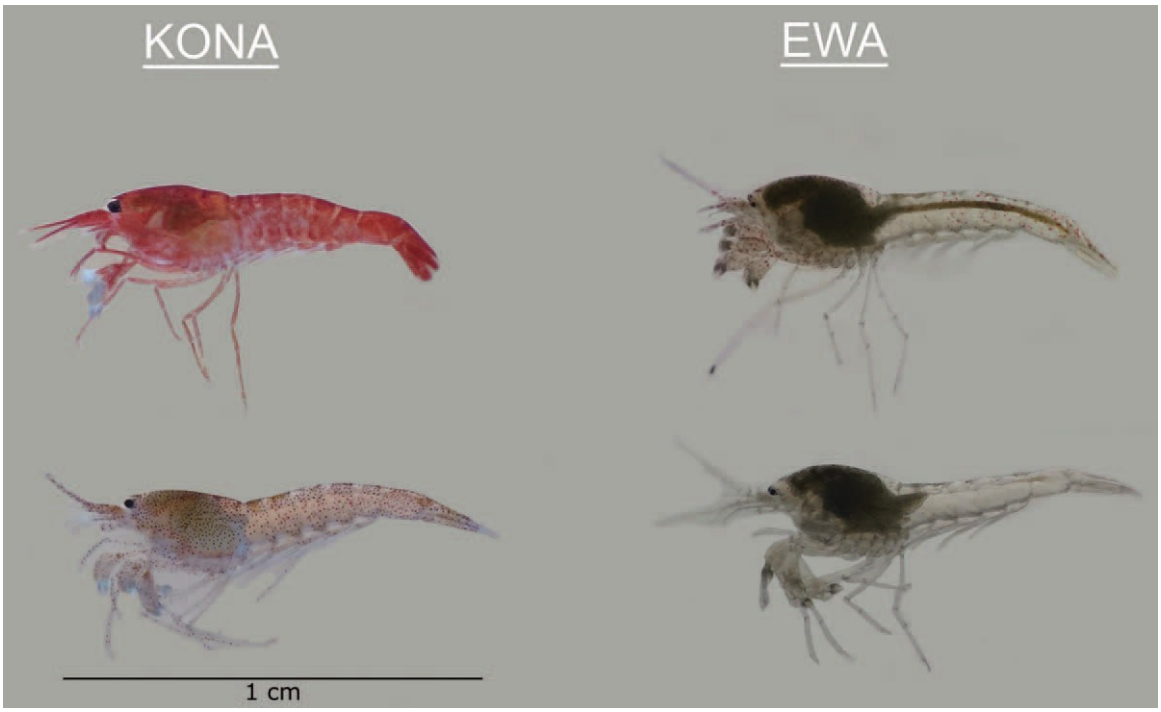

Figure 1. Red coloration of *Halocaridina rubra* varies both within (columns) and among (rows) individuals from laboratory colonies. Representative photographs taken from one of the most colorful (KONA: KONA site, West Hawaii lineage) and least colorful (EWA: EWA site, South Oahu lineage) colonies.

tains pools with shrimp that belong to a single genetic lineage (named after the island) that is distinct from any other site (see Fig. 2: EP site [Windward Oahu lineage], HM site [South Maui lineage], IH site [East Hawaii lineage], OWAI site [West Oahu lineage], WC site [East Maui lineage], KONA site [West Hawaii lineage], and EWA site [South Oahu lineage]) (Craft *et al.*, 2008). We also collected shrimp from the KBP site, which contains a mix of shrimp that are from one of two genetic lineages: either genetically identical to the South Oahu lineage (represented by EWA) or one of two genetic groups within the West Oahu lineage (represented by OWAI) (Craft *et al.*, 2008). We established laboratory colonies of these lineages by culturing them in separate 20-gallon aquaria under common conditions: 15-psu artificial seawater at 24 ₇C and exposed to a 12h∶12h light∶dark cycle for 1 week every month. These laboratory colonies have been maintained consistently under the conditions described above for nearly 14 years, since they were initiated in 2006. Porous volcanic rocks inoculated with naturally occurring microbial and microalgal communities from outdoor ponds were provided equally to each tank as a source of food and substrate. Shrimp grazed on these microbes and algae *ad libitum*, and no other food was routinely added to the tanks. Reproduction occurred consistently but sporadically for all colonies under these conditions (Havird *et al.*, 2015).

Carotenoid extraction and determination of red color pigment

To determine the pigmentary source of red coloration and test for differences between colonies, we performed a carotenoid extraction protocol following Weaver *et al.* (2018a). In 2018, we sampled shrimp from the KONA and EWA lab colonies (n 5 5 each) because they represent one of the most and least colorful lineages, respectively (Fig. 1). Our aim was to determine (1) whether carotenoids were the pigment responsible for red coloration and (2) whether we could detect any

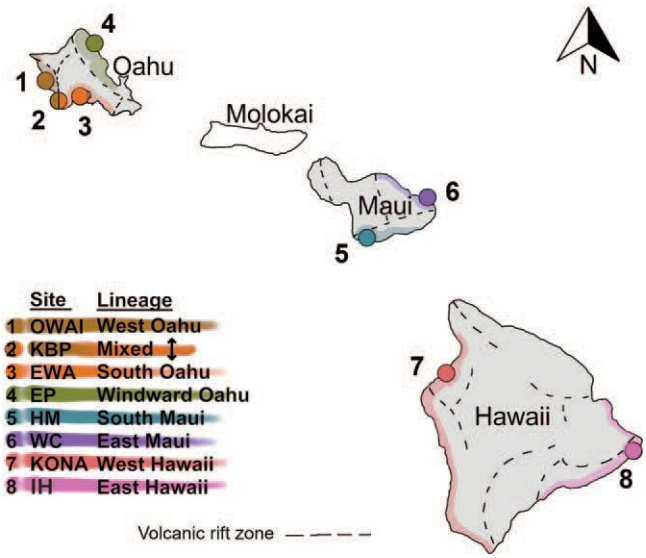

Figure 2. Map of collection sites for *Halocaridina rubra* used in establishing laboratory colonies. Each number represents a single site from a distinct genetic lineage, except for KBP, which is a mix of individuals from two lineages (OWAI site, West Oahu lineage; and EWA site, South Oahu lineage). The shaded regions show the approximate range of each lineage.

pigment in the least colorful lineage. We weighed individuals to the nearest 0.1 mg, flash froze them in liquid N_2, and stored them at 280 °C. To extract carotenoids, we sonicated shrimp in 1 mL of acetone for 10 s at 10 W, then stored samples under N_2 gas at 4 °C in the dark overnight. We then centrifuged the resulting samples at 10,000×*g* for 5 min, removed the supernatant containing the carotenoids to a new tube, capped the tubes with N_2 gas, and stored them at 280 °C. We performed a second extraction of the pellet with 500 mL of acetone under N_2 at 4 °C overnight. The supernatants from both extractions were pooled, then capped with N_2 gas and stored at 280 °C. We refer to this as the primary extract.

First, we sought to test for the presence of carotenoids and, if present, to identify the specific carotenoid composition per *H. rubra* individual. We injected 10 mL of primary extract from each EWA (n = 5) and KONA (n = 5) individual onto a Sonoma C18 column (250 mm×4.6 mm, 10 mm, ES Industries, West Berlin, NJ) installed on a Shimadzu (Columbia, MD) high-performance liquid chromatography (HPLC) system. We used the mobile phase solvents 80:20, methanol:0.5 mol L^{-1} ammonium acetate, 90:10, acetonitrile:H_2O, and ethyl acetate over a series of gradients, described by Weaver *et al.* (2018a). Carotenoids were separated and identified by comparison of retention times and absorbance of carotenoid standards measured at 450 nm by using a UV-visible light detector. Because most crustaceans store carotenoids in both their free and fatty-acid-esterified forms (Goodwin and Srisukh, 1949; Matsuno, 2001; Su *et al.*, 2018), we also performed a saponification of the primary extract using 0.2 mol L^{-1} KOH in methanol for 6 h, which removes the fatty-acid esters and results in all free carotenoids (Toomey and McGraw, 2007). We determined the relative composition of free and esterified carotenoids by calculating the ratio of free carotenoids measured before and after saponification.

Quantifying carotenoids from distinct lineages of Halocaridina rubra

In 2018 we also sampled 26 adult individuals from each of the 8 laboratory colonies. As mentioned above, individuals from KBP are from two distinct genetic lineages, but we did not determine to which lineage individuals belonged before processing for carotenoid content. We extracted carotenoids from individual shrimp as described previously, then measured total carotenoid content spectrophotometrically using a 96-well microplate reader. For most of the samples, we evaporated a 300-mL aliquot of primary extract from each individual to dryness under vacuum at 30 °C, then resuspended the samples in 300 mL of 100% EtOH. In cases where the primary extract was from the typically less colorful colonies (*e.g.*, EWA) or appeared to be relatively light in color, we concentrated the sample to increase the likelihood that the carotenoid concentration would be above the lower detection limit of the plate reader. In those instances, we evaporated a 600-mL aliquot of primary extract to dryness, then resuspended it in 300 mL of 100% EtOH. We measured the absorbance of each sample in duplicate at 470 nm and calculated the carotenoid content of the samples by using a standard curve of astaxanthin measured under the same conditions. We report the carotenoid content as micrograms per milligram wet weight of the individual shrimp.

To assess whether colony-specific levels of coloration observed in adults were consistent across developmental stages, we also measured carotenoid content of individual lecithotrophic (*i.e.*, maternal yolk-bearing) larvae from two of the most colorful colonies (EP, n = 14 and KONA, n = 11) and two of the least colorful colonies (EWA, n = 11 and KBP, n = 12). The difference in sample sizes is a result of the fact that larvae are more difficult to obtain because of the sporadic reproductive cycles of the laboratory colonies. The larvae were visually inspected as described in Havird and Santos (2016) and were classified as "early," being in either the Z_1 or Z_2 stage. Carotenoids were extracted as described above, but because larvae are much smaller (~1.5-mm length) than adults (~1-cm length), we evaporated the 1.5-mL primary extract to dryness under vacuum at 30 °C, then resuspended it in 100 mL of HPLC-grade acetone. We quantified the amount of astaxanthin present by using HPLC as described above, given that it can detect considerably lower amounts of carotenoids than the plate reader method. We report the astaxanthin content of larvae as micrograms per individual.

Carotenoid supplementation and restriction experiments

Although we maintained shrimp from each lineage under common conditions, they were kept in separate aquaria. Therefore, tank-specific differences in microbial or microalgal communities might provide different amounts of dietary carotenoids to different colonies. To test whether environmental limitations of dietary carotenoids might explain colony-level differences in coloration, we performed carotenoid-supplementation and carotenoid-restriction feeding experiments. We used shrimp from one of the most and one of the least colorful colonies, EP and EWA, respectively. Specifically, we supplemented 18 EWA shrimp with zeaxanthin, a yellow carotenoid naturally found in a microalgae diet. Shrimp and other crustaceans are known to enzymatically convert zeaxanthin to astaxanthin (Matsuno, 2001; Rhodes, 2007; Maoka, 2011; Weaver *et al.*, 2018a). We also fed 12 EP shrimp a carotenoid-restricted diet of nutritional yeast (Weaver *et al.*, 2018a). Shrimp were maintained on the experimental diets for 14 days, then processed for carotenoid extraction by using the aforementioned HPLC protocol.

Statistical comparisons of carotenoid content among colonies

Preliminary data analyses revealed significant heteroskedasticity in carotenoid content among colonies (Flinger-Kileen

test, $v^2 = 34.7$, $P < 0.001$). To account for this, we used the inverse variance of astaxanthin measures for each colony as a correction for heteroskedasticity and fit the data to a weighted least squares model to estimate the mean carotenoid content of each group. We compared group means by using Tukey's honest significant difference (HSD) test to control for multiple comparisons. Below, we report carotenoid content as the mean ± standard deviation unless otherwise noted. Statistical analyses were performed using R (ver. 3.6, R Core Team, 2017), and the results were visualized using ggplot2 (Wickham, 2016).

Phylogenetic analyses of candidate carotenoid metabolism genes in crustaceans

To gain insights into the molecular mechanisms of carotenoid metabolism in crustaceans, we searched the transcriptomes of *H. rubra* (Havird and Santos, 2016) and five other red anchialine shrimp species (*Metabataeus lohena*, *Metabataeus minutus*, *Caridina rubella*, *Antecaridina lauensis*, and *Halocaridines trigonopthalma*) (Genomic Resources Development Consortium *et al.*, 2014) for known carotenoid conversion genes. The genome of the marine copepod *Tigriopus californicus* (Barreto *et al.*, 2018) was also searched, as red color in this species was previously shown to arise from the conversion of yellow dietary carotenoids to astaxanthin (Weaver *et al.*, 2018a).

The *CrtS* gene from the basidiomycetous yeast *Xanthophyllomyces dendrorhous* and the *CYP2J19* gene from the bird *Quelea quelea* were used as queries in tBLASTn searches (Altschul *et al.*, 1997). We retained the top 3 unique hits from each search that had an e-value below $1e^{-10}$. Additional BLAST searches were conducted using the same queries in the National Center for Biotechnology Information nonredundant protein sequence database limited to crustaceans and amphibians. Discussions about red coloration in poison dart frogs inspired us to also search for carotenoid conversion homologs in amphibians (E. Twomey, Vrije Universiteit Brussel, pers. comm.).

We translated the retained hits from the searches and identified open reading frames by using ExPASy (Gasteiger *et al.*, 2005). The resulting amino acid sequences were aligned using MUSCLE (Edgar, 2004) as implemented in MEGA X (Kumar *et al.*, 2018). Additional protein sequences included in the alignment were (1) the known carotenoid hydroxylase protein sequences from plants and bacteria; (2) the putative copepod carotenoid hydroxylase proteins identified in Mojib *et al.* (2014); (3) the *CYP2J19* sequences from birds and turtles; (4) protein sequences of *CYP2J* family genes from birds, other reptiles, and humans identified in Twyman *et al.* (2016); and (5) the *CYP384A1* sequence identified from the spider mite *Tetranychus* sp. in Wybouw *et al.* (2019). Phylogenetic analyses were performed using Bayesian estimation, using Markov chain Monte Carlo (MCMC) implemented by MRBAYES 3.2.7 (Huelsenbeck and Ronquist, 2001). We set the amino acid substitution model parameter to mixed in order for MRBAYES to estimate the fixed-rate model that best fit the data; in our case, this was the Blosum62 model (Henikoff and Henikoff, 1992). We used a flat Dirichlet distribution for the priors and ran 2 independent runs of 4 chains each until the split standard deviation between the 2 runs was <0.01 (Huelsenbeck and Ronquist, 2001). We used a burn-in of 25% and retained posterior estimates every 1000 generations. We assessed convergence from the summary statistics calculated by MRBAYES and by visually examining the trace plots of the MCMC chains, using Tracer version 1.7.1 (Rambaut *et al.*, 2018). The resulting consensus tree was visualized using TreeGraph 2 (Stöver and Müller, 2010), and nodes with posterior probability (pp) support values <50% were collapsed into polytomies. Using a subset of the above sequences, we also estimated phylogenies using maximum likelihood, using RAxML version 8.2.12 (Stamatakis, 2014) with the Le and Gascuel (LG) model of amino acid substitution and a gamma distribution (LGGAMMA model). Nodal support was evaluated with 1000 rapid bootstraps (Stamatakis, 2014). Phylogenetic analyses were also performed on the alignment after trimming with gBlocks (Talavera and Castresana, 2007). Results from the maximum likelihood and gBlocks trimmed approaches are supplied in the see supplemental material (see Figs. S1–S4, available online).

Results

Astaxanthin is responsible for red coloration in Halocaridina rubra

We found that the red carotenoid astaxanthin was the major carotenoid accumulated in the tissues of adult *Halocaridina rubra* (Fig. A1). The majority of astaxanthin detected was esterified (86.7% ± 8.7% SD, $n = 10$), with free astaxanthin making up a smaller proportion (16.5% ± 6.2%, $n = 8$). The amount of free astaxanthin in two of the five individuals from the EWA colony was below the detection limit of our HPLC system.

Lineage differences in astaxanthin content of adults and larvae

While we detected carotenoids in nearly all individual adult shrimp from each colony, we found that carotenoid content varied significantly between a number of colonies (Fig 3A). Specifically, shrimp from WC and EP had the highest amount of carotenoids, but with large interindividual variation (WC = 0.75 ± 0.4 mg mg^{-1}, $n = 26$; EP = 0.59 ± 0.36 mg mg^{-1}, $n = 26$) while those from KBP and EWA had the lowest amount of carotenoids, with small interindividual variation (KBP = 0.13 ± 0.16 mg mg^{-1}, $n = 26$; EWA = 0.09 ± 0.09 mg mg^{-1}, $n = 26$). As mentioned previously, KBP is composed of individuals that are genetically identical either to the EWA lineage or to one of two genetic groups within the OWAI lineage.

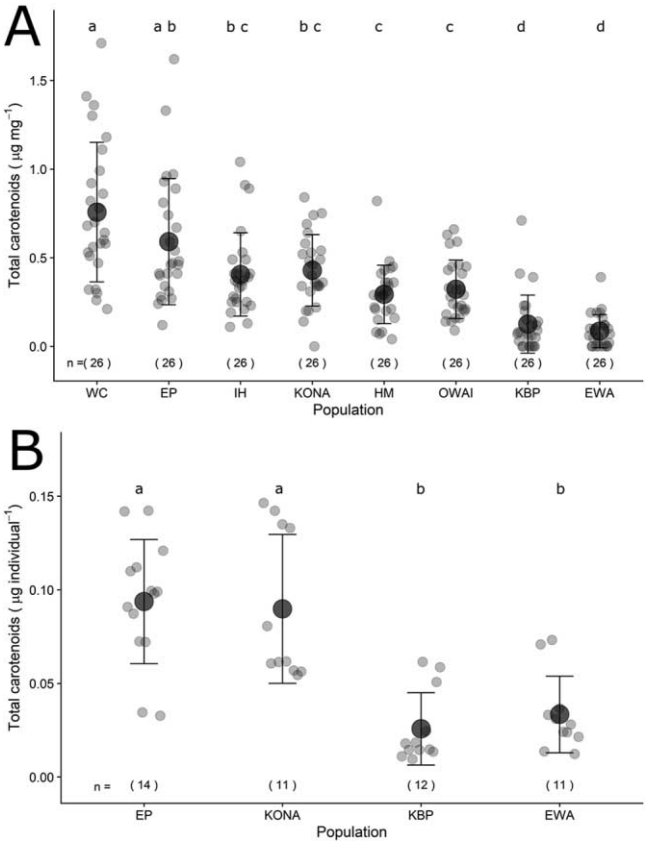

Figure 3. Carotenoid content of laboratory colonies of *Halocaridina rubra* adults (A) and larvae (B) sampled from genetically discrete lineages across the Hawaiian Islands. Larger dark circles and error bars show the mean and standard deviation. Smaller circles are individual sample measurements. Colonies that do not share a lowercase letter have statistically different carotenoid content (Tukey honest significant difference *post hoc*, $P < 0.05$). WC: WC site, East Maui lineage; EP: EP site, Windward Oahu lineage; IH: IH site, East Hawaii lineage; KONA: KONA site, West Hawaii lineage; HM: HM site, South Maui lineage; OWAI: OWAI site, West Oahu lineage; KBP: KBP site, mixed lineages; EWA: EWA site, South Oahu lineage.

Although we did not identify the lineage of any shrimp sampled from the KBP colony, we found that the carotenoid content of individuals from the KBP colony was intermediate between those of two lineages (OWAI = 0.32 ± 0.16 mg mg^{21}, n = 26; Fig. 3A). The mean values and standard deviations of measured carotenoid content for all colonies are available in Table A1.

Similar to adults, we found that the red carotenoid astaxanthin was the major carotenoid present in the lecithotrophic nonfeeding larvae of *H. rubra*. Carotenoid content of larvae from EP (n = 14), KONA (n = 11), KBP (n = 12), and EWA (n = 11) mirrored the patterns of carotenoid content of adults from those colonies (Fig. 3B). Namely, larvae from EP and KONA had nearly twice the carotenoid content of those from KBP and EWA, consistent with differences among adults (Tukey HSD, $P < 0.001$ in those comparisons; Fig. 3B *vs.* Fig. 3A).

Dietary limitation of carotenoids does not explain variation in astaxanthin content between colonies

We found that xanthophyll carotenoid supplementation did not increase astaxanthin content of EWA individuals (tank = 0.09 ± 0.09 mg mg^{21}, n = 26; supplemented = 0.09 ± 0.075 mg mg^{21}, n = 18, P = 0.99; Fig. 4). Similarly, carotenoid restriction for 14 days did not significantly reduce the mean astaxanthin content of adults from EP (tank = 0.59 ± 0.36 mg mg^{21}, n = 26; yeast = 0.47 ± 0.2 mg mg^{21}, n = 12, P = 0.47).

Crustaceans and amphibians lack CYP2J19 *but may use a carotenoid bioconversion enzyme similar to* CrtS

From the BLAST searches, we retained a total of 82 sequences from the species we investigated that had high similarity to *CrtS* from *Xanthophyllomyces dendrorhous* or *CYP2J19* from *Quelea quelea* after filtering to remove sequences with e-value scores >1e^{210} (see data available at the FigShare repository; see *Data Accessibility*). Each of the retained sequences contains the oxygen-binding, heme-binding, and pair-charge motifs diagnostic of cytochrome P450 enzymes (Wen *et al.*, 2001; see supplemental material). When combined with sequences from Mojib *et al.* (2014), Wybouw *et al.* (2019), and Twyman *et al.* (2016), the final dataset included 152 sequences. The consensus tree from the Bayesian MCMC analysis returned a well-supported (100% pp) monophyletic clade that contained all *CYP2J19* sequences from birds and turtles (Fig. 5A). Notably, none of the crustacean or amphibian sequences recovered from BLAST searches against *CYP2J19* grouped within this clade of putative carotenoid ketolase genes (Fig. 5C). Many of the crustacean sequences showed similarity to cytochrome P450 enzymes in the CYP2L1-like family (Fig. 5C). Our

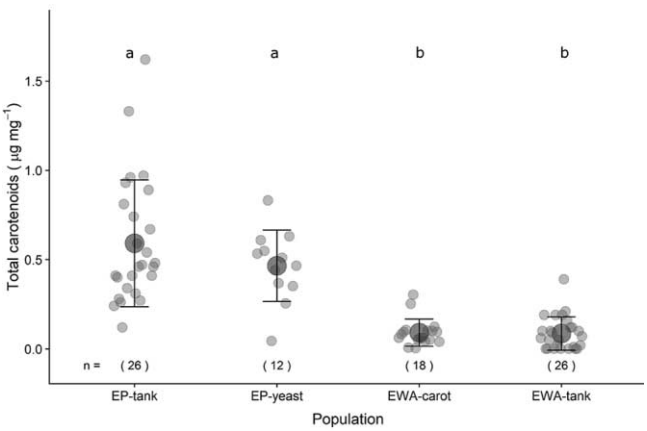

Figure 4. Comparison of carotenoid content of adult *Halocaridina rubra* kept in laboratory aquaria (tank) and after carotenoid supplementation (carot) and depletion (yeast). Larger dark circles and error bars show the mean and standard deviation. Smaller circles are individual sample measurements. Colonies that do not share a lowercase letter have statistically different carotenoid content (Tukey honest significant difference *post hoc*, $P < 0.05$). EP: EP site, Windward Oahu lineage; EWA: EWA site, South Oahu lineage.

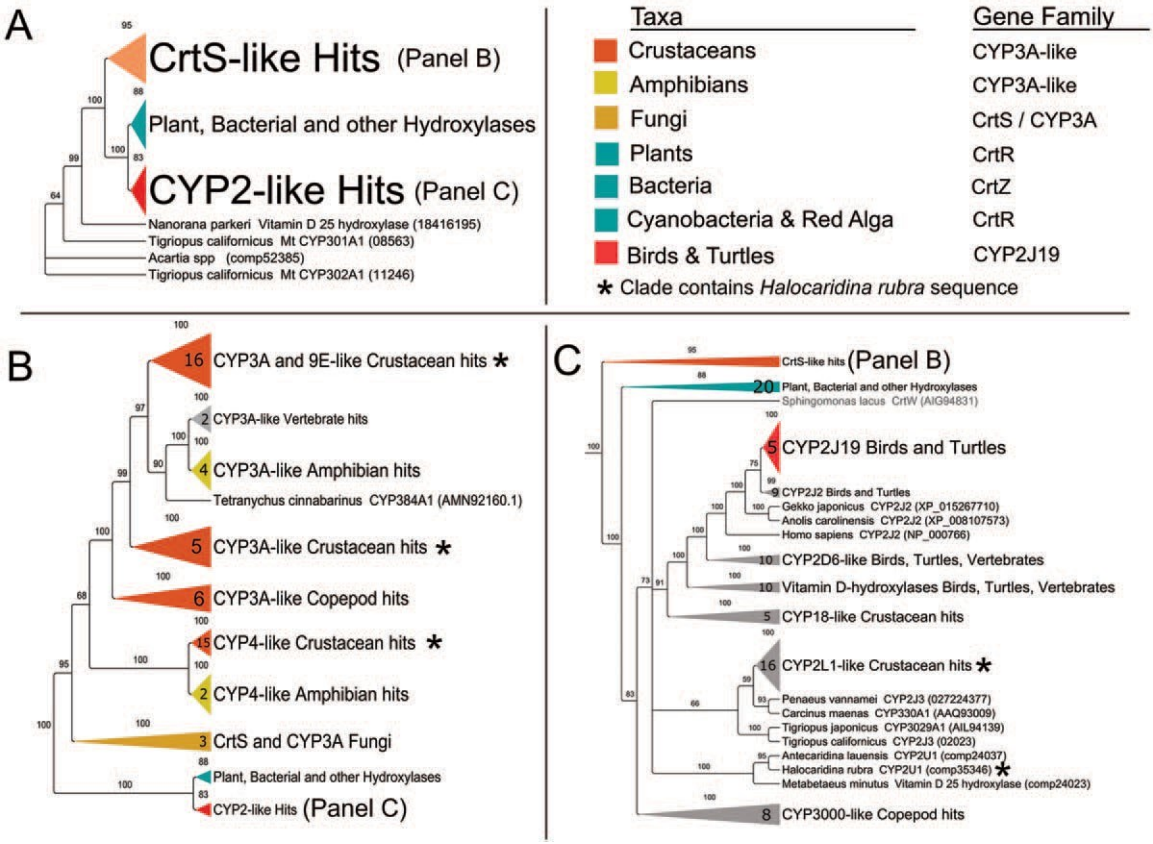

Figure 5. Estimated phylogeny of known carotenoid hydroxylases from plants, bacteria, cyanobacteria, fungi, and ketolases from birds and turtles and candidates from crustaceans and amphibians. (A) The phylogeny estimated using a Bayesian Markov chain Monte Carlo framework shows well-supported and separate clustering of CYP3-like and CYP2-like proteins. Crustacean and amphibian sequences returned from BLAST searches against *CrtS* cluster within the clade that contains *CrtS* (B), but results from BLASTing against *CYP2J19* do not fall within the clade that contains *CYP2J19* (C). Numbers at the nodes are the posterior probabilities, and the numbers in the triangles indicate how many sequences are in that clade.

phylogenetic analysis also recovered a well-supported monophyletic clade containing the known fungal carotenoid hydroxylase sequence *CrtS* (Fig. 5A). The best BLAST hits to *CrtS* from crustaceans and amphibians returned sequences similar to the CYP3A and CYP9E families of enzymes. These formed a strongly supported clade (100% pp) that was sister to the known fungal carotenoid hydroxylases in the CYP3A family (Fig. 5B). The topography of the resulting maximum likelihood phylogenies was similar to the Bayesian MCMC methods, but nodal support was lower toward the root of the tree (Figs. S1–S4, available online). Trees generated from trimmed and untrimmed alignments had similar topologies. All sequences, alignments, and expanded consensus trees from each method are provided at the FigShare repository (see *Data Accessibility*).

Discussion

Taxa that display red carotenoid-based coloration span the evolutionary tree and are found in terrestrial and aquatic habitats. However, despite its ubiquity, surprisingly little is known about the molecular and genetic basis for carotenoid coloration in many animals. Here we show that the atyid shrimp *Halocaridina rubra*, commonly referred to as ‘ōpae‘ula (literally, tiny red shrimp) by the native Hawaiian people, derives its characteristic coloration from the accumulation of the red ketocarotenoid astaxanthin. We found colony-specific differences in astaxanthin accumulation by adults (Fig. 3); we also found that the variation among colonies in astaxanthin content in this study closely matches the variation in chromatosome characteristics that give rise to differences in red coloration observed among the same laboratory colonies (Vaught *et al.*, 2014). In addition, we found that larvae hatch with colony-typical levels of astaxanthin in their tissues (Fig. 3B), likely *via* maternal deposition to eggs, which are also red. Taken together, because most of these colonies represent discrete genetic lineages (Craft *et al.*, 2008) and were maintained under common conditions, this suggests that lineage-typical astaxanthin accumulation is genetically based and possibly heritable (Nguyen *et al.*, 2014). However, we do not have a complete sampling of all pools within a lineage, which makes it unclear

how much variation in coloration exists within a lineage. Additionally, estimating heritability of this trait will require parent-offspring correlations in astaxanthin content, which we plan to pursue in future studies.

One hypothesis for why animals within a species vary in carotenoid content is based on trade-offs between using carotenoids as pigments and using them for physiological processes. The foundation of this idea is that carotenoids are a limited resource because they must be obtained from the diet (Weaver *et al.*, 2017; Koch and Hill, 2018). Because shrimp in our study were kept in separate aquaria, tank-specific differences in carotenoid availability may have contributed to differences in astaxanthin accumulation among colonies. However, differences in their coloration have been stable in captivity since 2006 (Vaught *et al.*, 2014), and carotenoid supplementation or restriction did not alter internal astaxanthin content from baseline levels (Fig. 4). Similar experiments in carotenoid-deficient copepods show that supplementation of dietary xanthophyll carotenoids (lutein, zeaxanthin) measurably increases astaxanthin content in as little as two days, and wild-type levels are reached within one week (Weaver *et al.*, 2018a, c). Supplementation of Pacific white shrimp, *Penaeus vannamei*, with precursor xanthophylls (lutein, zeaxanthin) has also been shown to nearly double astaxanthin content over 14 days (Vernon-Carter *et al.*, 1996). However, it is common in aquaculture for species such as shrimp to receive high concentrations of astaxanthin, directly, for four to eight weeks, due to the beneficial effects of supplementation on growth and survival, in addition to enhancing red coloration (Higuera-Ciapara *et al.*, 2006; Lim *et al.*, 2017; Wade *et al.*, 2017).

The lack of change in astaxanthin in our feeding trials leads us to speculate that variation in astaxanthin content of our *H. rubra* colonies is not shaped by environmental limitation of dietary carotenoids but instead is a genetically fixed trait. Supporting this, these laboratory colonies have been maintained under similar environmental conditions and grazing availability for nearly 14 years, which also suggests that there is a genetic basis for variation in coloration among the *H. rubra* lineages. However, the ultimate drivers of variation in red carotenoid accumulation likely also include some level of selective pressures from the environment. If this is the case, one possible selective pressure for red carotenoid accumulation in aquatic animals is the putative protection conferred by carotenoids against solar UV radiation exposure (Chalker-Scott, 1995; Davenport *et al.*, 2004; Sommaruga, 2010; de Carvalho and Caramujo, 2017; but see Schneider *et al.*, 2016).

The anchialine habitats that *H. rubra* occupies have complex hydrologies governed by their basin properties that vary within and across the islands on which they are found (Holthuis, 1973; Bailey-Brock and Brock, 1993). Thus, while all anchialine surface pools have direct connections to subterranean aquifers, some are inside caves and/or are in heavily canopied patches of forest that experience (at least in their current state) very low levels of UV radiation. Other pools found in relatively young lava fields are unprotected from full solar UV (Santos, 2006; Craft *et al.*, 2008). Although protection from UV exposure is one possible driver of increased carotenoid accumulation, this is likely not the case for *H. rubra*, since collection sites such as WC and EP are both within forest patches or caves with low UV reaching the pool surfaces (RJW and JCH, unpubl. data); yet shrimp from these sites accumulated the greatest amounts of astaxanthin in their tissues (Fig. 3A). Given that UV exposure does not drive carotenoid coloration in a predictable way in this system, future work should focus on investigating other potential environmental factors that may have selected for higher carotenoid accumulation in some lineages of *H. rubra*.

Several carotenoid metabolism genes that underlie red carotenoid phenotypes encode proteins involved in the uptake and transport (*SCARB1*; Toomey *et al.*, 2017), deposition (*FGGY*; Lopes *et al.*, 2016), and degradation of carotenoids (*BCO2*; Walsh *et al.*, 2012), in addition to the yellow to red carotenoid conversion enzymes discussed above. The possible genetic basis for carotenoid phenotypes of *H. rubra* is perhaps not surprising given the relatively high genetic divergence (*i.e.*, ~5% in mitochondrial loci) observed among lineages (Craft *et al.*, 2008), which approaches levels observed between distinct species in other crustacean taxa.

A recent study on the extent of species-specific carotenoid coloration in birds highlights how fixed genetic differences in carotenoid metabolism genes give rise to differences in color phenotypes. For example, golden-winged (*Vermivora chrysoptera*) and blue-winged (*Vermivora cyanoptera*) warblers show low divergence across their genomes but differ dramatically in the extent of yellow carotenoid-based feather color. In this case, Toews *et al.* (2016) showed that the genomes of these bird species are highly differentiated at the locus that encodes the carotenoid degradation gene, *BCO2*. Similarly, a transcriptomic approach in an African cichlid fish, *Tropheus duboisi*, revealed greater expression of *BCO2*; and lower expression of carotenoid uptake gene *SCARB1* was associated with white *versus* yellow skin (Ahi *et al.*, 2020). Variation among *H. rubra* lineages in the functionality or expression of *BCO2*, carotenoid bioconversion enzymes, or any of the other carotenoid metabolism proteins could result in the observed differences in carotenoid content in this study.

Results from our phylogenetic analyses suggest that crustaceans and amphibians capable of converting dietary xanthophylls to ketocarotenoids do so using an enzyme that is different from that of birds and turtles. The convergence on accumulating similar carotenoid products (ketocarotenoids) among many invertebrate and vertebrate animals suggests that different biochemical reactions, likely involving different cytochrome P450 genes, have been co-opted to produce the same final pigment products and phenotypes (Britton and Goodwin, 1982; Twyman *et al.*, 2018b; Wybouw *et al.*, 2019). While *CYP2J19* appears to be absent in the non-reptilian taxa investigated

here, we identified several candidate carotenoid bioconversion genes in the CYP3 family among the crustaceans and amphibians included in this study. Taken together, we suggest that crustaceans may serve as an attractive model to further elucidate the genetics and physiology that underpin variation in carotenoid coloration of non-avian animals.

Acknowledgments

This work was supported by National Science Foundation (NSF) awards DBI-1906751 and IOS-1701827 to RJW, NSF DEB-0949855 to SRS, and University of Texas at Austin funds to JCH. This article represents contributions 196 and 97 from the Auburn University Marine Biology Program and Molette Biology Laboratory for Environmental and Climate Change Studies, respectively. RJW and SRS conceived the study; RJW and BKG performed carotenoid analyses; RJW performed statistical analyses; RJW and JCH performed phylogenetic analyses and led the writing of the manuscript; all authors contributed critically to the drafts and gave approval to submission.

Data Accessibility

The data from this study and code for analyses are available at the FigShare digital repository (https://doi.org/10.6084/m9.figshare.11803908.v1).

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Appendix

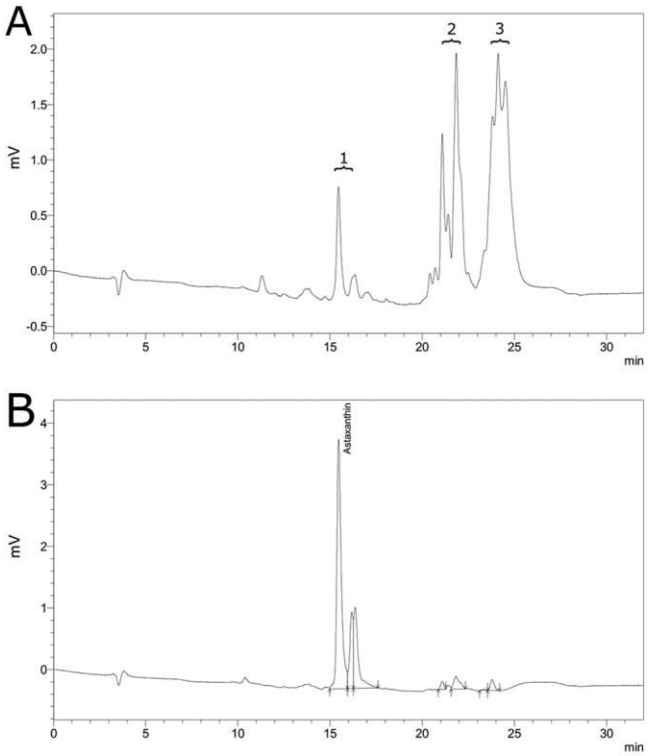

Figure A1. Representative chromatogram of carotenoids extracted from *Halocaridina rubra*. (A) Astaxanthin in its free form (1) and mono-esters (2) and di-esters (3) of astaxanthin comprise over 90% of the detectable carotenoids in *H. rubra*. (B) After saponification to remove esterified fatty acids, only free astaxanthin is detected.

Table A1

Total carotenoid content of Halocaridina rubra *laboratory colonies that represent discrete populations across its range*

Colony	Total carotenoids (mg mg^{21})	SD	*n*
Adults			
EP	0.591	0.356	26
EWA	0.086	0.093	26
IH	0.406	0.235	26
HM	0.294	0.165	26
KBP	0.126	0.163	26
KONA	0.429	0.202	26
OWAI	0.322	0.165	26
WC	0.758	0.394	26
Larvae			
EP	0.094	0.033	14
EWA	0.033	0.020	11
KBP	0.026	0.019	12
KONA	0.090	0.040	11

EP, Windward Oahu; EWA, South Oahu; IH, East Hawaii; HM, South Maui; KBP, mixed; KONA, West Hawaii; OWAI, West Oahu; WC, East Maui.