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Iridophores and Not Carotenoids Account for Chromatic Variation of Carotenoid-Based Coloration in Common Lizards (*Lacerta vivipara*)

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ABSTRACT: Carotenoids typically need reflective background components to shine. Such components, iridophores, leucophores, and keratin- and collagen-derived structures, are generally assumed to show no or little environmental variability. Here, we investigate the origin of environmentally induced variation in the carotenoid-based ventral coloration of male common lizards (*Lacerta vivipara*) by investigating the effects of dietary carotenoids and corticosterone on both carotenoid- and background-related reflectance. We observed a general negative chromatic change that was prevented by β -carotene supplementation. However, chromatic changes did not result from changes in carotenoid-related reflectance or skin carotenoid content but from changes in background-related reflectance that may have been mediated by vitamin A₁. An in vitro experiment showed that the encountered chromatic changes most likely resulted from changes in iridophore reflectance. Our findings demonstrate that chromatic variation in carotenoid-based ornaments may not exclusively reflect differences in integumentary carotenoid content and, hence, in qualities linked to carotenoid deposition (e.g., foraging ability, immune response, or antioxidant capacity). Moreover, skin carotenoid content and carotenoid-related reflectance were related to male color polymorphism, suggesting that carotenoid-based coloration of male common lizards is a multicomponent signal, with iridophores reflecting environmental conditions and carotenoids reflecting genetically based color morphs.

Keywords: carotenoid-based ornaments, condition-dependent signaling, honest signaling, structural coloration.

Introduction

Carotenoid pigments are responsible for bright yellow, orange, and red ornamental coloration that may often func-

tion as honest, condition-dependent signals (Olson and Owens 1998). Signal honesty arises because the ornamental incorporation of carotenoids is limited by (i) dietary carotenoid availability, given that animals cannot synthesize carotenoids (Hill 1994), and (ii) carotenoid requirements of other physiological functions, such as immune and antioxidant responses (Chew and Park 2004) or vitamin A synthesis (Olson 1989). Thus, carotenoid deposition into ornaments is frequently linked to animals' quality to deal with these limitations (e.g., foraging ability, health condition, or nutritional status; Hill 1990; von Schantz et al. 1999).

Variation in individuals' quality or condition is therefore assumed to induce color changes by promoting changes in carotenoid deposition (Andersson and Prager 2006). Because carotenoids exclusively absorb short-wavelength light, namely, in the violet-blue range, variation in their deposition leads to chromatic variation by altering the ratio between short- and long-wavelength reflectance (Shawkey and Hill 2005). However, carotenoids have a purely pigmentary effect (i.e., they absorb but do not emit UV or visible light) and typically need an underlying reflective surface to produce or enhance integumentary colors. Such reflective backgrounds consist of keratin- and collagen-derived matrices in birds (Prum and Torres 2003) and of iridophores and collagen fascia in reptiles, fish, and amphibians (Bagnara and Hadley 1973).

The spectral contribution of reflective backgrounds and their environmental susceptibility has been rarely considered (Kemp et al. 2011). Jacot et al. (2010) demonstrated that environmental conditions affect both carotenoid deposition and background components of great tits' plumage. Such effects are spectrally distinguishable because the

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keratin-derived structures of feathers reflect light almost uniformly (white background) and induce an achromatic variation that contrasts with the chromatic variation induced by carotenoids. However, some carotenoid-based ornaments possess a long-wavelength reflectance peak (e.g., Grether et al. 2005; Velando et al. 2006; Richardson et al. 2009), which indicates that background components may reflect certain wavelengths with more intensity than others (i.e., they produce color), and that could account for chromatic variation (fig. A1, available online). Further, carotenoid deposition may not always depend on environmental conditions, as, for instance, in species with genetically based color morphotypes (Dale 2000). Therefore, chromatic changes may not necessarily indicate changes in integumentary carotenoids and, hence, in animal qualities usually linked with carotenoid-based ornaments. Unraveling whether background components induce chromatic variation in response to environmental conditions is crucial to understanding the physiological and evolutionary mechanisms underlying carotenoid-based coloration.

Here we investigate these questions in the carotenoid-based coloration of male common lizards, *Lacerta vivipara* (Fitze et al. 2009). Male common lizards show discrete color morphotypes (Sinervo et al. 2007) but also chromatic variation in response to environmental conditions (Meylan et al. 2007; Cote et al. 2008, 2010; Fitze et al. 2009; San-Jose et al. 2012a). Carotenoid supplementation and subsequent increase in blood carotenoid levels do not affect coloration (Fitze et al. 2009; San-Jose et al. 2012a), which is consistent with discrete color morphotypes but contradicts studies showing environmental determination of coloration. This incongruence suggests that some integumentary components are environmentally flexible, whereas others reflect morphotypes. The integument of *L. vivipara* shows contiguous chromatophore cell layers (Breathnach and Poyntz 1966; Bryant et al. 1967), with the outermost layer formed by xanthophores and erythrophores, both containing carotenoids, a reflective second layer of iridophores, and an absorbing third layer of melanophores (Bagnara et al. 1968). Iridophores and melanophores provide carotenoids with a colored background (Grether et al. 2004), as evidenced by (i) the presence of a high long-wavelength reflectance peak (fig. 1) and (ii) the extraction of carotenoids with acetone, which leads to blue coloration (Fitze et al. 2009). This blue coloration additionally suggests that the background more likely results from light reflectance in iridophores, whereas melanophores may modulate iridophore-based reflectance (Grether et al. 2004). Iridophores contain purine crystals arranged into stacked platelets, which may produce different colors by constructive multilayer interference (Land 1972) and according to platelet spacing and size as well

as platelet and cytoplasm refractive index (Rohrlich and Porter 1972).

To investigate which integumentary components account for environmental plasticity in *L. vivipara*, we first ran an in vivo experiment treating lizards with two different carotenoid diets and with corticosterone, a known inductor of chromatic changes in this species that affects condition by increasing oxidative damage and enhances food requirements (Fitze et al. 2009; Cote et al. 2010). We investigated treatment effects on coloration, discerning between changes owing to carotenoid- and background-related reflectance. To confirm treatment effects on carotenoid-related reflectance, we also quantified carotenoid concentrations in the skin, blood, and liver and the hepatic concentration of vitamin A using high-performance liquid chromatography (HPLC). In this experiment, we also investigated which integumentary components determine color differences between morphotypes and whether morphotypes differ in the amount of carotenoids of the skin and other tissues. To confirm that iridophores determine background-related reflectance and might account for the encountered color changes, we ran an in vitro experiment manipulating skin osmolarity with different solutions of phosphate-buffered saline (PBS). Osmolarity changes directly affect the spacing of iridophore platelets, changing iridophore reflective properties and revealing how irido-

Figure 1: Average reflectance spectrum of ventral male common lizard coloration ($n = 36$). Spectral regions used for color parameter calculation are shaded, and the location of peak reflectance in the ultraviolet part, maximum negative slope (background slope) above 575 nm, and peak reflectance above 575 nm are indicated.

Table 1: Results from mixed models of reflectance parameters related with background components

Factor	<i>F</i>	df	<i>P</i>
Background reflectance ($R_{575-700}$):			
Time	12.74	1, 31	.001
Treatment	.99	3, 28	.414
Treatment $\times$ time	3.26	3, 31	.035
Molt status	9.11	1, 28	.005
Molt status $\times$ time	11.57	1, 31	.002
Ingested larvae	2.28	1, 28	.142
O score	4.00	1, 28	.055
W score	1.12	1, 28	.298
Maximum background reflectance ($R_{\max}$):			
Time	18.18	1, 31	<.001
Treatment	.84	3, 28	.486
Treatment $\times$ time	3.34	3, 31	.032
Molt status	12.00	1, 28	.002
Molt status $\times$ time	13.25	1, 31	.001
Ingested larvae	3.03	1, 28	.093
O score	4.31	1, 28	.047
W score	1.49	1, 28	.233
Background slope ($ b_{\max\text{neg}} $):			
Time	26.05	1, 32	<.001
Treatment	.45	3, 28	.717
Treatment $\times$ time	4.19	3, 32	.013
Molt status	9.84	1, 28	.004
Molt status $\times$ time	1.62	1, 31	.213
Ingested larvae	<.01	1, 28	.997
O score	1.20	1, 28	.283
W score	1.84	1, 28	.186

Note: Only fixed effects are given. Statistics of nonsignificant interactions correspond to values before backward elimination.

phores determine spectral reflectance (Bone and Denton 1971; Morrison et al. 1996).

Methods

Species Description and Preexperimental Methods

Lacerta vivipara is a small Euroasiatic lizard. Adult males exhibit conspicuous ventral coloration based on carotenoids; mainly, lutein (and its esters), zeaxanthin, and β -carotene (Fitze et al. 2009; San-Jose et al. 2012b). Carotenoid extraction with acetone produces bluish coloration, which discard other yellow-orange pigments such as pteridines (Fitze et al. 2009). In the Pyrenees, common lizard males exhibit six color morphotypes determined by a putative locus with three alleles (orange, *o*; yellow, *y*; and white, *w*; Sinervo et al. 2007). Males are classified as putative homozygotes in the presence of uniform orange (*oo*), yellow (*yy*), or white (*ww*) ventral coloration or as putative heterozygotes (*wo*, *wy*, *yo*) in the presence of mosaiclike coloration.

For the in vivo experiment, we collected 36 adult males

from two neighboring populations at Somport (central Pyrenees, Spain, 42°47'N, 0°31'W). For the in vitro experiment, we collected 12 adult males from Somport and Puerto de Ibañeta (western Pyrenees, Spain, 43°1'N, 1°19'W). Lizards were brought to the laboratory, where they were individually housed in terraria equipped with two shelters, a water pond, and peat soil as substrate (for further details, see San-Jose et al. 2012a). Snout-vent length (SVL; to the nearest 1 mm) and body mass (to the nearest 1 mg) were measured, and standardized photographs taken. We scored lizards as having 0, 1, or 2 putative *o* alleles (O score) and 0, 1, or 2 putative *w* alleles (W score; for further details see Sinervo et al. 2007).

In Vivo Experiment

We randomly assigned 8 lizards to a xanthophyll supplementation group (XAN), 8 to a β -carotene supplementation group (β CAR), 8 to a corticosterone administration group (CORT), and 12 to a control group (CONT). No significant differences existed among treatment groups in

Figure 2: Treatment effects on changes in parameters reflecting variation in background components: background reflectance (A), maximum background reflectance (B), and maximum background slope (C). Pre- and postexperimental means ($\pm$ SE) are plotted. Asterisks indicate significant changes within treatment groups (post hoc contrasts: two asterisks, $P_{adj} < .01$; one asterisk, $P_{adj} < .05$).

coloration, SVL, body mass, and color morph (all $P > .25$). Lizards were fed with carotenoids present in their natural diets and in blood, skin, and reserve tissues in doses within the natural range (Fitze et al. 2009; San-Jose et al. 2012b). The XAN group was fed with *Galleria mellonella* larvae injected with .03 mL of a solution of 200-mg lutein : zeaxanthin beadlets (5.58% lutein, .44% zeaxanthin; Hoffmann-La Roche, Basel, Switzerland) dissolved in 100 mL of distilled water. The β CAR group was fed with larvae injected with .03 mL of a solution of 160.45-mg β -carotene (7.5%) and 39.55-mg control beadlets dissolved in 100 mL of distilled water. The CORT and CONT groups were fed with larvae injected with .03 mL

of a solution of 200-mg control beadlets dissolved in 100 mL of distilled water. Lizards were fed every 2 days for 14 days. If lizards refused to eat, we left the larva in the terrarium and waited for 1 day before removing it. The corticosterone treatment consisted of daily application to the lizards' backs of 6.75 μ g corticosterone diluted in 4.5 μ L sesame oil for 14 days (Meylan et al. 2003; Gonzalez-Jimena and Fitze 2012). The XAN, β CAR, and CONT groups were treated with a control solution (4.5 μ L sesame oil).

At the start of the experiment, a blood sample was taken using a heparinized microcapillary. Blood was centrifuged (5 min, 8,900 g) and plasma stored at -80°C . At the end of the experiment, we collected a second blood sample from a subset of 26 males (XAN: $n = 6$, β CAR: $n = 5$, CORT: $n = 6$, CONT: $n = 9$). Lizards of the subsample were thereafter anesthetized (.02 mL/g of medetomidine : ketamine [1 : 50]) and decapitated to extract ventral skin and liver. Tissues were rinsed with PBS to avoid contamination with blood carotenoids, weighed (to the nearest .001 mg), and stored at -80°C . Concentrations of lutein, zeaxanthin, and β -carotene in plasma, skin, and liver and vitamin A₁ and A₂ concentrations in liver were analyzed using HPLC following the protocol described by San-Jose et al. (2012b).

In Vitro Experiment

One week after capture, lizards were euthanized and their ventral skin removed as described above. Skins were divided into three pieces, except for one small male whose skin could only be cut in two pieces. One skin piece per male was assigned to a treatment of increasing osmolarity (IO group, $n = 12$ pieces), which was expected to reduce platelet spacing and displace peak reflectance toward shorter wavelengths (i.e., toward less orange colors; Morrison et al. 1996), to a treatment of decreasing osmolarity (DO group, $n = 12$), which was expected to augment platelet spacing and displace peak reflectance toward longer wavelengths (i.e., toward more orange colors; Land 1972), or to a control treatment (CO group, $n = 11$). Skin pieces were first immersed in standard PBS solutions (295 mOsm/L). Thereafter, they were put into PBS solutions of increasing osmolarity (1.5 $\times$, 442.5 mOsm/L; 2 $\times$, 590 mOsm/L; 3 $\times$, 885 mOsm/L), decreasing osmolarity (0.5 $\times$, 147.5 mOsm/L; 0.25 $\times$, 73.75 mOsm/L; 0 $\times$, i.e., distilled water, 0 mOsm/L), or into four different flasks of standard PBS solutions (control pieces). Twenty minutes after immersion, skin pieces were placed on a black photographic cloth to measure reflectance (see below).

Figure 3: Mean $\pm$ SE (line and shading) of pre- and postexperimental reflectance spectra for the CONT, CORT, β CAR, and XAN groups.

Color Measurements

We measured coloration with a USB4000 spectrometer (Ocean Optics, Dunedin, FL), a standard reflection probe (QR400-7-UV/VIS-BX, Ocean Optics), and a deuterium and tungsten-halogen light (DT-MINI-2-GS, Ocean Optics). Using OOIBase software (Ocean Optics), we measured reflectance relative to diffuse white (WS-1, Ocean Optics) and black standards. For the *in vivo* experiment, we measured pre- and postexperimental reflectance in the middle of the same three belly scales of the left central longitudinal line (second scale below the collar scales, mid scale of the belly, and second scale before the anal scale). Skin pieces (*in vitro* experiment) were measured on three predetermined central scales. For each measurement, the average of the three scales was used for the statistical analyses. To account for molt effects on coloration, we classified males of the *in vivo* experiment as premolting

($n = 7$) or nonmolting ($n = 29$). No male of the *in vitro* experiment was molting.

Using RCLR (Montgomerie 2008), we calculated parameters describing spectral intensity and shape. We calculated background reflectance ($R_{\text{background}}$), maximum background reflectance (R_{max}), and background slope (absolute value of the maximum negative slope, $|b_{\text{maxneg}}|$). These parameters were quantified between 575 and 700 nm, which is outside the range where carotenoids absorb light (fig. 1; Jacot et al. 2010). Hence, they exclusively measure carotenoid-independent spectral variation resulting from background components (melanophores and iridophores; Grether et al. 2004). To investigate effects on carotenoid content, we calculated reflectance in the violet-blue range (violet-blue reflectance: $R_{400-515}$) where carotenoids absorb light (Shawkey et al. 2006). We calculated absolute carotenoid chroma ($R_{\text{violet-blue}}/R_{\text{background}}$), which is

Table 2: Results from mixed models of parameters reflecting variation in the wavelength where carotenoids absorb light

Factor	<i>F</i>	df	<i>P</i>
Violet-blue reflectance ($R_{400-500}$):			
Time	12.64	1, 35	.001
Treatment	.34	3, 28	.795
Treatment $\times$ time	.69	3, 29	.566
Molt status	.01	1, 28	.920
Molt status $\times$ time	1.80	1, 33	.188
Ingested larvae	.30	1, 28	.586
O score	4.86	1, 28	.036
W score	2.22	1, 28	.147
Absolute carotenoid chroma ($R_{400-500}/R_{575-700}$):			
Time	35.44	1, 31	<.001
Treatment	.35	3, 28	.787
Treatment $\times$ time	3.10	3, 31	.041
Molt status	9.15	1, 28	.005
Molt status $\times$ time	16.30	1, 31	<.001
Ingested larvae	.66	1, 28	.423
O score	.37	1, 28	.547
W score	5.72	1, 28	.024
Hue:			
Time	6.19	1, 31	.018
Treatment	3.56	3, 28	.027
Treatment $\times$ time	3.00	3, 31	.045
Molt status	3.32	1, 28	.079
Molt status $\times$ time	13.25	1, 31	.001
Ingested larvae	1.11	1, 28	.301
O score	.15	1, 28	.702
W score	2.10	1, 28	.159

Note: Only fixed effects are given. Statistics of nonsignificant interactions correspond to values before backward elimination.

negatively correlated with saturation and accurately reflects carotenoid content when background components vary achromatically (Jacot et al. 2010). In the presence of colored backgrounds, absolute carotenoid chroma may poorly predict integument carotenoid concentration because chromatic variation may not exclusively result from carotenoids (fig. A1). We calculated hue using Endler's (1990) segment classification method to make results comparable with previous studies (Fitze et al. 2009; Cote et al. 2010) and UV reflectance (between 300 and 400 nm; R_{UV}) and the spectral position of maximum reflectance in the UV range ($\lambda_{UV\text{ peak}}$), which may be affected by background components and carotenoids (Jacot et al. 2010). All color measurements were highly repeatable (repeatability based on two repeated measures: $F_{10,11} > 6.14$, $P < 0.01$, $r > 0.72$).

Statistics

Statistical analyses were run using R 2.14.2 (R Development Core Team 2008) and JMPIN 8.0.2 (SAS Institute,

Cary, NC). If necessary, variables were transformed to fulfill model assumptions. To investigate which parts of the reflectance spectrum depend on skin carotenoids, we ran Pearson's correlations between color measurements and skin carotenoid concentrations. Correlations were based on individuals of the CONT, CORT, and β CAR groups ($n = 20$), but not of the XAN group, where treatment affected skin carotenoid concentration (see "Results"). To control for multiple testing here and in all post hoc tests, we adjusted *P* values (P_{adj}) following Benjamini and Hochberg (1995). Moreover, we also estimated the study-wide false discovery rate (*q* values; "qvalue" R function; Storey and Tibshirani 2003) to test for spurious significant *P* values. All significant *P* values remained significant at a *q* value of ≤ 0.05 , and thus only *P* values are reported.

We tested treatment effects on coloration (in vivo experiment) using linear mixed models with restricted maximum likelihood estimation and lizard as random factor. As fixed effects, full models included time (before/after experiment), treatment, molt status, number of ingested larvae, O and W scores, and all two-way interactions be-

Figure 4: Treatment effects on changes in parameters reflecting variation in the wavelength where carotenoids absorb light: violet-blue reflectance (A), absolute carotenoid chroma (B), and hue (C). Pre- and postexperimental means ($\pm$ SE) are plotted. Asterisks indicate significant changes within treatment groups (post hoc contrasts: two asterisks, $P_{\text{adj}} < .01$; one asterisk, $P_{\text{adj}} < .05$).

tween main effects with time and treatment and between O and W scores. Denominator degrees of freedom were computed by dividing residual degrees of freedom into between- and within-subject portions (BETWITHIN option). Higher-order interactions were not considered since they may provide unreliable parameter estimates given group sample sizes. Final models were obtained by backward elimination of nonsignificant interactions ($P > .10$) and verified by re-adding excluded terms one at a time. For simplicity of the presentation, only main effects and significant interactions are reported and discussed. To account for the potential bias in parameter estimates that could result from unbalanced group sample sizes, results were verified using bootstrap methods based on 1,000 rep-

licates (R “pbkrtest” package). Bootstrap tests yielded qualitatively identical results and are not reported.

We also used mixed models to analyze treatment effects on plasma lutein and zeaxanthin concentrations. Because common lizards show only small traces of plasma β -carotene (San-Jose et al. 2012b), we tested whether its presence (rather than its concentration) was time- and treatment-dependent using a generalized linear mixed model with binomially distributed errors and lizard as random factor. Treatment effects on skin and hepatic concentrations of carotenoids and vitamin A were analyzed with ANCOVAs, using type III sums of squares to account for unbalanced sample sizes. Treatment was modeled as main factor and number of ingested larvae and O and W scores as covariates. The assumption of homogeneous within-group regression slopes held for all the ANCOVA models (all treatment-covariate interactions $P > .09$).

Osmolarity treatment effects on coloration (in vitro experiment) were analyzed using mixed models with lizard, skin patch (nested within lizard), and their interactions with n th solution (i.e., number of solution into which tissues were immersed; IO group: $1\times = 1$, $3\times = 4$; CO group: $1\times = 1$, $0\times = 4$) as random and using osmolarity treatment (IO, DO, and CO groups), n th solution,² and their interactions with treatment as fixed effects. The n th solution was standardized prior to model fitting, and the interpretation of osmolarity effects was based on 95% confidence intervals (Quinn and Keough 2002).

Results

Relationships between Skin Carotenoid Concentration and Coloration

Skin β -carotene concentration negatively correlated with violet-blue reflectance ($r = -.65$, $n = 20$, $P_{\text{adj}} = .042$). None of the other correlations between skin concentrations of β -carotene, lutein, or zeaxanthin and coloration were significant (all $P_{\text{adj}} > .20$; table B1, available online).

Effects of Corticosterone and Carotenoid Supplementation on Coloration

The temporal change in parameters reflecting background components significantly differed among treatments (treatment $\times$ time; table 1). The CONT, XAN, and CORT groups showed a significant decrease in all parameters reflecting background components, while no significant change existed in the β CAR group (fig. 2). This indicated that the long-wavelength reflectance peak became smaller and flatter and that coloration faded in these groups but not in the β CAR group (fig. 3). The O score was signifi-

cantly and negatively related to maximum background reflectance (estimate $\pm$ SE = $-.11 \pm .05$; table 1), and a similar but marginally significant correlation existed in background reflectance ($-4.80 \pm 2.4\%$). There were no significant correlations between morph scores and background slope (table 1). Molt-status-affected reflectance parameters related with background components (table 1). During the experiment, premolting lizards showed a significant decrease in background reflectance ($-5.97 \pm 1.55\%$; $t_{31} = 3.86$, $P_{\text{adj}} < .001$) and maximum background reflectance ($-1.17 \pm .29\%$; $t_{31} = 4.38$, $P_{\text{adj}} < .001$). Independent of time, background slope was significantly lower in premolting ($-.17 \pm .05$) than in nonmolting lizards (table 1).

None of the applied treatments affected violet-blue reflectance (table 2; fig. 4A). Absolute carotenoid chroma significantly increased (i.e., coloration became less saturated) in the CONT, XAN, and CORT groups, while no change was observed in the β CAR group (table 2; fig. 4B). Hue significantly increased (became less orange) in the CONT and XAN groups, and it did not change in the β CAR and CORT groups (table 2; fig. 4C). Morph scores were significantly correlated with violet-blue reflectance (O score: $-2.92 \pm 1.32\%$) and absolute carotenoid chroma (W score: $.10 \pm .04$) but not with hue (table 2). Molting induced a significant increase in absolute carotenoid chroma over time ($.30 \pm .07$; $t_{31} = 4.32$, $P_{\text{adj}} < .001$) and hue ($.14 \pm .04^\circ$; $t_{31} = 3.40$, $P_{\text{adj}} = .004$). UV reflectance and the location of the UV peak were not significantly affected by treatment or molt and were not related with morph scores (all $P > .23$).

Effects of Corticosterone and Carotenoid Supplementation on Carotenoid Concentration

At the start of the experiment, treatment groups did not significantly differ in plasma lutein and zeaxanthin concentrations or in the β -carotene presence (all $P > .13$). Treatments significantly affected the change in plasma lutein concentration (time: $F_{1,22} = .23$, $P = .64$; treatment: $F_{3,19} = 6.12$, $P = .004$; treatment $\times$ time: $F_{3,22} = 6.60$, $P = .002$). Plasma lutein concentration significantly increased in the XAN group (post hoc contrast: $7.2 \pm 1.6 \mu\text{g/mL}$, $t_{22} = 3.99$, $P_{\text{adj}} = .006$), while no significant change existed in the CONT, β CAR, and CORT groups (all $P_{\text{adj}} = .42$). There were no significant treatment effects on plasma zeaxanthin concentration or β -carotene presence (all $P > .13$). The number of ingested larvae and morph scores were not significantly related to plasma carotenoids (all $P > .15$).

Treatment groups did not significantly differ in their skin β -carotene concentration (fig. 5A), which was significantly and negatively related with the W score (estimate $\pm$ SE = $-.002 \pm .001 \mu\text{g/mg}$; table 3). The XAN group showed significantly higher lutein skin concentration than the other groups (table 3; fig. 5B), and a nonsignificant tendency existed in zeaxanthin skin concentration (table 3; fig. 5C). Skin lutein and zeaxanthin concentrations increased with the number of ingested larvae (lutein: $.037 \pm .02 \mu\text{g/mg}$; zeaxanthin: $.011 \pm .005 \mu\text{g/mg}$; table 3) and were not correlated with morph scores (table 3).

The hepatic concentration of β -carotene did not significantly differ between treatments (fig. 5D), and it was

Figure 5: Treatment effects on skin and hepatic concentrations ($\mu\text{g/g}$) of β -carotene, lutein, and zeaxanthin (mean $\pm$ SE). Different letters indicate statistically significant (adjusted) contrasts. The asterisk indicates square-root transformation.

Table 3: Results from ANCOVAs on skin and liver concentrations of β -carotene, lutein, and zeaxanthin

Factor	β -carotene			Lutein			Zeaxanthin		
	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>
Skin:									
Treatment	.93	3, 19	.73	4.23	3, 19	.019	2.76	3, 19	.07
<i>W</i> score	4.49	1, 19	.047	<.01	1, 19	.94	.89	1, 19	.35
<i>O</i> score	1.55	1, 19	.23	.98	1, 19	.33	.25	1, 19	.62
Ingested larvae	.47	1, 19	.50	4.56	1, 19	.046	4.94	1, 19	.039
Liver:									
Treatment	2.17	3, 19	.13	3.27	3, 19	.044	3.25	3, 19	.045
<i>W</i> scores	1.01	1, 19	.33	6.53	1, 19	.019	4.84	1, 19	.040
<i>O</i> scores	2.55	1, 19	.13	.15	1, 19	.70	<.01	1, 19	.99
Ingested larvae	.79	1, 19	.38	2.03	1, 19	.17	2.45	1, 19	.13

not correlated with morph scores or the number of ingested larvae (table 3). Treatments significantly differed in the hepatic concentration of lutein and zeaxanthin (table 3). The former was significantly higher in the XAN group than in the other groups (fig. 5E), and the latter was higher in the XAN group than in the β CAR and CORT groups but did not differ from the CONT group (fig. 5D). The hepatic concentration of lutein and zeaxanthin increased with the *W* score (lutein: $9.18 \pm 3.59 \mu\text{g}/\text{mg}$; zeaxanthin: $.789 \pm .36 \mu\text{g}/\text{mg}$) and were not significantly related with the *O* score or the number of ingested larvae (table 3).

The hepatic concentration of vitamin A_1 but not of A_2 significantly differed among treatment groups ($F_{3,19} = 3.31$, $P = .041$; $F_{3,19} = .41$, $P = .74$, respectively). Vitamin A_1 concentration was significantly higher in the β CAR group than in the CONT ($.16 \pm .06 \mu\text{g}/\text{mg}$, $t_{19} = 2.89$, $P_{\text{adj}} = .024$) and CORT groups ($.16 \pm .06 \mu\text{g}/\text{mg}$, $t_{19} = 2.64$, $P_{\text{adj}} = .024$) and tended to be higher than in the XAN group ($.13 \pm .06 \mu\text{g}/\text{mg}$, $t_{19} = 2.07$, $P_{\text{adj}} = .052$). The hepatic vitamin A_1 and vitamin A_2 concentrations were not significantly correlated with morph scores or the number of ingested larvae (all $P > .25$).

Effects of Osmolarity on Coloration

Experimental changes in osmolarity significantly affected the reflectance spectra and all but one color parameter of the biopsies (table 4; fig. B1, available online). Background reflectance significantly decreased in the IO group compared to the CO and DO groups (fig. 6A), whereas the DO group did not differ from the CO group. Maximum background reflectance showed a decrease in the IO group that significantly differed from the CO group at high osmolarity ($3 \times$; fig. 6B; see also fig. B1). The DO and CO group showed no significant differences. Background slope showed a concave change in the DO group and a convex change in the IO group, and no significant change existed

in the control group (table 4, fig. 6B; see also fig. B1). In comparison to the CO group, background slope in the DO group was significantly lower at osmolarities below $1 \times$, while in the IO group background slope was significantly lower only at the highest osmolarity ($3 \times$; fig. 6C). There were significant slope differences among treatments in violet-blue reflectance (table 4), but confidence intervals indicated that none of the osmolarity treatments significantly differed from the CO group (fig. 6D). Absolute carotenoid chroma increased in the IO group and differed from the CO group at highest osmolarity (fig. 6E). Increasing osmolarity led to increasing hue and UV reflectance, in the former differing from the CO and DO groups (fig. 6F), and in the latter differing at the highest osmolarity from the DO but not the CO group (fig. 6G). No significant interaction between treatment and solution number was found for the spectral position of the UV peak.

Discussion

Our results showed that β -carotene intake prevented the chromatic changes observed in control and in xanthophyll- and corticosterone-treated common lizards. In contrast to findings in previous studies (McGraw et al. 2002a, 2004), HPLC analyses revealed that the observed chromatic changes were not due to integumentary deposition of β -carotene. Spectral changes exclusively occurred at long wavelengths (i.e., in background reflectance, maximum background reflectance, and background slope) and not at wavelengths where carotenoids absorb light (i.e., in the violet-blue range; Jacot et al. 2010). These results therefore show that integumentary components providing carotenoids with a reflective background (i.e., iridophores or melanophores; Grether et al. 2004) but not carotenoids accounted for the observed chromatic changes.

Melanophores may induce chromatic changes when lo-

Table 4: Osmolarity treatment effects on ventral coloration

	<i>F</i>	df ^a	<i>P</i>
Background reflectance ($R_{575-700}$):			
<i>n</i> th solution	100.77	1, 5.71	<.001
Treatment	30.25	2, 20.18	<.001
<i>n</i> th solution $\times$ treatment	141.96	2, 19.1	<.001
Maximum background reflectance ($R_{\max}$):			
<i>n</i> th solution	11.98	1, 6.97	.011
<i>n</i> th solution ²	20.60	1, 10.25	.001
Treatment	.17	2, 25.71	.847
<i>n</i> th solution $\times$ treatment	45.09	2, 19.99	<.001
<i>n</i> th solution ² $\times$ treatment	27.25	2, 10.23	<.001
Background slope ($ b_{\max\text{neg}} $):			
<i>n</i> th solution	21.32	1, 7.51	.002
<i>n</i> th solution ²	<.01	1, 9.96	.938
Treatment	52.26	2, 46.94	<.001
<i>n</i> th solution $\times$ treatment	29.82	2, 21.26	<.001
<i>n</i> th solution ² $\times$ treatment	62.81	2, 47.74	<.001
Violet-blue reflectance ($R_{400-500}$):			
<i>n</i> th solution	24.40	1, 11.03	<.001
Treatment	1.96	2, 20.91	.166
<i>n</i> th solution $\times$ treatment	6.06	2, 22.24	.008
Absolute carotenoid chroma ($R_{400-500}/R_{575-700}$):			
<i>n</i> th solution	112.75	1, 9.22	<.001
Treatment	9.66	2, 21.09	.001
<i>n</i> th solution $\times$ treatment	35.19	2, 20.56	<.001
Hue:			
<i>n</i> th solution	35.67	1, 10.93	<.001
Treatment	40.62	2, 21.63	<.001
<i>n</i> th solution $\times$ treatment	50.21	2, 22.1	<.001
UV reflectance ($R_{300-400}$):			
<i>n</i> th solution	41.59	1, 10.16	<.001
Treatment	8.16	2, 20.34	.003
<i>n</i> th solution $\times$ treatment	14.02	2, 21.03	<.001
UV peak ($\lambda_{\text{UV max}}$):			
<i>n</i> th solution	15.31	1, 5.95	.008
Treatment	4.55	2, 19.94	.024
<i>n</i> th solution $\times$ treatment	2.33	2, 15.13	.131

Note: Only fixed effects from linear mixed models are given.

^a Kenward-Rogers adjusted denominator degrees of freedom.

cated below xanthophores, erythrophores, and iridophores by absorbing the light that is not effectively absorbed or reflected by them (see fig. 13 in Grether et al. 2004). Thus, decreased melanophore absorbance (through decreased melanin content or dispersion) induces an increase in reflectance that is more apparent at longer wavelengths, leading to a flatter background slope and to zero or a positive change in maximum reflectance. In contrast, increased melanophore absorbance leads to a decrease in reflectance and, hence, to an increase in background slope and to zero or a negative change in maximum reflectance (Grether et al. 2004). In the in vivo experiment, both maximum background reflectance and background slope decreased, which is not congruent with chromatic changes expected from

melanophores and discards that they accounted for the observed chromatic changes.

The chromatic changes were, however, in line with predictions from changes in iridophore reflectance (Grether et al. 2004). The in vitro manipulation of skin osmolarity and crystal spacing showed that iridophores affect the relative contribution of short and long wavelengths by modifying the height and shape of the long-wavelength reflectance peak. These results are congruent with the changes observed in vivo. More specifically, reduced background reflectance, maximum background reflectance, and maximum background slope observed in vivo are in line with reduced background reflectance, maximum background reflectance, and maximum background slope observed in

Figure 6: Effects of osmolarity on coloration. Fitted lines for each treatment group and 95% confidence intervals are given. Different lines indicate the three osmolarity treatment groups. Abscissas reflect subsequent solutions and the osmolarity for each solution and treatment. The asterisk indicates log transformation.

high-osmolarity solutions ($3\times$; fig. 6B, 6C) and, hence, with decreased crystal spacing. This suggests that changes in crystal spacing and, hence, iridophores accounted for the chromatic changes observed in vivo.

Our findings show that iridophores are flexible integumentary components that respond to environmental

factors (e.g., dietary availability of β -carotene), which suggests that iridophores may account for condition-dependent signaling in *Lacerta vivipara*. It has been suggested that iridophores are under hormonal and neural control, both inducing contractile activity of the actin filaments that links purine crystals (Rohrlich and Porter 1972; Rohr-

lich 1974). As suggested for the fish neon tetra (*Parachanna innesi*; Lythgoe and Shan 1982), the reflectance changes observed in the CONT, XAN, and CORT groups could have been a response to handling stress. In fact, despite the spectral changes observed in the CORT lizards, we observed that there was no hue change within this group in contrast with the change toward less orange hue values observed in the CONT and XAN lizards (fig. 4C). This finding is in line with previous studies in *L. vivipara* showing a positive effect of corticosterone on ventral hue but not in other color parameters (Fitze et al. 2009; Cote et al. 2010) and supports that stress and stress-related hormones may modulate iridophore-based reflectance in *L. vivipara*. The lack of change in β CAR indicates that β -carotene may have prevented the negative effects of handling stress. Direct effects of β -carotene on iridophores are unlikely, given the absence of treatment differences in β -carotene content. However, β -carotene supplementation led to increased vitamin A₁ concentrations, suggesting that supplemented β -carotene was preferentially used for vitamin A₁ synthesis within enterocytes (Simpson 1983) and, thus, that the β -carotene effect on coloration was mediated by vitamin A₁. Previous studies have shown that vitamin A₁ is related with ontogenetic changes in fish chromatophores (Miwa and Yamano 1999). Moreover, vitamin A₁ depends on the dietary availability of retinol and carotenoid precursors and is implicated in different biological functions (immune response, development, growth, and fertility; Olson 1989), which supports a potential condition-dependent linkage between coloration and vitamin A₁. However, future studies are needed to completely understand the influence of vitamin A₁ in iridophores.

In contrast to iridophore-related reflectance, carotenoid-related reflectance may show no environmental flexibility, which is in contrast with the general belief that carotenoids mainly determine the environmental component of carotenoid-based signals (Badyaev and Hill 2000). To date, none of the studies manipulating dietary carotenoid intake in lizards has found significant effects on carotenoid-related reflectance (Olsson et al. 2008; Fitze et al. 2009; Steffen et al. 2010), suggesting that mechanisms controlling carotenoid deposition may be different in lizards than in birds and fishes. Our results further demonstrated that xanthophyll intake resulted in no color improvement in relation to the CONT lizards, despite that it enhanced skin, blood, and hepatic xanthophyll concentrations. These findings suggest that factors different from availability may control carotenoid or, at least, xanthophyll incorporation into chromatophores. Additional support comes from the observed correlations between skin β -carotene concentration and *W* score and between violet-blue reflectance and *O* score, which suggest that orange and yellow males incorporate more carotenoids into the

integument than white males. The linkage between integumentary carotenoids and carotenoid-based reflectance with the genetically based morphs may support a tight genetic determination of carotenoid incorporation and explain the absence of environmental effects (Sinervo and Zamudio 2001; Svensson et al. 2001; Huyghe et al. 2010). Different components in the integument of the common lizard may therefore convey different information about individual phenotype; carotenoids may reflect the genetic context, whereas iridophores may reflect condition-dependent variation (e.g., vitamin A status). Thus, ventral, carotenoid-based coloration of common lizards may be a multiple-message signal, where a single signal, perceived coloration, provides different messages (Grether et al. 2004; Vercken et al. 2008).

In conclusion, our study shows that environmentally induced chromatic variation in carotenoid-based ornaments does not necessarily result from differences in integumentary carotenoid content but may be explained by changes in iridophores. To our knowledge, this is the first experimental proof that integumentary components other than carotenoids account for condition-dependent chromatic variation of carotenoid-based ornaments. Therefore, our results suggest that carotenoid-based ornaments do not always reflect differences in carotenoid deposition and, thus, in individual capacity of dealing with the costs of carotenoid deposition. We provide evidence that carotenoid-based coloration may function as multiple-message signals, where different integumentary components reveal different aspects of individual phenotype, supporting that carotenoid-based signals are more complexly determined than previously thought (Kemp et al. 2011). Our findings indicate that chromatic variation in carotenoid-based coloration needs careful interpretation in species with integumentary iridophores (i.e., fish and amphibians; Bagnara 1966; Bagnara et al. 1968) as well as in bird species where keratin- and collagen-derived reflective structures could contribute to chromatic variation (Keyser and Hill 1999, 2000; McGraw et al. 2002b; Prum and Torres 2003; Prum 2006). Our study, therefore, highlights that special caution is required when deriving evolutionary theories about signal content from chromatic variation and suggests that only detailed studies on the integumentary components responsible for observed variation in reflectance spectra may allow the derivation of accurate conclusions.

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