

Inter-population variation of carotenoids in Galápagos land iguanas (*Conolophus subcristatus*)

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Abstract

Carotenoids have received much attention from biologists because of their ecological and evolutionary implications in vertebrate biology. We sampled Galápagos land iguanas (*Conolophus subcristatus*) to investigate the types and levels of blood carotenoids and the possible factors affecting inter-population variation. Blood samples were collected from populations from three islands within the species natural range (Santa Cruz, Isabela, and Fernandina) and one translocated population (Venecia). Lutein and zeaxanthin were the predominant carotenoids found in the serum. In addition, two metabolically modified carotenoids (anhydrolutein and 3'-dehydrolutein) were also identified. Differences in the carotenoid types were not related to sex or locality. Instead, carotenoid concentration varied across the localities, it was higher in females, and it was positively correlated to an index of body condition. Our results suggest a possible sex-related physiological role of xanthophylls in land iguanas. The variation in the overall carotenoid concentration between populations seems to be related to the differences in local abundance and type of food within and between islands.

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1. Introduction

Carotenoids are a group of natural fat-soluble pigments synthesized by plants, algae, and photosynthetic bacteria, where they are primarily involved in photosynthetic processes (e.g. Frank and Cogdell, 1996). Animals are incapable of synthesizing carotenoids and can only incorporate them through their diet. For this reason, these

substances are considered as limited compounds. In animals, carotenoids carry out different functions, such as providing bright colouration and they serve as antioxidants and immunostimulants (Ong and Tee, 1992; Britton et al., 1995). More than 600 carotenoids have been chemically characterized and many diverse health-related biological functions and actions have been ascribed to these compounds (e.g., Olson and Owens, 1998; Møller et al., 2000; Blount et al., 2003; Faivre et al., 2003).

Recent research has shown that variation among populations in diet may covary with differences in carotenoid

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blood concentrations, and that this variation may affect the colour expression in skin and plumage (Grether et al., 1999; Negro et al., 2000). Types and levels of carotenoids may also be influenced by other factors. For example, in the American kestrel (*Falco sparverius*) the carotenoid-based colour of the skin and the carotenoid concentration of the plasma vary seasonally, suggesting a potential trade-off between the use of these compounds in colour expression and as physiological mediators such as immunostimulation and antioxidant activity (Negro et al., 1998).

The multiple factors that affect the metabolic pathways of carotenoids, in terms of their absorption, breakdown, transport, and storage, can vary between species (Yeum and Russell, 2002). For example, some bird species can accumulate carotenoids chemically unchanged (e.g. the Eurasian kestrel *F. tinnunculus*: Casagrande et al., in press) whereas others can store them in a metabolically modified form in specific tissues (e.g., some estrildid finches: McGraw and Schuetz, 2004).

Up to now, most studies of carotenoids have examined fish, birds, and mammals (Goodwin, 1984; Olson and Owens, 1998; Møller et al., 2000), whereas reptiles, especially herbivorous reptiles, have received little attention (see Czczuga, 1980; Raila et al., 2002). Of the more than 7800 species of squamate reptiles (lizards, snakes and their relatives) less than 2% are herbivorous (Espinoza et al., 2004). Herbivorous species are expected to obtain more carotenoids per unit mass of food than omnivores and carnivores, as plants are the primary providers of these compounds (Goodwin, 1980, 1984).

In this work we investigate the type and the blood concentration of carotenoids in several populations of *Conolophus subcristatus*, one of two species of terrestrial iguanas endemic in the central and western Galápagos Islands (Snell et al., 1984). Adult land iguanas from Galápagos are generally herbivorous, feeding on different vegetable species. In particular, they feed primarily on leaves and flowers of shrub plants such as *Lantana* and *Cordia*; the pads, flowers and fruits of *Opuntia* cactus are also important in their diet, with grass, leaves, and twigs being of less significance (Carpenter, 1969). In addition, in a previous work, the diet was found to vary according to both sampling site and season (Christian et al., 1984). Here, we discuss the inter-population variation of carotenoids as a possible correlate of different food sources among the islands.

Currently, *C. subcristatus* is listed as a threatened species by the World Conservation Union (Baillie and Groombridge, 1996). Galápagos land iguanas have in fact experienced various disturbances by direct and indirect effects of human activity so that several populations became dramatically reduced in size or were extirpated (Snell et al., 1984; Mattison, 1989; Cogger and Zweifel, 1998). For this reason, besides the general lack of data on carotenoids in reptiles, another point of interest for the investigation of the type and levels of carotenoids in *Conolophus* is related to

the ability of these substances to prevent infections and diseases (Kim et al., 2000; Ribaya-Mercado and Blumberg, 2004), which is in general a desirable attribute for an endangered species.

2. Materials and methods

2.1. Sample collection and study area

Land iguanas (*C. subcristatus*) were sampled within their natural range (Santa Cruz, Fernandina, and Isabela) in addition to a single translocated population (Venecia) (Snell et al., 1984). Samples from Santa Cruz and Venecia were collected in December 2003. Eleven males and seven females were sampled in Santa Cruz in the areas of Cerro Dragon and Cerro Montura. Sixteen males and 15 females were sampled in Venecia, a small islet proximate to Cerro Dragon. During a second trip (July 2004), blood samples were collected from iguanas of Isabela and Fernandina islands. We obtained samples from two localities at Isabela: Bahía Cartago (43 males and 29 females) and Bahía Urbina (22 males and 11 females). Eleven males and 14 females were sampled at Cueva Norte, Fernandina. All samples were collected and exported with the approval of the Galápagos National Park (Ecuador) and were included in export–import CITES permits granted to Gabriele Gentile.

Blood (1–2 mL) was drawn from either the brachial or caudal veins. Blood samples were either immediately centrifuged after collection to obtain serum, or they were taken using heparinized syringes and centrifuged later to obtain plasma. To test for potential differences between carotenoid concentrations as measured in serum or plasma, 10 blood samples were collected and processed following both procedures. Morphological data collected from each individual included snout-vent length (cm) and wet mass (g). Gender was determined by visual inspection of the animal (e.g. the presence of hemipenis). Juveniles were not considered in this study.

An index of body condition was computed as $(\text{body mass}/\text{snout-vent length}^3) \times 10^6$ (the ratio was multiplied for 10^6 in order to reduce the number of decimals). This index has been shown to effectively describe the physical condition of marine iguanas (e.g. Romero and Wikelski, 2001), with specimens at risk of starvation having indices of less than 25 and those of maximum fatness having indices of 60.

2.2. Measurement of carotenoids

For the isolation of carotenoids the same protocol developed for egg yolk carotenoids extraction was used (Saino et al., 2002). The identification of carotenoids was carried out by High Performance Liquid Chromatography (HPLC) and mass spectra analysis following the protocol of Stradi et al. (1995) using samples collected in Santa Cruz

($n=6$, 3 males and 3 females) and Venecia ($n=6$, 3 males and 3 females). The quantification of the total carotenoids content was calculated by means of a Beckman DU 7400 spectrophotometer. The serum or plasma (100–200 μL) was diluted with absolute methanol (1:5) and the flocculent proteins were precipitated by centrifugation at $12000 \times g$ for 5 min. Carotenoids concentration was estimated as micrograms per milliliter of serum or plasma using the standard absorbance curve of lutein (alpha-carotene-3,3'-diol; Sigma-Aldrich), which represented the predominant carotenoid in land iguanas.

2.3. Statistical analyses

The effects of both sampling site and sex on carotenoid concentration were analysed with an Ancova model, using the body condition index as a covariate. The interaction between sampling site and sex tested for geographical covariation in sexual variation of carotenoid concentration. Post hoc comparisons were performed using the Tukey's Honest Significant Difference test (Tukey, 1953; Zar, 1998) for unequal sample sizes, to test for specific between-group differences. Since raw data of carotenoid concentration did not meet the normality assumption, we transformed them by using the square-root transformation. The heteroscedasticity assumption was not met by carotenoid concentration even after the square-root transformation. However, the test is robust for heteroscedasticity if results are statistically significant (Ito, 1980). The analysis of data by non-parametric tests (Mann–Whitney, Kruskal–Wallis, sequential Bonferroni correction) gave similar results (data not shown). However, parametric tests control

better for possible effects of non-measured confounding variables and by considering at the same time all factors and variables involved provide a more comprehensive scenario of the effects (e.g. Sokal and Rohlf, 1995). Data are presented as mean in the untransformed scale \pm standard error. Confidence limits ($\pm 95\%$) are also shown as suggested by Sokal and Rohlf (1995) for the transformed variables. The repeatability (r) of carotenoid concentration between serum and plasma was calculated according to Lessells and Boag (1987); r is the intraclass correlation coefficient (Sokal and Rohlf, 1995) obtained by a one-way Anova with carotenoid concentration as the response variable. All analyses were performed using the STATISTICA package (Version 5.1, StatSoft, Padova, Italy).

3. Results

The HPLC analysis revealed that polar xanthophylls were the only carotenoids occurring in the serum of land iguanas (Fig. 1). In particular, lutein (II) was the most abundant, but zeaxanthin (III) was also present (see Table 1 for values on total carotenoids concentration). Two metabolically derived carotenoids were also identified, namely anhydrolutein (IV) and 3'-dehydrolutein (I). The same carotenoid pattern was found in Santa Cruz and Venecia, in both sexes.

The carotenoid concentrations measured with the two procedures in serum and plasma were highly repeatable ($r=0.82$, $p=0.002$). Therefore, the subsequent analyses were carried out considering all the samples collected.

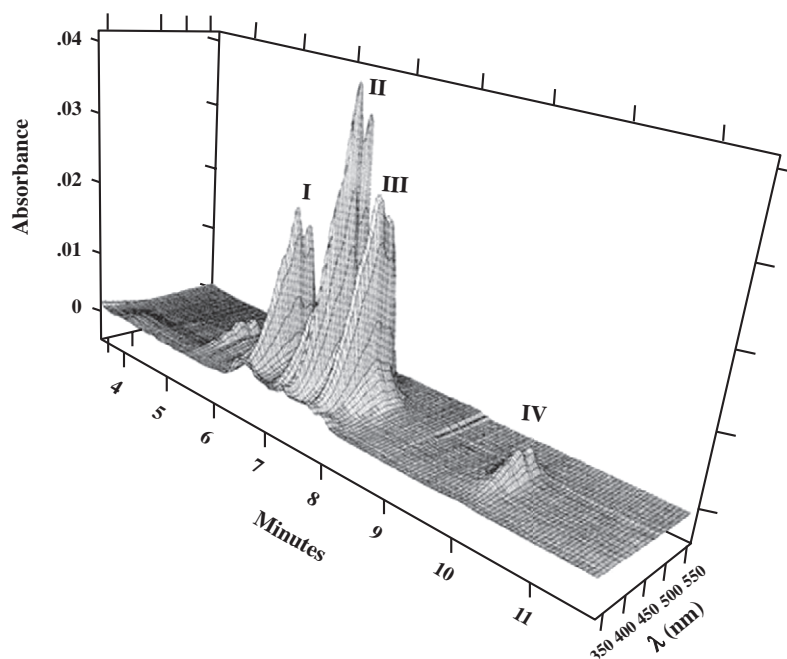


Fig. 1. Three-dimensional chromatogram showing the carotenoid pattern of land iguanas: (I) 3'-dehydrolutein; (II) lutein; (III) zeaxanthin; (IV) anhydrolutein.

Table 1

Total carotenoids concentration and body condition index of land iguanas (m=male, f=female; SC=Santa Cruz, VC=Venecia, CAR=Bahía Cartago, URB=Bahía Urbina, FER=Fernandina)

Area	Sex	Total carotenoids concentration			Body condition index			
		Mean±s.e.	−95%	+95%	Mean±s.e.	−95%	+95%	N
SC	m	4.05±1.19	1.39	6.70	44.41±5.10	33.06	55.76	11
	f	6.48±1.63	2.50	10.46	30.10±4.69	18.62	41.58	7
VC	m	4.96±0.89	3.07	6.84	38.93±0.86	37.10	40.76	16
	f	6.40±1.21	3.80	9.00	38.89±1.12	36.48	41.29	15
CAR	m	5.03±0.30	4.42	5.63	55.08±0.77	53.53	56.64	43
	f	8.14±0.47	7.18	9.11	54.31±1.44	51.36	57.27	29
URB	m	7.76±0.75	6.20	9.33	60.21±1.15	57.82	62.61	22
	f	16.14±1.02	13.87	18.41	57.52±1.98	53.12	61.93	11
FER	m	6.82±1.04	4.52	9.13	50.41±1.20	47.74	53.08	11
	f	9.95±1.32	7.11	12.80	47.24±1.55	43.89	50.59	14
Total		7.15±0.32	6.51	7.79	50.41±0.80	48.83	51.98	179

Xanthophyll concentration increased with increasing body condition ($F_{1,168}=12.44$, $p<0.001$; Fig. 2) and it was significantly higher in females than in males (females = $9.14\pm0.56 \mu\text{g mL}^{-1}$, males = $5.69\pm0.32 \mu\text{g mL}^{-1}$; $F_{1,168}=39.85$, $p<0.001$). The effect of the sampling site on the xanthophyll concentration was significant ($F_{4,168}=8.31$, $p<0.001$), being higher in animals from Bahía Urbina (Isabela) than in those from other sites (Fig. 3). A significant interaction between sampling site and sex was also found ($F_{4,168}=2.51$, $p=0.044$). The pair-wise comparisons with Tukey's HSD test showed that significant sex-related differences in total carotenoid concentration occurred only in Isabela (Bahía Cartago, males = $5.03\pm0.30 \mu\text{g mL}^{-1}$, females = $8.14\pm0.47 \mu\text{g mL}^{-1}$, $p=0.017$; Bahía Urbina, males = $7.76\pm0.75 \mu\text{g mL}^{-1}$, females = $16.14\pm1.02 \mu\text{g mL}^{-1}$, $p=0.0003$). Among males, the concentration of total carotenoids was significantly higher in Bahía Cartago than in Santa Cruz ($4.05\pm1.19 \mu\text{g mL}^{-1}$; $p=0.047$); similarly,

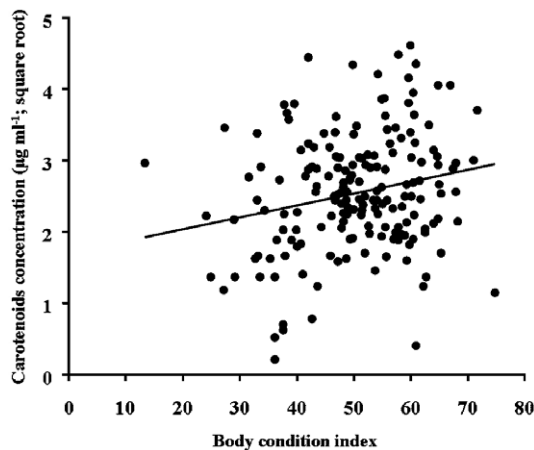


Fig. 2. Relationship between body condition and carotenoids concentration ($F_{1,177}=8.57$, $R^2=0.05$, $p<0.01$, $y=0.017x+1.70$; s.e. of the estimate=0.81).

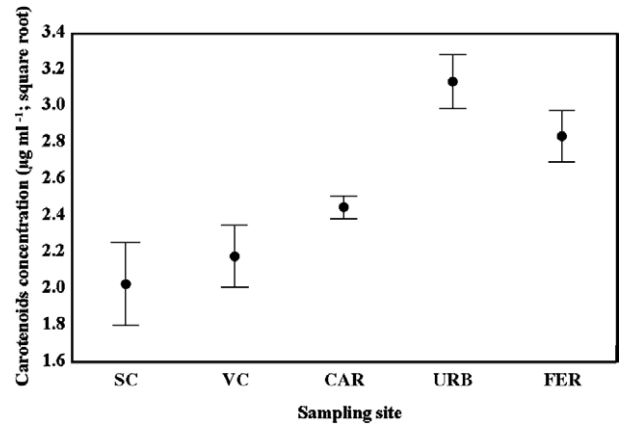


Fig. 3. Inter-sampling site differences in carotenoids concentration. Data are shown as mean±s.e.

the females of Bahía Urbina showed the highest concentration when compared with the females of the other sites (always $p<0.01$).

4. Discussion

4.1. Carotenoid patterns and physiological implications

Our study showed that land iguanas accumulate carotenoids and, in particular, polar xanthophylls. The two dietary carotenoids, lutein and zeaxanthin, are known to have important properties as antioxidants and stimulators of both cell-mediated and humoral immune responses in vertebrates (e.g. Mortensen et al., 1997; Kim et al., 2000; Blount et al., 2003). Lutein and zeaxanthin were found together with two metabolically modified xanthophylls (anhydrolutein and 3'-dehydrolutein), the relative abundances of which were different among the samples analysed by HPLC. Because of the antioxidant function of carotenoids, we are currently investigating whether such differences in abundance may be correlated to differences in the level of oxidative stress.

To date, no evidence of anhydrolutein and 3'-dehydrolutein was found in previous studies on carotenoids in reptiles (see e.g. *Lacerta agilis*, *Lacerta vivipara*, and *Anguis fragilis* in Czezug, 1980; *Iguana iguana* in Raila et al., 2002). Anhydrolutein and dehydrolutein are known to derive from lutein and zeaxanthin, respectively, and are rarely reported in both plants and animals (Barua and Das, 1975). Anhydrolutein is a dehydration product of dietary lutein in humans (Khachik et al., 1992, 1995) and in some birds, such as the estrildid zebra finch, *Taeniopygia guttata* (McGraw et al., 2002; McGraw and Schuetz, 2004). In this species, the analysis of the digestive system failed to show the formation of anhydrolutein in the stomach or its presence in the crop, while only trace amounts were detected in the gizzard (McGraw et al., 2002); substantial levels of anhydrolutein were found only in the duodenum of these birds. The authors suggested that the liver may also be

a site of anhydrolutein synthesis in zebra finches. The chemical formation of the 3'-dehydrolutein has been shown to occur in chickens from zeaxanthin by means of pigment radiolabel experiments (Schiedt, 1998). This represents an uncommon yellow carotenoid that has been found also in the feathers of some bird species (e.g. Stradi et al., 1996; McGraw and Schuetz, 2004).

Different transport mechanisms discriminating among the various carotenoids are known to be present at the same time in the intestine of adult birds (Parker, 1996) as well as in the avian embryo, this latter being able to discriminate among different carotenoids during their distribution from the yolk to the developing tissues (e.g. Surai et al., 2001). Furthermore, many birds select almost exclusively xanthophylls at the intestinal level (Goodwin, 1984; Brush, 1990). It is conceivable that reptiles and birds may share a similar absorption system for carotenoids at the intestinal level, as suggested by Raila et al. (2002).

4.2. Effects of sampling site and sex

The blood carotenoid concentration in land iguanas varied geographically and sexually. Our results suggest that geographic variation is related mostly to large-scale between-island effects, even though smaller scale within-island, or local conditions, also have a significant influence. In fact, in Isabela, where carotenoids concentrations were the highest, samples from two populations living in two apparently isolated areas (Bahía Cartago and Bahía Urbina) showed significant differences. Bahía Cartago is a small hill in the eastern side of Isabela, rising from a wide lava field. Instead, individuals sampled from Bahía Urbina were all collected in a sandy and vegetated area in the western coast of the same island. It seems plausible that the differences in carotenoid levels reflect the differences in local abundance and type between the areas. In this context, it is important to note that different parts of the same plant species, such as fruits and leaves, can provide different quantities of xanthophylls (e.g. Castenmiller and West, 1998).

The positive relationship between carotenoid concentration and body condition also suggests a role of food availability in determining the variation in carotenoid concentration in land iguanas. Food shortage has been shown to determine a decrease in body condition and an increase in hormonal stress in marine iguanas (*Amblyrhynchus cristatus*, Romero and Wikelski, 2001). Among-sampling site differences in food abundance can be expected due to the sampling periods. In fact, the samples from Santa Cruz and Venecia were collected at the end of the dry season while those from Isabela and Fernandina were taken just after the end of the rainy season (see Snell et al., 1984 for data on seasonality). Therefore, the higher xanthophyll content measured in land iguanas from Isabela and Fernandina could reflect a seasonal increase of vegetation abundance that occurs after rains. Populations of land iguanas distinct from those sampled in this study have

shown large differences in population-level energetic patterns, presumably based on density and food availability (Snell and Christian, 1985).

In birds with carotenoid-based colours, carotenoid concentration is generally higher in males than in females (in the plumage, e.g. Stradi et al., 1995, 1997; in both blood and skin, e.g. Negro et al., 1998). Here, the blood carotenoid levels did not show such a relationship, on the contrary, our data indicate that male iguanas exhibit lower xanthophyll concentrations than females, although only in Isabela was this difference significant. Further investigation is needed to clarify whether this pattern, if confirmed, may indicate a sex-related physiological role of xanthophylls.

Our work is the first study on carotenoids in *Conolophus*. Results here presented are preliminary and some questions remain open. For example, we are aware of the importance of measuring skin carotenoids to determine whether carotenoids are responsible for the yellow-brown skin pigmentation in *Conolophus*. However, because of the invasiveness of this procedure in this strictly protected species we were unable to include these measures in this preliminary research. Because the occurrence of carotenoids in the skin of lizards has been provided by Czeuczuga (1980), who reported high contents of different xanthophylls in the skin of three lacertid lizards, it is conceivable that an involvement of carotenoids in skin colours can also occur in *Conolophus*. We hope to address this issue in the future, when skin samples from dead individuals become available.

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