Dewlap Color Variation Based on Pterin and Carotenoid Pigments in Three Subspecies of *Anolis jubar* of the Cuban Southern Coast

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Dewlap coloration of anoles is diverse and both carotenoid and pterin pigments play a role in generating this color variation. In the present study, we examined the carotenoid and pterin concentrations responsible for generating color variability in the proximal, central, and marginal dewlap sections in three subspecies of *Anolis jubar* of the Cuban southern coast (*A. j. oriens, A. j. maisiensis,* and *A. j. albertschwartzi*). With the exception of pterins—likely drosopterins—(entirely absent in *A. j. albertschwartzi* and in the scales proximal to the dewlap base in the three subspecies), the dewlap colors were produced by the combination of the two pigment classes. Carotenoid levels differed by section in *A. j. oriens* and *A. j. maisiensis*, being highest in the central section of dewlap. *Anolis j. oriens* had higher pterin concentration than *A. j. maisiensis* in both central and marginal sections. Carotenoid concentrations were significantly higher than pterin concentrations, excepting the central section of *A. j. oriens*, in which no significant differences were found between pigments. Our findings demonstrate subspecies-level variation in dewlap pigmentation in Cuban *A. jubar* and have implications for cellular mechanisms responsible for phenotypic variation.

THE bold colors that animals display have attracted the attention of biologists for over 100 years (Darwin, 1871; Wallace, 1889; Poulton, 1890). Recently, however, it has become clear that an understanding of the function and evolution of these bold colors can only be achieved through an understanding of their mechanistic bases (Hill and McGraw, 2006). In general, the mechanistic basis to color generation relies on interactions between chemical pigments (and their light-absorbing properties) and structural light-reflecting properties of the cells and tissues in which the pigments reside. Two of the pigment classes commonly found in animal integument are carotenoids and pterins. Carotenoids are well known in physiology, behavior, and evolution studies for their roles in visual communication as condition-dependent signals (Kodric-Brown and Brown, 1984; Hill, 1991; Lozano, 1994). Pterins are much less known but may also serve a signaling role in visual communication (McGraw, 2006a).

Adult males in many species of iguanian lizards exhibit colorful pigmented dewlaps that have evolved exclusively for signaling (Cooper and Greenberg, 1992; Macedonia et al., 2003). The dewlaps of male *Anolis* lizards are among the most elaborate color traits in reptiles, and the color of a dewlap may carry information about species identity and sex (Sigmund, 1983; Losos, 2009). These dewlaps are most commonly red, orange, or yellow in color (Nicholson et al., 2007) and these colors have been shown to be derived from pterins (Ortiz et al., 1962; Ortiz and Williams-Ashman, 1963; Ortiz and Maldonado, 1966; Steffen and McGraw, 2007) and carotenoids (Macedonia et al., 2000; Steffen and McGraw, 2007). How these pigment classes interact to generate intraspecific color variation has rarely been tested, especially in reptiles (but see Steffen and McGraw, 2009). Pterin pigments are hydrophilic compounds made up of nitrogen rings that are synthesized in purine salvage pathways (Brown, 1985; McGraw, 2006a). On the other hand, carotenoids (lipid-soluble) are composed of hydrocarbon chains containing terminal carbon rings and constitute the largest group of natural pigments (McGraw, 2006b). Unlike pterins, animals cannot synthesize carotenoids and must obtain them from the diet (Olson and Owens, 1988; McGraw et al., 2005). Because both pigment classes can confer a range of red-yellow hues to dewlaps of *Anolis*, their relative importance in creating these color patterns is not always evident. Moreover, these pigment classes differ in the way they are produced or obtained and may differ in the types of physiological advantage they confer (McGraw, 2006a, 2006b). Therefore, the determination of the pigment basis of dewlap color variability is important to better understand their biochemical, physiological, and evolutionary relevance.

The pigment identities responsible for generating color in the dewlaps of a few Caribbean anoles are known. Pteridines have been identified in the dewlap and body of Puerto Rican lizards (Ortiz and Maldonado, 1966). Carotenoids as well as pteridines have been identified in the dewlap and body of Jamaican *Anolis* lizards, from the ''*grahami* series'' (Macedonia et al., 2000). Additionally, Steffen and McGraw (2007) identified and quantified the carotenoid and pterin content in two species of *Norops* (beta-*Anolis*), one of which is *Norops* (*Anolis*) *sagrei* from Florida (historically found in Cuba). However, no work has been done on dewlap pigment profiles of anoles currently endemic to Cuba.

In the present study, we analyze and compare the yellow-orange-red dewlap color variability in three subspecies of *Anolis jubar* of Cuban southern coast (*A. j. oriens, A. j. maisiensis,* and *A. j. albertschwartzi*; Fig. 1) based on the determination of pterin and carotenoid pigments. Here we test the idea that differences in three subspecies' dewlap colors are the result of differences in carotenoid and pterin

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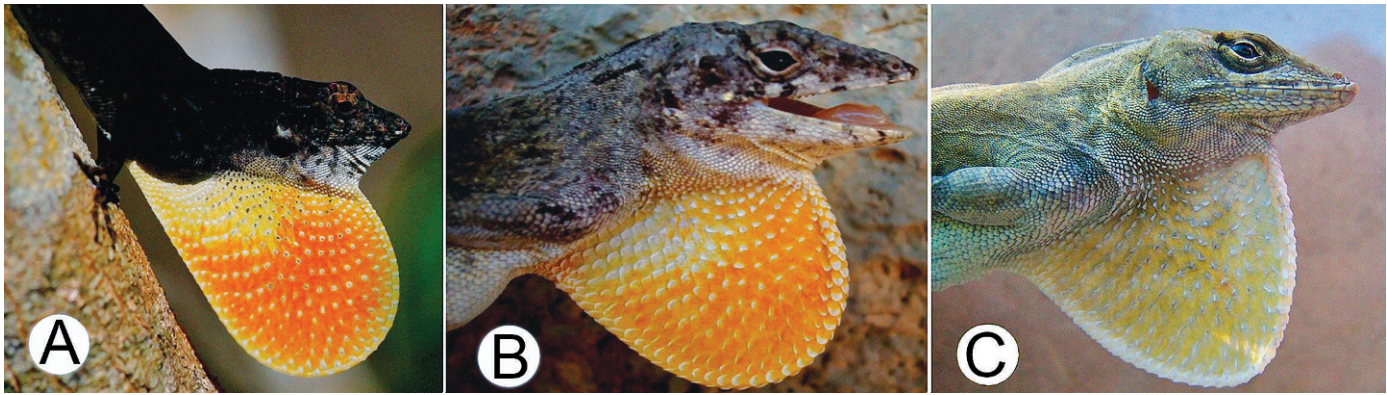[3] Institute of Ecology and Systematic (IES), Carretera Varona, km 3.5, Boyeros, A. P.8029, C.P. 10800, La Habana, Cuba; E-mail: zoologia.ies@ama.cu.
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Fig. 1. (A) *Anolis jubar oriens* collected at Siboney-Juticí Ecological Reserve (Siboney, Santiago de Cuba). (B) *A. j. maisiensis* collected at Maisí Ecological Reserve (Punta de Maisí, Guantánamo). (C) *A. j. albertschwartzi* collected at Baitiquiri Ecological Reserve (San Antonio del Sur, Guantánamo).

concentrations. This paper provides the first quantitative comparison of subspecies-level variation in dewlap pigmentation of anoles. The results have implications for future systematic and evolutionary investigations.

MATERIALS AND METHODS

Study subspecies and populations.—Five male lizards from each subspecies were collected with permission and licenses from the Ministry of Science, Technology and Environment (CITMA) authorities at three Ecological Reserves (ERs): *A. j. oriens* were collected at Siboney-Juticí ER (Siboney, Santiago de Cuba), *A. j. maisiensis* were collected at Maisí ER (Punta de Maisí, Guantánamo), and *A. j. albertschwartzi* were collected at Baitiquiri ER (San Antonio del Sur, Guantánamo). The dominant vegetation and habitat in the Siboney-Juticí and Maisí ERs is coastal xerophytic scrub, while the vegetation and habitat for Baitiquiri ER is a microphyllous evergreen forest. Five adult males of each subspecies were collected and transported live to the Morris Lab (Department of Biology, University of Oriente, Santiago de Cuba) and sacrificed the following day.

Dewlap pigment analyses.—Dewlap tissue was removed from lizards as described by Macedonia et al. (2000). The animals were fully anesthetized with chloroform and sacrificed. The dewlap skin was excised from the body, and the hyoid cartilage underlying the skin was removed with forceps. Samples were taken from three different sections of the dewlap tissue: (i) the scales below the throat and proximal to the base of dewlap, (ii) the central section, and (iii) the marginal section (scales along the dewlap margin). The tissue was rinsed with water to rid samples of spilled blood that may contaminate skin samples. These tissues (about 5 mg) were initially stored in Eppendorf tubes containing 70% ethanol which were placed in the dark in a $-40°C$ freezer to prevent pigments from oxidizing or going into solution with alcohol. Less than one week later, the tubes containing the pigments were moved into a 4°C refrigerator to thaw and prepared for pigment extraction.

The tissues from each dewlap fraction were homogenized with a mixer mill and the pigments were extracted in hexane. Skin pteridines were extracted from the carotenoid-stripped tissue with 30% acidified ethanol (with HCl to pH 2) overnight. Pigment quantification was performed by absorbance spectrophotometry (Grether et al., 2001) on an Ultrospec III equipment (Pharmacia-LKB, Sweden). The

spectrophotometric scans on each solvent fraction recorded from 400 to 600 nm provided λ_{max} values for calculating concentrations. The λ_{max} values also provided confirmation that the pigments in the solvent fractions were in fact pterins (drosopterins in positive samples) and carotenoids, likely xanthophylls (Raila et al., 2002), as evidenced in other anoline lizards. Carotenoid concentration was determined from absorbance of extracts at the peak of absorption (≈455 nm) in hexane using an extinction coefficient $E_{1cm}^{1\%}$ of 2,350 (Britton, 1985). Colored pterins (red drosopterins) were quantified at the peak of absorption (478–495 nm) using an extinction coefficient $E_{1cm}^{1\%}$ of 10,000 (Wilson and Jacobsen, 1977). If yellow sepiapterins, xanthopterins, and riboflavins, as well as orange carotenes were present as potential confounding pigments they were not concentrated enough to affect the λ_{max} values for drosopterin or carotenoids.

Statistical analysis.—We used factorial analysis of variance (ANOVA) to investigate the effect of subspecies and dewlap tissue section on carotenoid levels and on pterin levels in separate models: 1) carotenoid concentration as dependent variable and 2) pterin concentration as dependent variable with subspecies and dewlap sections as factors. To minimize the departure from parametric assumptions of normality and homoscedasticity, data on carotenoid and pterin concentrations were $\log_{10}$-transformed. To compare the means of pigment concentrations within dewlap sections and subspecies the Student's *t*-test was used. To investigate which dewlap sections differed significantly from each other, *post-hoc* Bonferroni multiple-comparison tests were performed. All analyses were performed using SPSS (version 12.0/2003 for Window, SPSS Inc.). Note: as drosopterins seem to be entirely absent in *A. j. albertschwartzi* and in the section proximal to dewlap base in all subspecies, the factorial ANOVA of pterin is solely composed of the central and marginal sections of the subspecies *A. j. oriens* and *A. j. maisiensis*.

RESULTS

The results of the two separate factorial ANOVAs are shown in Table 1. We found significant main effects in carotenoid concentrations by subspecies. Carotenoid concentrations were highest in *A. j. oriens* (654.06 ± 37.00 μg/g) followed by *A. j. maisiensis* (478.07 ± 37.00 μg/g) and lowest in *A. j. albertschwartzi* (35.33 ± 37.00 μg/g). There was also a significant subspecies by dewlap section effect, which shows that

Table 1. 3 × 3 Factorial ANOVA of Carotenoid Concentration (µg/g; Dependent Variable), with Subspecies and Dewlap as Factors, and Pterin Concentration (µg/g; Dependent Variable), with Subspecies and Dewlap as Factors. Note: as pterins seem to be entirely absent in *A. j. albertschwartzi* and in the region proximal to dewlap base in all subspecies, the analysis comprises solely the central and marginal regions of subspecies of *A. j. oriens* and *A. j. maisiensis*.

Pigment	Variation source	DF	SS	MS	F ratio	P
Carotenoids	Subspecies	2	14.17	7.08	268.21	<0.001
	Dewlap region	2	0.45	0.22	8.44	<0.001
	Subspecies * Region	4	0.30	0.07	2.80	<0.05
Pterins	Subspecies	1	3.46	3.46	108.90	<0.001
	Dewlap region	1	1.07	1.07	33.70	<0.001
	Subspecies * Region	1	0.03	0.03	0.81	ns

DF = degrees of freedom, SS = sums of squares, MS = means squares, F ratio = variance ratio test. ns = non-significant at $P = 0.05$.

carotenoids were distributed differently in the dewlap sections (proximal, central, and marginal), depending on the subspecies under evaluation (Fig. 2, Table 1). Tissue sections of the dewlap differed significantly in carotenoid concentrations, depending on the subspecies under evaluation. For example, carotenoid concentration did not differ by section in *A. j. albertschwartzi* ($F = 1.70$, $P = 0.2186$), but carotenoid levels differed by section in *A. j. oriens* and *A. j. maisiensis,* being highest in both subspecies in the central section with values of 904.2±62.22 µg/g ($F = 3.94$, $P = 0.0484$) and 712.09±5.74 µg/g ($F = 10.64$, $P = 0.0022$), respectively. In *A. j. oriens*, the central dewlap section had significantly higher carotenoid concentrations than the proximal dewlap section (*post-hoc* Bonferroni multiple comparisons test, mean difference = −445.8±137.7, $P = 0.021$), but the central section carotenoid concentration did not significantly differ from the marginal section (*post-hoc* Bonferroni multiple comparisons test, mean difference = 304.8±137.7, $P = 0.143$). In *A. j. maisiensis*, the central dewlap section had significantly higher carotenoid concentrations than the proximal dewlap section (*post-hoc* Bonferroni multiple comparisons test, mean difference = −416.6±74.9, $P < 0.001$), and the central section carotenoid concentration had significantly higher carotenoid concentrations than the marginal section (*post-hoc* Bonferroni multiple comparisons test, mean difference = 285.8±74.9, $P = 0.007$).

Pterin concentrations differed according to subspecies (Fig. 2, Table 1). Pterin concentrations in *A. j. oriens* (233.200± 19.20 µg/g) were higher than pterin concentrations in *A. j. maisiensis* (22.80±19.20 µg/g). Pterin concentrations also differed according to dewlap section (Fig. 2). The central section had higher mean pterin concentrations (202.07± 19.20 µg/g) than the marginal section (53.93±19.207 µg/g). There was no subspecies-by-dewlap section interaction. In *A. j. oriens*, the central dewlap section had significantly higher pterin concentrations than the marginal dewlap section (*post-hoc* Bonferroni multiple comparisons test, mean difference = 414.4±81.2, $P < 0.001$). In *A. j. maisiensis*, the central dewlap section had significantly higher pterin concentrations than the marginal dewlap section (*post-hoc* Bonferroni multiple comparisons test, mean difference = 30.0±6.5, $P = 0.002$).

DISCUSSION

We found significant pigment concentration differences among and within subspecies of *Anolis jubar*. In two of the three subspecies (*A. j. oriens* and *A. j. maisinensis*) pterins as well as carotenoids were present but carotenoids were present in higher concentrations than pterins in each section of the dewlap. Pterins, which are red in color may interact with carotenoids, which are yellow in color, and may be the pigments responsible for the orange appearance of dewlaps in these two subspecies. Interestingly, Macedonia et al. (2000) reported that Jamaican species of *Anolis* whose dewlaps are prominently colored with orange or red (e.g., *A.*

A

B
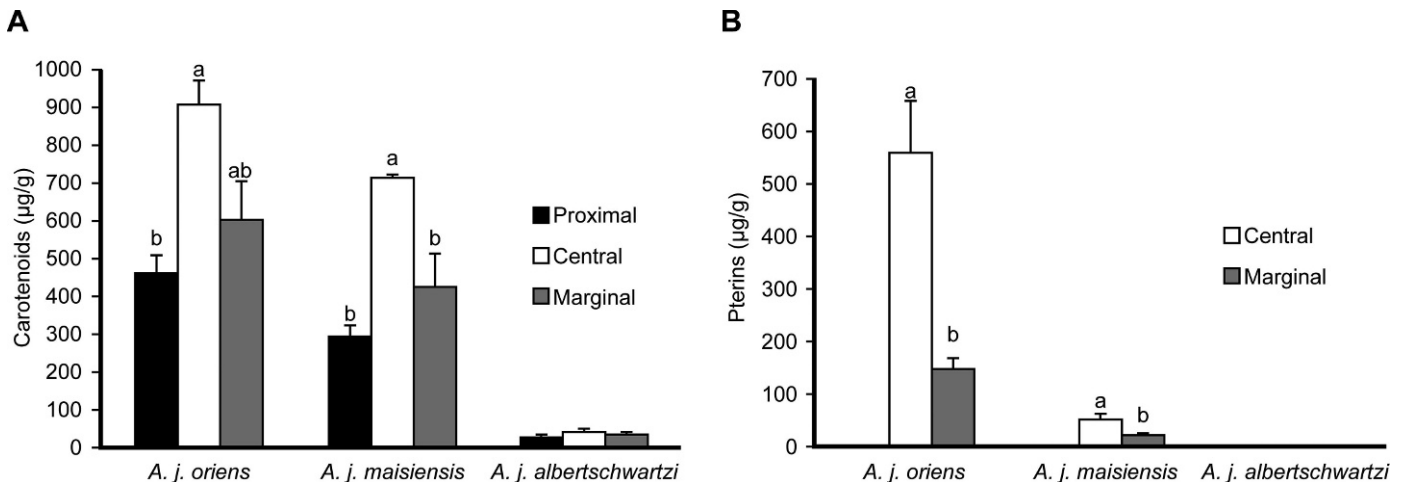
Fig. 2. (A) Comparison of carotenoid concentrations (µg/g) in three subspecies of *Anolis jubar*. (B) Comparison of pterin concentrations (µg/g) in three subspecies of *Anolis jubar*. Lowercase letters represent significant differences in pigment concentrations between dewlap sections (using *post-hoc* Bonferroni multiple comparisons) for each subspecies. See text for details.

grahami, *A. opalinus*, *A. valencienni*) exhibited proportionately more drosopterins than those species lacking orange or red (e.g., *A. conspersus* and *A. garmani*). The dewlap of *A. j. albertschwartzi* lacks pterins which may explain its light yellowish appearance. Furthermore, the dewlap of *A. j. albertschwartzi* differs from the other two subspecies studied here because it inhabits evergreen forests, and its light yellow appearance might be related to its need to be maximally conspicuous in a dark green light environment (Fleishman, 1992). Changes in the spectral environment could potentially shift the optimal ratio of drosopterins to carotenoids for maximizing visual contrast and cause drosopterin production to evolve through the sensory-drive process (Reimchen, 1989; Endler, 1992).

In general, the differences observed in pigment concentrations as well as in the drosopterin to carotenoid ratios allow an explanation for the perceived dewlap colorations of these subspecies of *A. jubar*. The richest orange-yellow dewlap colors of *A. j. oriens* had the highest carotenoid and pterin content in both central and marginal sections. The second richest yellow dewlap of *A. j. maisiensis* also had an intermediate carotenoid concentration but a low pterin concentration, and the lightest-yellow dewlap of *A. j. albertschwartzi* had the lowest carotenoid content and lacked pterin. The results of section-specific pigment concentrations in the dewlap of these three subspecies correlated with preliminary data obtained from imaging analysis (unpubl. data).

Our results demonstrate that pterin concentrations differed significantly between the three subspecies of *A. jubar*, and carotenoid concentrations were similar between *A. j. oriens* and *A. j. maisiensis*, both of which differed from *A. j. albertschwartzi*. In this context, the comparative biochemistry of animal coloration may shed light on the selective mechanisms through which coloration evolves. Particularly, pterins have received little mention in a sexual selection and signal evolution context (Grether et al., 2001). However, when studying the Trinidadian guppy (*Poecilia reticulata*) populations, Grether et al. (2005) considered mechanisms of color production in yellow carotenoids versus red pterins, and reported that pterin production is relatively insensitive to the diet and comes under strong genetic control.

Steffen and McGraw (2007) speculated that diet should have little to do with the pigment patterns observed in dewlaps of two similarly colored species (*Norops humilis* and *Norops* [*Anolis*] *sagrei*) because they both are generalist insectivores that consume phytophagous insects. This speculation also seems reasonable for the three subspecies of *A. jubar* considered in our work. Nicholson et al. (2007) studied potential hypotheses explaining evolution dewlap color variation in Caribbean *Anolis* and reported a different color description for the dewlap of *A. jubar* than was found in populations we studied. Therefore, our results could reflect more variation in dewlap pigmentation for subspecies of *A. jubar* found in Cuba. Animal coloration has been reported as a powerful model for studying the genetic mechanisms that determine phenotype, and its variation can offer important insights into the process of population divergence and speciation (Hubbard et al., 2010; Ng and Glor, 2011; Stapley et al., 2011). Thus, future research should focus on molecular phylogenetic studies which help us to clarify the morphological and biochemical differences found in Cuban subspecies of *A. jubar*, and particularly, the possible speciation of *A. j. albertschwartzi*. It would be also of

interest to study the relationship between habitat lighting and color signal evolution in subspecies of *A. jubar*, as well as the potential benefits, including the immunomodulatory and antioxidant roles, of pigment use in these animals.

In conclusion, the dewlap colorations (Fig. 2) were produced by the combination of carotenoid and pterin pigments. Pterins were absent in the tissue proximal to the dewlap base in the three subspecies of *A. jubar* and entirely absent from the dewlap of *A. j. albertschwartzi*. Carotenoid concentrations differed by section in *A. j. oriens* and *A. j. maisiensis*, being highest in the central section of the dewlap. *Anolis j. oriens* had higher pterin concentrations than *A. j. maisiensis* in both central and marginal sections. Carotenoids were significantly higher than pterin concentrations in the three dewlap sections studied in *A. j. maisiensis* and *A. j. oriens*, with the exception of the central section of *A. j. oriens* in which carotenoid concentrations were not significantly different from pterin concentrations.

Animals, including reptiles, use a variety of pigments to color their visual signals. These pigment varieties differ in the way they are produced or obtained and may differ in the types of physiological advantage they confer. Together with studies of ecology and population genetics, quantitative pigment analyses of dewlap coloration can help to illuminate the cellular mechanisms responsible for phenotypic variation in dewlap coloration among species of anoles.

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