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Contributions of pterin and carotenoid pigments to dewlap coloration in two anole species

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Abstract

Animals can acquire bright coloration using a variety of pigmentary and microstructural mechanisms. Reptiles and amphibians are known to use two types of pigments—pterins and carotenoids—to generate their spectrum of colorful red, orange, and yellow hues. Because both pigment classes can confer all of these hues, the relative importance of pterins versus carotenoids in creating these different colors is not always apparent. We studied the carotenoid and pterin content of red and yellow dewlap regions in two neotropical anole species—the brown anole (*Norops sagrei*) and the ground anole (*N. humilis*). Pterins (likely drosopterins) and carotenoids (likely xanthophylls) were present in all tissues from all individuals. Pterins were more enriched in the lateral (red) region, and carotenoids more enriched in the midline (yellow) region in *N. humilis*, but pterins and carotenoids were found in similar concentrations among lateral and midline regions in *N. sagrei*. These patterns indicate that both carotenoid and pterin pigments are responsible for producing color in the dichromatic dewlaps of these two species, and that in these two species the two pigments interact differently to produce the observed colors.

Keywords: Anole; Carotenoids; Dewlaps; Lizards; *Norops*; Pigments; Pterins

1. Introduction

A conspicuously colored integument is common among reptiles (Bagnara and Hadley, 1973). Especially in lizards, colors are used as sexual or social signals and may honestly reveal aspects of an individual's quality as a rival or mate (e.g. Cooper and Greenberg, 1992; Kwiatkowski and Sullivan, 2002; Stuart-Fox et al., 2003). Work on other animals, such as birds (reviewed in Hill and McGraw, 2006), has shown that, to better understand how and why these colors have evolved as signals, it is important to determine their molecular basis. This is because different types of color-producing mechanisms (e.g. pigments, microstructures) involve different physiological challenges (McGraw, 2005).

Among the more elaborate color traits in reptiles that carry potential information as social signals are the dewlaps of male lizards. These dewlaps are most commonly red, orange, or yellow in color and have been previously shown to be derived from two types of pigments: pterins (Ortiz et al., 1962) and carotenoids (Macedonia et al., 2000). Pterin pigments are nitrogen-rich, UV-fluorescent compounds that animals synthesize from basic purine (e.g. guanine) precursors (McGraw, 2006). In contrast, carotenoids are lipid-soluble accessory photosynthetic pigments in plants that animals must acquire from the diet (Goodwin, 1984). In fact, both types of pigments can generate this range of red–yellow hues in dewlaps, so one cannot determine which pigment types are responsible for color without biochemical tests.

Prior studies of *Anolis* spp. have shown that yellow-colored dewlaps contained only carotenoids, but that species with orange or red throat colors used pterins (three drosopterins known as drosopterin, isodrosopterin, and neodrosopterin) in their throat fans (Ortiz, 1962; Ortiz et al., 1962; Ortiz and Williams-Ashman, 1963; Ortiz and Maldonado, 1966; Macedonia et al., 2000). However, little work has been done on pigment profiles within individual species when multiple color types are displayed in a dewlap. Many male lizards will combine patches

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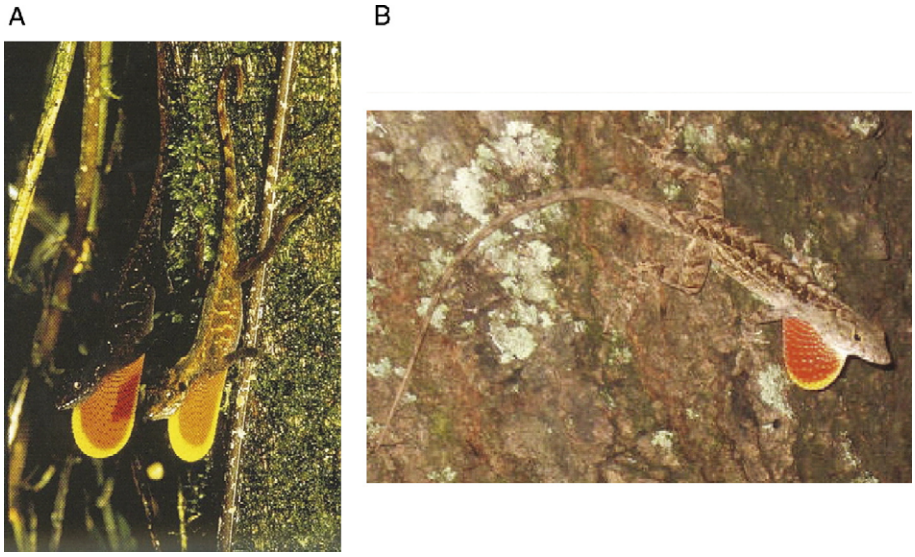

Fig. 1. A. Left: Two male Costa Rican Ground Anoles, *N. humilis*. These lizards display in light gaps of lowland tropical forests of Central America. Copyright Christian d'Orgeix, 2005. Used by permission. B. Right: Male Florida Brown Anole, *N. sagrei*. This lizard displays in full shade of disturbed environments and hardwood-hammock forests throughout Florida and Cuba. Copyright Ann Paterson, 2005. Used by permission.

or borders of yellow with red in their dewlap, and in these cases it is possible that animals use different colored pigments from the same class (pterin or carotenoid) to create the different colors, or that, as has been shown interspecifically, the different colors are in fact due to the different pigment classes.

To investigate how color is produced in different regions of the dewlap, we analyzed and compared concentrations of carotenoids and pterins between central and outer regions in two species of anoles that generally have red (in the lateral regions) and yellow (in the mid-line region) colors (see Fig. 1A and B). *Norops sagrei*, the brown anole, is an invasive species historically found in Cuba, but now found commonly throughout Florida. It is a denizen of disturbed habitats, as well as hardwood-hammock forests throughout much of the state. *N. sagrei* displays in partial shade of an elevated perch, or in forest gaps surrounded by areas of 'long wavelength' light. In contrast, *Norops humilis*, the ground anole, is a common native of lowland rainforests throughout Costa Rica and Panama. It is a cryptic dweller of primary and secondary forests, but readily exhibits courtship and territorial displays from the deeply shaded forest floor (Guyer and Donnelly, 2005).

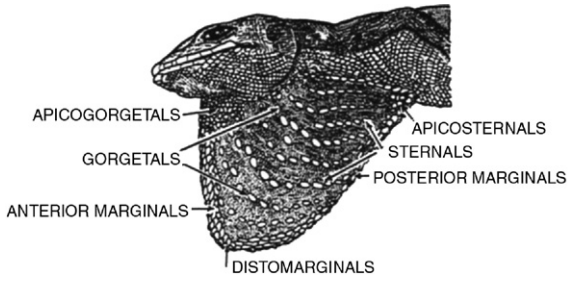

Fig. 2. Adult male *Norops* with dewlap spread, showing location of dewlap scales with terminology proposed by Fitch and Hillis, 1984. We use lateral region to refer to area including gorgetal scales, and midline region to refer to the marginal (anterior, disto-, and posterior) region. See text for rationale.

2. Materials and methods

Lizards used in this study were obtained in two different ways. Male brown anoles (*N. sagrei*) were purchased from a pet store (Glades Herp, Bushnell, FL,USA), where employees capture the lizards locally. They were shipped to one of us (JES) and sacrificed two days later. Male ground anoles (*N. humilis*) were collected at La Selva Biological Station in Costa Rica, transported live to the United States (exportation permit # 003-2005-OFAU), and sacrificed the following day.

Dewlap tissue was removed from lizards as described by Macedonia et al. (2000). Five adult males of each species were fully anaesthetized with chloroform. When animals were completely unconscious, and respiration rates slowed, the spinal cord of each lizard was cut at the base of the neck with surgical scissors. The dewlap skin was excised from the body, and the hyoid cartilage underlying the skin was removed with forceps.

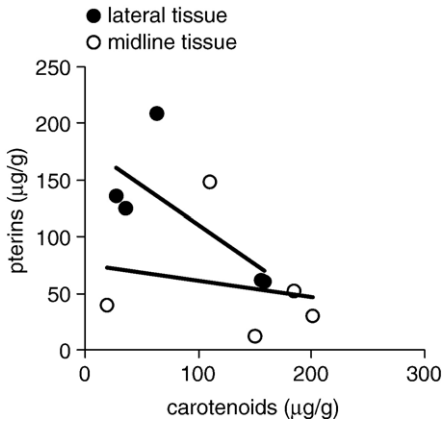

Fig. 3. Regression of pterin concentrations vs. carotenoid concentrations (both expressed in units of micrograms per gram of tissue) in dewlaps of 5 *N. sagrei*. Lateral tissue, $r^2=0.536$, $df=1$, $P=0.16$. Midline tissue $r^2=0.030$, $df=4$, $P=0.78$.

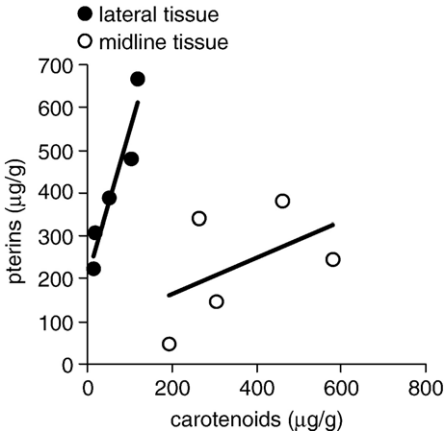

Fig. 4. Regression of pterin concentrations vs. carotenoid concentrations (both expressed in units of micrograms per gram of tissue) in dewlaps of 5 *N. humilis*. Lateral tissue, $r^2=0.910$, $df=1$, $P=0.012$. Midline tissue $r^2=0.245$, $df=4$, $P=0.396$.

Yellow dewlap tissue from the mid-line was separated from red lateral tissue by cutting with a sharp razor blade. The tissue was rinsed with water to rid samples of spilled blood that may contain pigments and contaminate skin samples. These tissues were then stored at −80 °C in Eppendorf tubes containing 70% ethanol for 1 week, until they were sent to KJM for pigment analysis.

Concentrations of carotenoid and pterin pigments were measured in each tissue region using the following method: 3–5 mg of tissue was removed from yellow (midline) and red (lateral) tissue samples and ground using a mixer mill (McGraw, 2005) in the presence of 1% NH_4OH. The ground material and solvent were transferred to a fresh tube, at which point *tert*-butyl methyl ether (TBME) was added to partition carotenoids from pterins. The solution was then shaken for 1 min, centrifuged, and the two solvents separated for pterin and carotenoid quantification using absorbance spectrophotometry (*sensu* Grether et al., 2001). Full-spectrum spectrophotometric scans on each solvent fraction provided λ_{max} values for calculating concentrations ($\lambda_{max}=455$ nm for the carotenoid fraction and 490 nm for the pterin fraction); λ_{max} values also provided secondary confirmation that the pigments in the two solvent fractions were in fact pterins (likely predominated by red drosopterins; see evidence above in other anoline lizards) and carotenoids (likely xanthophylls, such as lutein and zeaxanthin; Raila et al., 2002).

We used linear regression to investigate correlations between concentrations of each pigment type within the same dewlap region. We used two-way analyses of variance (ANOVAs) to investigate the effect of species and dewlap tissue region (red lateral vs. yellow midline) on carotenoid levels and on pterin levels (in separate models). Data on carotenoid and pterin concentrations were log-transformed to meet assumptions of parametric statistics (normality and homogeneity of variance). All analyses were performed using SPSS statistical software.

Table 1
2×2 factorial ANOVA of carotenoid concentration (µg/g) (dependent variable), with species and dewlap as factors

Source	DF	SS	*F* Ratio	Prob>*F*
Species	1	0.02346	0.2153	0.6489
Region	1	0.92924	8.526	0.01
Species*Region	1	0.98612	9.0479	0.0083

DF = degrees of freedom, SS = sums of squares, *F* ratio = variance ratio test, Prob>*F* = probability that observed variance ratio is greater than critical *F* value.

Table 2
2×2 factorial ANOVA, of pterin concentration (µg/g) (dependent variable), with species and dewlap as factors

Source	*DF*	SS	*F* Ratio	Prob>*F*
Species	1	2.4437	21.8825	0.0003
Region	1	0.36856	3.3003	0.088
Species*Region	1	0.01157	0.1036	0.7517

DF = degrees of freedom, SS = sums of squares, *F* ratio = variance ratio test, Prob>*F* = probability that observed variance ratio is greater than critical *F* value.

Finally, there has been only one paper published to date which attempts to establish names for the different areas of the dewlap (and the scales and tissues therein). Fitch and Hillis (1984) describe the scales along the margin (edge, or lateral region) of the dewlap, and differentiate between anterior, posterior, and disto-marginal regions and scales, as well as the gorgetal region (the scales below the throat on the base of the dewlap, and in successive rows outward, separated by bare skin that are usually brightly colored). These definitions are problematic, however, because they appear to define and describe the regions relative to the observer's spatial orientation of the anatomical signal (i.e. from the side) and not to the animal itself. Furthermore, at least one term (gorgetal) is obscure, and is not found in most anatomical dictionaries or text books. For this paper, we use the words 'midline' and 'lateral' to refer to the marginal and gorgetal regions, respectively, because these synonyms are standard terms found in most anatomical textbooks (see Fig. 2). Lateral means in the direction away from, or farther from, a midline bisecting the animal.

3. Results

3.1. Correlations between pigment types within colored tissue regions

Both pterins and carotenoids were detected in tissues from both dewlap regions from all animals of both species. In red dewlap tissue from *N. sagrei*, carotenoid concentration was negatively correlated with pterin concentration, but not significantly so (Fig. 3; $r^2=0.536$, $df=1$, $N=10$, $P=0.16$). In *N. sagrei's* yellow dewlap tissue, carotenoid concentration did not correlate with pterin concentration ($r^2=0.030$, $df=4$, $P=0.78$).

In the red dewlap tissue from *N. humilis*, carotenoid concentration correlated positively and significantly with pterin concentration (Fig. 4; $r^2=0.910$, $df=1$, $P=0.012$). In *N. humilis'* yellow dewlap tissue, carotenoid concentration did not correlate with pterin concentration ($r^2=0.245$, $df=4$, $P=0.396$).

Table 3
Comparison of pigment concentrations in each dewlap region of *N. sagrei*

Region	Pigment	Mean conc. (S.E.)	Pigment	Mean conc. (S.E.)	*F*	*P*
Red	Carotenoid	118.46 (27.439)	Pterin	88.28 (28.747)	0.97	0.35
Yellow	Carotenoid	131.3 (32.088)	Pterin	56.74 (23.821)	2.46	0.15

Mean conc. = mean concentration of pigment per region, expressed as micrograms of pigment per gram of tissue. S.E. = standard error. *F* = observed variance ratio. *P* = probability that observed value is due to chance.

3.2. Differences in carotenoid content between species and dewlap regions

We found a significant species-by-dewlap region interaction for carotenoids (Table 1), which shows that carotenoids were apportioned differently to the red lateral vs. yellow midline dewlap regions in the two species. While species did not differ significantly in carotenoid concentrations (Table 1), tissue regions of the dewlap significantly differed in carotenoid concentrations, depending on the species under consideration (Table 1). For example, carotenoid concentration did not differ by region in *N. sagrei* (red region mean ±SE=118.5±27.4 μg/g; yellow region=131.3±32.1; $F=0.0038$, $P=0.95$), but carotenoid concentration did differ by region in *N. humilis*, being highest in yellow tissue (red region=61.7±48.1 μg/g; yellow region=361.2±70.6 μg/g; $F=18.06$, $P<0.009$).

3.3. Differences in pterin content between species and dewlap regions

Species differed in pterin concentrations (Table 2), but there was no effect of tissue region or the species-by-tissue region interaction (Table 2). *N. humilis* had higher pterin concentrations than *N. sagrei* in both lateral and midline dewlap regions. In *N. sagrei*, pterin concentrations did not differ between regions (red region=88.3±64.3 μg/g; yellow region=56.7±23.8 μg/g; $F=0.91$, $P=0.37$), but in *N. humilis* pterin concentrations nearly differed significantly between regions (red region=413.1±76.5 μg/g; yellow region=232.1±61.8 μg/g; $F=2.94$, $P=0.12$).

3.4. Comparing pterin versus carotenoid levels within dewlap regions and species

In the red portion of *N. sagrei's* dewlap, we found no significant difference between carotenoid and pterin concentrations. In the yellow portion of *N. sagrei's* dewlap, carotenoid concentration was higher than pterin concentration, but this difference was not statistically significant (Table 3).

In the red portion of *N. humilis'* dewlap, pterin concentration was significantly higher than carotenoid concentration (Table 4). In the yellow portion of *N. humilis'* dewlap, we found no significant difference between carotenoid and pterin concentrations.

4. Discussion

These results suggest that, for both species, each color in the dichromatic dewlap (red and yellow) is produced by combinations of the two pigment classes: carotenoids and pterins. Moreover, this analysis shows that, despite the apparent chromatic similarity between species' dewlaps, colors observed are produced by different pigment concentrations and ratios, and future research needs to investigate whether this represents underlying differences in the two species' signal use.

In *N. humilis*, the concentration of carotenoids in the lateral (red) portion of the dewlap correlated positively with the concentration of pterins, but carotenoid concentration did not correlate significantly with pterin concentration in the midline (yellow) portion of the dewlap. Moreover, carotenoid concentrations differed significantly between the lateral and midline dewlap regions, while pterin concentrations nearly differed significantly. Pterins were more concentrated than carotenoids in the lateral portion of the dewlap, while carotenoids were more concentrated in the midline portion of the dewlap. Thus, it appears that red color in the lateral portion of the dewlap is predominantly due to pterins, but that carotenoids are still present (and more so in animals with more pterins) and might combine with pterins to generate the brightest colors in this species.

In *N. sagrei*, pterin concentrations did not correlate significantly with carotenoid concentrations in either the lateral or midline dewlap regions. Furthermore, there was no significant difference between pterin and carotenoid concentrations in lateral or midline regions of the dewlap. Despite this, the lateral portion of the dewlap appears red-orange while the midline portion appears yellow in dewlap tissues sampled. Thus, there appears to be greater potential for variation in pigment deposition in these tissue regions compared to those in *N. humilis*. Moreover, it is possible in these animals that amounts of orange (carotenes) or red (ketocarotenoids) carotenoids are present to help create red color, though they clearly are not dominant in the tissue (as would be indicated by a unique spectral shape for carotenoid absorbance in this species compared to *N. sagrei*). We attempted high-performance liquid

Table 4
Comparison of pigment concentrations in each dewlap region of *N. humilis*

Region	Pigment	Mean conc. (S.E.)	Pigment	Mean conc. (S.E.)	*F*	*P*
Red	Carotenoid	61.72 (21.521)	Pterin	413.06 (76.512)	21	0.002*
Yellow	Carotenoid	361.22 (70.61)	Pterin	232.12 (61.792)	1.88	0.21

Mean conc. = mean concentration of pigment per region, expressed as micrograms of pigment per gram of tissue. S.E. = standard error. *F* = observed variance ratio. *P* = probability that observed value is due to chance. *denotes statistical significance at *P* value=0.05.

chromatographic (HPLC) analyses of these pigments to identify particular types in these two anole species, but these proved unsuccessful due to the fact that dewlap carotenoids are highly esterified.

Pterin, but not carotenoid, concentrations differed significantly between the two species. Coupled with all other region-specific differences in pigmentation between the two species, this raises the question of why these two anoles differed in pigment profiles. Grether et al. (2005) have similarly considered the balancing costs and benefits of color production via yellow carotenoids and red pterins in the orange spots of Trinidadian guppy (*Poecilia reticulata*) populations. Mechanistically, they have shown that pterin production is relatively insensitive to the diet and instead comes under strong genetic control (Grether et al., 2005). The fact that these lizards have similar diets (e.g. phytophagous insects) is consistent with the fact that diet should have little to do with the pigment patterns we observed in their dewlaps. Pigment differences between species may also reflect the lighting environment inhabited by each species and the relative value of pterins and carotenoids for coloration. For example, the poor light environment in which *N. humilis* displays may favor the accumulation of more pterin pigments to ensure adequate color production. Guppy populations appear to do this via scaling total pigment amounts, while maintaining identical ratios of pterins:carotenoids (Grether et al., 2001), but these two *Norops* sp. clearly show more flexibility in pigmentation, by varying pigment amounts independently, even within specific dewlap tissue regions.

Admittedly, the findings presented here are derived from a small sample size of each species, and future research should focus on increasing sample sizes and incorporate the use of UV–visible spectrometry to understand how pigments contribute to observed colors. We also point out that the role of dewlap color *per se* as a social signal has not been properly studied in *Norops* lizards, and whether intra- and/or inter-sexual selection maintains these bright colors should be an exciting avenue to pursue. Finally, because the pigments identified in *Norops* dewlap tissue are similar to pigments used in a variety of avian species, many of which use these pigments as condition-dependent signals, future research should investigate the mechanistic costs and benefits (including their antioxidant role; McGraw, 2005) of pigment use in these animals.

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