The Role of Heritable and Dietary Factors in the Sexual Signal of a Hispaniolan Anolis Lizard, Anolis distichus

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The diversity of sexual signals is astounding, and divergence in these traits is believed to be associated with the early stages of speciation. An increasing number of studies also suggest a role for natural selection in driving signal divergence for effective transmission in heterogeneous environments. Both speciation and adaptive divergence, however, are contingent on the sexual signal being heritable, yet this often remains assumed and untested. It is particularly critical that the heritability of carotenoid-based sexual signals is investigated because such traits may instead be phenotypically plastic indicators of an individual's quality that exhibit no or little heritable variation. We present the first study to investigate the relative contribution of genetic and environmental factors to the striking diversity of dewlap color and pattern in Anolis lizards. Using a breeding experiment with Anolis distichus populations exhibiting different dewlap phenotypes, we raise F1 offspring in a common garden experiment to assess whether dewlap color is inherited. We follow this with carotenoid supplementation to investigate the influence of dietary pigments to dewlap color variation. We find significant differences in several aspects of dewlap color and pattern to persist to the F2 generation (fathers: N = 19; F1 males: N = 50; P < 0.01) with no change in dewlap phenotype with carotenoid supplementation (N = 52; P > 0.05). These results strongly support that genetic differences underlie dewlap color variation, thereby satisfying a key requirement of natural selection. Our findings provide an important stepping-stone to understanding the evolution of an incredibly diverse signal important for sexual selection and species recognition.

Key words: carotenoid, dewlap, geographic variation, heritability, pigmentation, signal divergence

There is a spectacular diversity of signals involved in sexual selection. In many cases, such as the nuptial coloration of cichlids and auditory calls in frogs, signal divergence is believed to be associated with the early stages of the evolution of reproductive isolation and speciation (e.g., Masta and Maddison 2002; Boake 2005; Mendelson and Shaw 2005; Boul et al. 2007; Maan and Seehausen 2010). A growing number of studies suggest that signals can also diverge adaptively to optimize their detectability among signaling environments. In birds, for example, both song and sexually dimorphic coloration appear to diverge adaptively among habitats in response to selection for effective signal transmission (e.g., Marchetti 1993; Slabbekoorn and Smith 2002; Tobias et al. 2010; Uy and Stein 2007). These 2 processes may also act in combination, with the early stages of speciation resulting from adaptive divergence of signals. Although heritability of signal variation is a central assumption of both speciation and adaptation, this hypothesis is often untested. In many cases, particularly among vertebrates, heritability must be assumed because the type of experiments (e.g., common garden, reciprocal transplant) or observations (e.g., multi-generational studies of genotype and phenotype in natural populations) required to test it are infeasible or impractical.

In many species where heritability of sexual signals has been assessed, studies have recovered a strong role for both genetic and environmental factors. This is particularly evident in visual signals that are carotenoid based. Carotenoid pigments are often responsible for the yellow, red, and orange coloration seen in the sexual signals of birds, fish, and reptiles, including a number of well-studied signals such as the plumage coloration of great tits (Parus major) (Slagsvold and Lifjeld 1985; Partali et al. 1987) and house finches (Carpodacus mexicanus) (Brush and Power 1976; Hill 1992; Hill et al. 2002), guppy spots (Poecilia reticulata) (Grether et al. 1999) and the dewlap of Anolis lizards (Steffen and McGraw 2007, 2009). Unlike pteridines, another pigment that has been found in sexual signals and can also appear yellow, red or orange, carotenoids cannot be synthesized de novo and instead must be obtained from diet (Goodwin 1984). For this reason, the color of a carotenoid-based signal is largely determined by the individual's ability to acquire and utilize dietary...
carotenoids (reviewed in Olson and Owens 1998; Svensson and Wong 2011). In addition to directly contributing to signal coloration, carotenoids are also an important antioxidant and have been associated with immune function, parasite resistance, and overall health (e.g., Blount et al. 2003; Faire et al. 2003; McGraw and Ardia 2003; Grether et al. 2004; Dijkstra et al. 2007; Baeta et al. 2008; reviewed in Olson and Owens 1998; Svensson and Wong 2011). Due to this dual role, an individual’s carotenoid-based signal may honestly advertise its overall fitness, rather than merely indicating the presence of loci that result in an intrinsically attractive signal (Olson and Owens 1998). Such phenotypically plastic color variation is likely to exhibit little heritable variation (Kodric-Brown and Brown 1984) and should, therefore, not be considered an adaptive response to natural selection or used to diagnose species or incipient species (Slagsvold and Lifjeld 1985; Hill 1993).

Nevertheless, color divergence in many carotenoid-based sexual signals may also be determined by genetic factors. For example, the color saturation of the guppy’s spots, the color of the house finch’s patch, and the brightness of great tits’ breast plumage all exhibit low heritability and are, instead, highly dependent on the amount of carotenoids consumed, whereas the size of the guppy’s orange spots, the ventral patch size of house finches and the color of great tits’ breast plumage are all highly heritable signals (Brush and Power 1976; Kodric-Brown 1989; Houde 1992; Hill 1993; Grether 2000; Brooks and Endler 2001; Karino and Hajjima 2001, 2004; Evans and Sheldon 2012).

We here present the first study to investigate the relative contribution of genetic and nonheritable environmental factors to the divergence of a striking and widely studied visual signal: the dewlap of Anolis lizards. The dewlap is a throat fan that is extended during species-specific visual displays that may also involve stereotypical sequences of headbobs, push-ups, and tail wags (reviewed in Jenssen 1977). Dewlap color differences are often used to diagnose species and incipient species (Underwood and Williams 1959; Williams 1965; Schwartz 1968). More recently, research suggests that dewlap color variation can be an adaptive response to variation in signaling environments and may contribute to speciation (Leaf and Fleshman 2002, Leaf 2004; Ng and Glor 2011; Ng et al. 2013). Although heritability of dewlap color is often assumed (e.g., Stellar and White 2010), we are unaware of previous studies that have experimentally tested this.

We use a combination of common garden experiments and dietary supplementation to test the genetic and environmental factors contributing to dewlap color and pattern in a highly polymorphic species of Hispaniolan Anolis lizard, Anolis distichus. Geographically distinct populations of this species have dewlaps that range from pale white to deep maroon and nearly every shade of red, orange, and yellow in between (Schwartz 1968). This variation has been used to diagnose subspecies of A. distichus (Schwartz 1968), some of which appear to experience some degree of reproductive isolation where they come into contact (Case and Williams 1984; Ng and Glor 2011; Glor and Laport 2012).

More recently, we have recovered evidence that dewlap color diverges adaptively in response to variation in signaling environments (Ng et al. 2013). Anolis distichus inhabits a range of habitats, from xeric lowlands to high-elevation cloud forests (Schwartz 1968), and dewlap color strongly correlates with these environments; in xeric habitats, male A. distichus exhibit smaller, brighter yellow dewlaps than the relatively large, less bright orange dewlaps of those populations inhabiting mesic habitats (Ng et al. 2013). Although this repeated pattern of dewlap divergence with environmental divergence is consistent with adaptive divergence of dewlap coloration (Ng et al. 2013), it is also possible that the observed variation in dewlap color may merely be a reflection of differences in the availability of carotenoids or other dietary resources necessary for signal coloration. Such geographic variation in carotenoid availability along with associated signal color differences has been shown in guppies (Grether et al. 1999; Grether et al. 2001), and thus, it is critical to investigate the influence of genetic and environmental factors to signal coloration to understand whether any observed correlation with different environments is driven by natural selection or merely a plastic response to varying environmental factors.

We here use 2 approaches to test the relative contributions of heritable and dietary factors to A. distichus dewlap coloration. First, we test whether dewlap color is inherited by conducting experimental crosses involving 2 A. distichus subspecies that differ in dewlap color and raising the resulting offspring in a common garden environment. Second, we test whether dewlap color variation results from differences in carotenoid consumption by conducting dietary manipulation experiments with the laboratory-reared offspring produced by our experimental crosses. Our study is the first to test the effect of carotenoid supplementation on coloration in lizards using offspring that are the result of matings in the laboratory environment. As parents have been maintained under the same laboratory conditions, this importantly reduces the potentially confounding effect of maternally acquired signal coloration through carotenoid deposition in eggs (e.g., McGraw et al. 2005; Biard et al. 2007; Biard et al. 2009). In addition, with a breeding design that includes half-sib offspring, our experiment also controls for any other potential confounding maternal effects, such as epigenetic factors, that may influence the phenotype.

Material and Methods

Breeding Group Establishment, Husbandry, and Egg Collection

In January 2010, we collected A. distichus from 2 populations in the Dominican Republic; each population represented a different subspecies and differed in dewlap color and pattern. We collected 24 male and 61 female Anolis distichus ignigularis (predominantly orange dewlaps with dark yellow margins) from Hato Mayor province, and 25 male and 57 female Anolis distichus properus (pale yellow dewlaps) from La Altagracia province (Figure 1). Although these localities were located well within the ranges of each
subspecies, these 2 subspecies do come into contact elsewhere in their ranges. In an AAALAC-accredited laboratory, we set up 2 types of crosses: 1) “pure crosses” where the breeding group consisted of individuals representing the same subspecies, and 2) “hybrid crosses” where each sex in the breeding group represented a different subspecies (Table 1). Each breeding group consisted of 1 male and 1–3 females housed in plexiglass enclosures (Lee’s Kritter Keepers, 36.8 cm length × 22.2 cm width × 24.8 cm height) with organic potting soil as substrate, 2 sterilized wooden dowels for perches, and artificial ivy leaves for cover. We misted each cage twice daily, supplying anoles with drinking water and maintaining cage humidity at approximately 85%. To mimic seasonality, anoles were maintained at a room temperature of 83°F and a light cycle of 10 h light/14 h dark from December to March (winter cycle) prior to the April–November breeding season during which we maintained a room temperature of 85°F with a light cycle of 14 h light/10 h dark. Anoles were fed 2-week-old (approximately 1/4″) Acheta crickets (Fluker Farms, LA) twice a week during the winter cycle and thrice weekly during the breeding season. Crickets were dusted every feeding with a multivitamin supplement (Herptivite, Rep-Cal) and once a week with a 1:1 mix of the multivitamin supplement and a calcium additive (Calcium powder, Rep-Cal).

During the breeding season, each breeding group was provided with a 32-ounce lidded yogurt container with entry holes cut out of the lid and side, and partly filled with moist vermiculite for egg oviposition. We checked these containers for eggs once every 2 weeks and transferred all calcified eggs to separate Solo® deli cups (4.5″ diameter), with prepunched holes on the side for ventilation (Superior Shipping Supplies, California) and filled with moist vermiculite (water potential = −150 kPa; Packard et al. 1987). Eggs were incubated at 85°F and stored adjacent to a small fan timed to run for 10 min twice daily to encourage airflow. We checked for new hatchlings daily. All hatchlings were uniquely toe clipped before being transferred to custom-built plexiglass cages (29.6 cm length, 10.3 cm width, 35.7 cm height) that housed up to 6 hatchlings. All hatchlings were fed the same diet of pinhead Acheta crickets (approximately 3/16″) daily until they were large enough to eat the same diet as adults. All hatchlings were maintained at the same temperature and misting regime as adult anoles. All procedures described follow protocols that were approved by the University of Rochester Institutional Animal Care and Use Committee.

To try to maximize the number of offspring produced, males from cages that produced no or very few eggs were swapped with one another, or with those from more productive cages, up to 4 times periodically throughout the breeding season. For most of the 31 males moved, this strategy successfully increased egg production, with the exception of 2 males that produced fewer or the same number of fertile eggs with each new breeding group.

Figure 1. Photographs of example F1 male offspring from each cross type at 4, 6, 9, and 12 months of age, and their respective fathers: (a) pure Anolis distichus properus cross, (b) pure Anolis distichus ignigularis cross, (c) A. d. properus ♂ × A. d. ignigularis ♀, (d) A. d. ignigularis ♂ × A. d. properus ♀. Figure can be seen in color online.
Breeding design used in study and the number of year-old F₁s used in inheritance analyses

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<td>Hybrid crosses</td>
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Each breeding group consisted of 1 male and up to 3 females. Pure cross groups comprised males and females from the same subspecies (i.e., same dewlap color), whereas hybrid crosses comprised males and females from different subspecies (i.e., different dewlap color). Colored circles represent the dewlap phenotype (which is inferred in females [which lack dewlaps] based on the phenotype of co-occurring males); yellow (light) colored circles represent *Anolis distichus ravitergum*, which typically have yellow dewlaps, and orange (dark) colored circles with a yellow (light) border represent *Anolis distichus ignigularis*, which typically have orange dewlaps with dark yellow borders (color online). The total number of F₁s produced from each cross that reached a year of age is shown in the table, in addition to the number of year-old full sibs and half sibs from each cross, as well as the number of year-old F₁ individuals that did not have any full siblings or half siblings (nonsibs). Full/half sibs indicate the number of year-old F₁ individuals for which it was unclear whether they were full sibs or half sibs.

Paternity Analysis

As anoles are known to store sperm (Fox 1963; Conner and Crews 1980; Calsbeek et al. 2007), and because some parental individuals were in more than 1 breeding group during the course of the breeding season, we conducted parentage analyses to identify the father of each offspring. We genotyped all parents and F₁ males using 7 microsatellite markers (DISTA2B12, DISTB85, DISTCC7, DISTBC4, DISTAG1, DISTAH6, and BREV2E9; Ng et al. 2010) and assigned paternity using an exclusion-based approach using the software FAP v3.6 (Taggart 2007). Four F₁ males whose fathers could not be confirmed were excluded from our analyses of dewlap color development and inheritance (see following sections).

Characterization of Dewlap Color and Pattern

To quantify dewlap color and pattern throughout development, we obtained 2 types of data for all male hatchlings at 4, 6, 9, and 12 months of age: 1) high-resolution photographs of extended dewlaps using a Nikon D70 or D90 digital SLR camera, and 2) quantitative data on spectral reflectance of each dewlap’s center and edge using a spectrometer. Dewlaps were first measured at 4 months of age because dewlaps of 3-month-old hatchlings were often too small to extend and measure. All photographs of dewlaps were taken with lizards positioned on their left side and their dewlap fully extended with forceps. Each photograph was taken with a card containing color standards (X-Rite Mini ColorChecker Card) and a 1-cm² grid as the background. Using these photographs, we quantified the relative amount of orange in the dewlaps of 12-month-old offspring. First, we measured dewlap size for each male in ImageJ (Abramoff et al. 2004) with the 1-cm² grid in each photograph used as a scale. Each dewlap’s size was measured twice and averaged prior to analyses. We then standardized the color of each photograph to the color-standard card in the photograph using the “curves adjustment” function in Adobe Photoshop v.12 and then used the “select color range” function to extract the absolute size of the orange portion of the dewlap. The ratio of the size of the orange area to the whole dewlap was subsequently used as a measure of the relative amount of orange in the dewlap.

We obtained dewlap reflectance spectra using the same methods outlined in Ng and Glor (2011) and Ng et al. (2013): dewlaps were measured with respect to a Spectralon white standard (Labsphere) using an Ocean Optics USB4000 spectrometer with a pulsed Xenon light source (PX-2, Ocean Optics) and a 400 µm reflectance probe encased in a black anodized aluminum sheath with a 45° angle tip to prevent specular reflection (glare) (Endler 1990). All dewlap measurements were taken twice from the right side of the extended dewlap and averaged prior to analyses. We reduced each spectrum to median values at 10 nm intervals from 300 nm to 700 nm to smooth out any artifactual spikes in the spectral recordings (Cuthill et al. 1999). We then extracted 4 variables from each reflectance spectrum to characterize differences in dewlap color: 1) brightness, 2) cut-on wavelength, and 3–4) relative reflectance in the UV range (365 nm) and long wavelength (655 nm). We quantified dewlap brightness as the area under the spectral curve from 300 nm to 700 nm. As we were interested in identifying differences in spectral shapes, we corrected all reflectance spectra for brightness by making the area under the curve equal to 1.0 (Endler 1990) before extracting relative reflectance and cut-on wavelength. Cut-on wavelength is the midpoint between
the baseline and maximum reflectance, and it differentiates spectra with slopes that gradually rise with increasing wavelength (longer cut-on wavelength; e.g., orange) from those with more steeply rising slopes (shorter cut-on wavelength; e.g., yellow; Cummings 2007).

We gathered data from a total of 80 F₁ males (pure crosses: prop N = 28, ign N = 29; hybrid crosses: ign ♂ × prop ♂ N = 7, prop ♂ × ign ♂ N = 16). Forty-nine individuals were measured at all 4 ages, but due to either mortality during the experiment or misidentifying the sex of an individual at a young age, 13 were measured 3 times, 10 were measured twice, and 8 were only measured once. All the collected spectral data is available from Dryad (doi:10.5061/dryad.pk718).

Statistical Analyses

Inheritance of Dewlap Color and Pattern

To investigate dewlap color inheritance, we used means for all 4 dewlap color reflectance values calculated from all 12-month-old offspring from the same father (fathers: N = 19; F₁ males: N = 50; Table 1). We used an analysis of variance (ANOVA) with Tukey’s HSD post hoc test to investigate differences in dewlap color between offspring and father, and among offspring of different cross types. Levene’s test conducted prior to the ANOVA, using the leveneTest function (car package) in R statistical computing framework (R Core Team 2012), showed that our data met the assumption of equal variances among groups.

Characterization of Dewlap Color Development

To investigate whether there were significant changes in dewlap color throughout an individual’s lifespan, we used linear mixed-effects model analyses (restricted maximum likelihood [REML]). We performed REML analyses using the lme function (nlme package) in R. As sibs and half sibs are not independent data points, we used F₁ s nested within their father as a random effect.

Carotenoid Supplementation

We investigated the effect of dietary pigments to dewlap color and pattern by experimentally manipulating carotenoid consumption of F₁ males. We used a matched pair design whereby 26 pairs of F₁ males, matched by their cross type, were randomly given either a carotenoid supplement (treatment) or distilled water (control) 3 times a week for 4 weeks. Whenever possible, siblings or half siblings were paired to receive different treatments. Although the duration required for supplementation to influence dewlap color coloration is unknown, the duration of our experiment has been shown to be a sufficient length of time to see a significant change in beak coloration from carotenoid supplementation in birds (Blount et al. 2003; McGraw and Ardia 2003; Baeta et al. 2008). No previous studies of carotenoid supplementation in lizards have found the type of dramatic impacts on coloration that are observed in birds and fish (Olsson et al. 2008; Fitz et al. 2009; Steffen et al. 2010; San-Jose et al. 2012; Weiss et al. 2012; San-Jose et al. 2013). As anole dewlap coloration is due to a group of carotenoids named xanthophylls, such as lutein and zeaxanthin (Macedonia et al. 2010; Steffen et al. 2010), we used a xanthophyll powder supplement consisting of 1.8% lutein and 0.2% zeaxanthin extracted from marigolds (20 g xanthophyll activity/kg; Oro Glo 20, Kemin Industries, Inc.). We stored the carotenoid powder in the dark at 4°C, and fresh carotenoid supplement was prepared immediately prior to each treatment by mixing xanthophyll powder with distilled water. Although the quantity of carotenoids naturally consumed by anoles is unknown, we followed Olsson et al. (2008), who conducted carotenoid supplementation experiments in another iguanian lizard (Ctenophorus pictus), by feeding treatment males 20 μg carotenoids (50 μl of solution) each treatment using a pipette and confirming all was ingested. We used the same method to provide control males with 50 μl distilled water. We supplemented a greater amount of carotenoids than that provided in a previous experiment with Anolis sagrei (Steffen et al. 2010) because this experiment did not observe any change in coloration in response to supplementation and because food-choice experiments find that zebra finches consume 20–40 μg carotenoids daily and maintained their beak coloration on this diet (McGrath and Ardia 2003; McGrath et al. 2003). Although zebra finches are larger than A. distichus, and likely consume far more calories than lizards and other ectotherms on a daily basis, we know little about how the amount of carotenoids in the diets of these species might differ. We monitored the health of the animals in both the supplementation and control groups by measuring their weight each week. We photographed dewlaps and quantified dewlap color 1 day before the experiment and the day after the treatment period finished. To check the validity of our results, we quantified all dewlaps again 3 months after the experiment, during which all anoles were fed their usual diets without carotenoid supplementation.

Our use of laboratory-reared F₁ males, whose diet we have been controlling since hatching, eliminates the possibility of males utilizing stored excess carotenoids to color their dewlap (Surai et al. 2001; Rajasingh et al. 2006; Svensson and Wong 2011). Furthermore, for the duration of the treatment, and the week prior, we stopped dusting crickets with multivitamin powder, which contains beta-carotene, to remove another potential source of carotenoids. Although it is unknown whether beta-carotene is deposited into A. distichus dewlap skin, if beta-carotene is used for dewlap pigmentation, the effect of removing it from the diet would still be detected in our experiment.

We used paired t-tests to investigate change in dewlap color between the start and end of the experiment and to determine whether there was a significant difference in dewlap color change between the treatment and control groups. We accounted for multiple comparisons by adjusting P values using sequential Bonferroni correction (Rice 1989). We note that the differences of interest here are expected regardless of the integumentary component responsible for the color change (San-Jose et al. 2013). For those individuals whose dewlaps had orange pigmentation, we also examined whether the relative size of the orange portion changed over the treatment period.
Results

Dewlap Inheritance

There were significant differences in dewlap color between wild-caught *A. d. ignigularis* and *A. d. properus* fathers. First, spectral data recovered significant differences between the dewlaps of these 2 subspecies at 655 nm, cut-on wavelength, and brightness at both the center and edge (P < 0.00001; Figure 2b–d, Supplementary Table S2). The 2 subspecies, however, did not differ significantly in UV reflectance (Figure 2a, Supplementary Table S2). Second, the relative amount of orange in the dewlap was also strongly differentiated between wild-caught males of the 2 subspecies (P < 0.00001; Figure 2c, Supplementary Table S2). We herein focus our discussion of dewlap color inheritance only on the traits that differ significantly between the 2 parental subspecies.

The significant differences in dewlap color observed between wild-caught fathers persisted in the offspring of pure crosses raised in the laboratory for all traits (655 nm: center and edge, P < 0.0001; cut-on wavelength: center and edge, P < 0.0001; center brightness, P < 0.01; relative amount of orange, P < 0.0001), except brightness at the edge of the dewlap (P > 0.05; Figures 2b–d and 3, Supplementary Table S2). That is, laboratory-reared *A. d. ignigularis* *F*1 males exhibited orange dewlaps, had a higher relative reflectance at 655 nm, and were less bright in the center than laboratory-reared *F*1 *A. d. properus* even at 4 months of age (Figure 3b–d).

The dewlaps of pure laboratory-reared *F*1 males were more similar to the dewlaps of their wild-caught fathers than they were to either the fathers or pure laboratory-reared sons from the other subspecies. The *A. d. properus* *F*1s exhibited the same characteristic pale yellow dewlap of *A. d. properus* fathers with either no orange or a diffuse orange portion in the center of the dewlap (Figure 1). No significant differences were found between *A. d. properus* *F*1s and *A. d. properus* fathers in reflectance at 655 nm, cut-on wavelength, or brightness at either the center or edge of the dewlap (P > 0.05) or the relative amount of orange in the dewlap (Figures 2b–d and 3, Supplementary Table S2). Pure *A. d. ignigularis* *F*1 males also exhibited dewlaps characteristic of *A. d. ignigularis* fathers, with an orange center and dark yellow margin (Figure 1). We found no significant difference in cut-on wavelength at either the center or edge of the dewlap, or the relative amount of orange in the dewlap of *A. d. ignigularis* *F*1 males and *A. d. ignigularis* fathers (P > 0.05; Figure 2c,e, Supplementary Table S2). However, we found that the dewlaps of *A. d. ignigularis* *F*1 males were significantly less bright (center: P < 0.01; edge: P < 0.001) and had a lower reflectance at 655 nm (center: P < 0.01; edge: P < 0.05) than *A. d. ignigularis* fathers (Figure 2b,d, Supplementary Table S2). Although *A. d. ignigularis* *F*1 dewlaps remained significantly different at 655 nm from *A. d. properus* fathers (center: P < 0.0001; edge: P < 0.001), they did not significantly differ in brightness (Figure 2b,d, Supplementary Table S2).

All hybrid *F*1 males exhibited dark yellow dewlaps with a basal orange patch of varying size that qualitatively looked more similar to *A. d. ignigularis* dewlaps than to *A. d. properus* dewlaps (Figure 1). Indeed, we found that center cut-on wavelength and relative amount of orange in the dewlap did not significantly differ between the dewlaps of hybrid *F*1 males and wild-caught *A. d. ignigularis* fathers (P > 0.05; Figure 2b,e, Supplementary Table S2). Furthermore, we found significant quantitative differences between the dewlaps of *F*1 hybrids and their *A. d. properus* fathers in cut-on wavelength (P < 0.0001) and 655 nm (P < 0.05) at the center of the dewlap (Figure 2b,e, Supplementary Table S2), as well as the relative amount of orange in the dewlap (P < 0.01; Figure 2e, Supplementary Table S2). Brightness at the center of hybrid *F*1 dewlaps, and both reflectance at 655 nm and cut-on wavelength at the edge of the dewlap were intermediate between the 2 subspecies (Figure 2b–d, Supplementary Table S2).

Characterization of Dewlap Color Development

Our time series of dewlap color development showed significant change in dewlap color over time in cut-on wavelength in the center of the dewlap (P < 0.01), and relative reflectance at 655 nm at both the dewlap’s center and edge (P < 0.0001), but no significant change in dewlap brightness (Figure 3b–d, Supplementary Table 1). Although we found significant change over time in UV reflectance (center P < 0.05, edge P < 0.01; Supplementary Table 1), this trait did not show a clear increase or decrease over time (Figure 3a). At 12 months of age, UV reflectance was similar among all cross types (Figure 3a).

Carotenoid Supplementation

After experimental manipulation of carotenoid supplementation, dewlaps were not significantly different in color from the start of the experiment in either the treatment or control group. Although the edge of the dewlap became marginally significantly brighter in the treatment group by the end of the experiment (P < 0.05), this result was no longer significant after Bonferroni correction (P > 0.006). The loss of significance after Bonferroni correction was further supported with the spectral data collected 3 months after the experiment; we found no significant differences in edge brightness since either the start or end of the experiment (P < 0.05). The relative size of the orange portion of the dewlap also did not change during the carotenoid experiment in either the control or treatment group (P > 0.05), nor were there any significant differences in weight between the 2 groups (P > 0.05).

Discussion

Dewlap Color and Pattern Are Heritable

Our common garden breeding experiment shows that several aspects of *A. dimitius* dewlap color and pattern are inherited. Significant differences in various components of the dewlap phenotype observed between wild-caught *A. d. properus* and *A. d. ignigularis* fathers were also recovered in the offspring of pure crosses reared in a common garden environment. We also found hybrid *F*1 offspring to be more...
Figure 2. Boxplots of the dewlap color of fathers (P) and mid-offspring dewlap color at 12 months of age (F₁): (a) relative reflectance at 365 nm, (b) relative reflectance at 655 nm, (c) cut-on wavelength, (d) brightness at both the center and edge of the dewlap, and (e) relative amount of orange. Subscript fonts represent different subspecies: i = Anolis distichus ignigularis, p = Anolis distichus properus, with the father stated first in all F₁ crosses. Sample sizes of each group are as follows, with numbers in parentheses representing the number of independent samples after removing half sibs and full sibs: Pᵢ N = 9, Pᵢ N = 10, F₁ᵢ × p N = 22 (7), F₁ᵢ × i N = 7 (3), F₁ᵢ × p N = 4 (2), F₁ᵢ × i N = 17 (8). Different letters on each boxplot indicate groups that significantly differ in the respective dewlap color variable based on Tukey’s HSD test (P < 0.05), whereas the same letter represent groups that do not significantly differ in the respective dewlap color variable. Graphs with no letters indicate no significant differences among groups (P > 0.05).
phenotypically similar to *A. d. ignigularis* than *A. d. properus*, exhibiting dark yellow dewlaps with basal orange patches rather than pale yellow dewlaps (Figures 1, 2b,c, and 3). This suggests that alleles responsible for dark yellow and orange dewlap colors in *A. distichus* are dominant or partially dominant. Moreover, because reciprocal hybrid crosses did not differ in dewlap color or pattern, our results suggest that loci responsible for dewlap color and pattern are autosomal rather than sex linked. Together, the results from our breeding experiment strongly suggest that genetic differences underlie dewlap color differences between *A. distichus* subspecies, rather than maternal effects or environmental factors. This discovery is consistent with the hypothesis that variation in dewlap color observed across the range of *A. distichus* is an adaptive response to variation in signaling environments rather than the result of underlying variation in carotenoid consumption (Ng et al. 2013).

Although we found strong evidence that dewlap color is under genetic control, we also found that dewlaps of laboratory-reared *A. d. ignigularis* sons were significantly brighter and had a lower relative reflectance at 655 nm than their wild-caught *A. d. ignigularis* fathers. As we are unable to score the mothers of this experiment (due to their lack of dewlaps), we are unable to determine whether this difference is due to different alleles being passed to the *F₁* generation from the mother or whether dewlaps may be influenced by an unmeasured environmental factor, such as the conditions fathers experienced in their native range. Despite the differences in dewlap color of laboratory-reared *A. d. ignigularis* *F₁* males relative to their wild-caught *A. d. ignigularis* fathers, the dewlap color of *A. d. ignigularis* *F₁* males remained significantly different from that of both laboratory-reared *A. d. properus* *F₁* offspring and wild-caught *A. d. properus* fathers.

Our time series investigating dewlap color development shows that dewlap differences between subspecies are clearly evident in 4-month-old juveniles. In particular, differences associated with orange coloration in the dewlap (cut-on wavelength, relative reflectance at 655 nm, and relative amount of orange in the dewlap) were consistently significantly different between subspecies at all ages. Our time series also suggests ontogenetic change in dewlap color. This was particularly prominent in cut-on wavelength at the center of the dewlap, which exhibited significant change with age such that differences in cut-on wavelength between pure *A. d. properus* and the other cross types increased from 4 months to 12 months of age; dewlaps of *A. d. properus* remained pale yellow, whereas the dewlaps of all other crosses became more orange/red (Figures 1 and 3c).

**Carotenoid Supplementation Does not Influence Dewlap Color or Pattern**

Our carotenoid supplementation experiment further suggests the important role of genetic factors in *A. distichus* dewlap color by recovering no significant differences in dewlap phenotype between the start and end of the treatment, in either the individuals that received carotenoid supplementation or the control individuals. Our findings are comparable with the study by Steffen et al. (2010) on *A. sagrei* despite supplementing anoles with a higher level of carotenoids. This suggests that geographic variation in pigment availability or consumption by adult males does not explain dewlap color variation in the wild. It is unknown why supplementary carotenoids did not influence *A. distichus* dewlap color despite the likelihood that *A. distichus* dewlaps contain carotenoids like most other *Anolis* species whose dewlap skin has been examined (Ortiz et al. 1962; Ortiz et al. 1963; Macedonia et al. 2000; Steffen and McGraw 2007).

One possible reason for the lack of dewlap color change with carotenoid supplementation is that dewlap phenotype may only respond to supplementation at a certain age or during development. For example, Fitze et al. (2003) showed that in great tit nestlings (*P. major*), only those carotenoids ingested within 6 days of hatching had an effect on plumage coloration. In addition, although our common garden design involving parents being fed identical diets strongly suggests that maternal effects do not explain the differences in dewlap color and pattern between *A. d. ignigularis* and *A. d. properus*, our experiment does not rule out the possibility that, in the wild, differences in carotenoid deposition in eggs from mothers may have some influence on dewlap color.

Another possibility is that carotenoids may not be present in *A. distichus* dewlap skin and/or carotenoids may not be primarily responsible for *A. distichus* dewlap color. Previous pigment analyses on *Anolis* dewlap skin suggest it likely that, like most other anoles, *A. distichus* dewlaps have both pteridines and carotenoids contributing to dewlap coloration (Ortiz et al. 1962, 1963; Macedonia et al. 2000; Steffen and McGraw 2007). However, the concentration and presence of carotenoids in explaining dewlap coloration can vary across species (Ortiz et al. 1963; Macedonia et al. 2000; Steffen and McGraw 2007) and even between sexes (Steffen and McGraw 2009). As an extreme example, the blue and mauve dewlaps of *Anolis conspersus* and *Anolis valencienni*, respectively, are devoid of carotenoids.
Figure 3. Dewlap color of F₁ males from each of the 4 breeding groups at 4, 6, 9, and 12 months of age: (a) relative reflectance at 365 nm, (b) relative reflectance at 655 nm, (c) cut-on wavelength, and (d) brightness at both the center and edge of the dewlap. Breeding group abbreviations: i = Anolis distichus igniarius, p = Anolis distichus properus, with the father stated first in each cross. Bars indicate standard error.
In addition, a comparison of *A. sagrei* and *A. humilis*, which have similar dimorphically colored dewlaps, revealed different concentrations of pteridines and carotenoids in both the red and yellow portions of the dewlaps, suggesting differences in their importance in explaining respective dewlap colors (Steffen and McGraw 2007). Although most anole dewlap skin examined thus far have shown carotenoids to be present and primarily responsible for yellow coloration (Ortiz et al. 1963; Macedonia et al. 2000), it is possible that the yellow coloration in the *A. distichus* dewlap is due to a yellow pteridine, sepiapterin, which has also been found in the dewlaps of some Jamaican and Puerto Rican anoles (Ortiz and Maldonado 1966; Macedonia et al. 2000). Future investigations of *A. distichus* dewlap skin are needed to ascertain the presence and concentration of carotenoids.

**Conclusion**

To our knowledge, our study is the first to use experimental crosses and rearing of laboratory-bred offspring in a common garden environment to test the hypothesis that natural variation in anole dewlap color and pattern is heritable. The fact that several aspects of dewlap color and pattern are heritable satisfies a core prediction of natural selection (Lewontin 1970) and is consistent with the hypothesis that natural selection can drive dewlaps to diverge adaptively among populations in response to variation in signaling conditions (Leal and Fleishman 2002; Leal 2004; Ng et al. 2013). In other cases, selection may be acting on dewlap color variation for enhanced species recognition in the presence of sympatric congeners (Rand and Williams 1977; Losos 1985; Macedonia and Stamps 1994; Vanhooydonck et al. 2009) or for reinforcement (Lambert et al. 2013; Webster and Burns 1973). The fact that our study finds that heritable variation exists between *A. distichus* populations representing 2 subspecies that appear to hybridize where they come into contact (Ng J, unpublished data) suggests that divergence at loci associated with dewlap color and pattern differences may be involved in the early stages of species differentiation. Whether speciation results directly from divergent natural selection on dewlap color and pattern requires more detailed study, including assessment of fitness of males with alternative dewlap phenotypes in nature and where geographically distinct populations come into contact and hybridize in nature.

**Supplementary Material**

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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