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Spectral Reflectance of Blanding's Turtle (*Emydoidea blandingii*) and Substrate Color-Induced Melanization in Laboratory-reared Turtles

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***Abstract.*—The color pattern of an animal may be involved with crypsis, social signaling, and thermoregulation. We studied substrate color-induced melanization in laboratory-reared Blanding's Turtles (*Emydoidea blandingii*) and compared spectral reflectance of hatchlings, laboratory-reared individuals, and wild caught adults. The dorsal ground color (background color of the head and carapace) of individuals reared on black substrates for 210 d remained dark or darkened while those reared on a white substrate lightened. The ventral integument regions (gular and plastral scutes) were not affected by the substrate color on which turtles were reared. Our data, and comparisons with other studies, indicated that different physiological control systems of the melanization process appear to exist among species and among tissue regions within individuals. Ground color of adults was lighter than that of hatchlings and in individuals reared on a black substrate suggesting that they experienced natural substrates that were not entirely black. The more yellow-orange gular regions of adults relative to hatchlings and laboratory-reared turtles might indicate ontogenetic changes or dietary carotenoid deficiencies experienced during rearing. Adult males tended to be darker than females and may have brighter orange yellow throats suggesting dichromatism. Ultraviolet reflectance was nearly absent in hatchlings, laboratory-reared individuals, and in adults suggesting that Ultra Violet light is not important for communication.**

Key Words.—melanization; pigmentation; spectral reflectance

Introduction

Color patterns of the integument of an animal can be involved in a range of functions (Cooper and Greenberg 1992). Ultraviolet reflectance and bright or contrasting color patterns that reflect within the visual spectrum may be used in social signaling within and between sexes (Endler 1992). Color patterns that obscure the outline of an animal or that resemble the color and texture of the environment may facilitate crypsis thereby reducing the probability of detection by predators or prey (Cooper and Greenberg 1992; Cuthill et al. 2005). Pigmentation that facilitates radiative warming, particularly within the infrared wavelengths, can serve an important function during thermoregulation (Trullas et al. 2007). The appearance of the integument of an individual may change over time as individuals move among environments, as social contexts change, or when the thermal environment is temporally variable.

Temporal changes in pigmentation of the integuments of some reptile species have been well studied. Color patterns of many lizards and snakes may be relatively constant throughout life (Olsson et al. 2013); however, many lizard species and some snake species can change the degree of darkness or pigmentation patterns to facilitate background matching or thermoregulatory capabilities. Such changes can occur over time periods that range from seconds to minutes (Cooper and Greenberg 1992; Rosenblum 2005; Olsson et al. 2013).

The functions of color patterns of freshwater turtles are less well understood than in other reptile groups. Dichromatism, or seasonal dichromatism, of the head region or in eye color has been shown to occur in a few species and could be important to intersexual communication (Moll et al. 1981; Ernst and Lovich 2009). A dark colored carapace would facilitate radiative warming and, combined with a light plastron, presumably represents cryptic countershading while the striping of the head and extremities and the mottling of the shell could break up the outline of the animal (Rowe et al. 2014a). Among populations within some species, the degree of melanization of the carapace and soft skin regions is greatest (turtles are darker) in populations that reside on dark-bottomed environments when compared to those living on light-bottomed environments (Rowe et al. 2006a; McGaugh 2008). In a range of species from various North American turtle groups, substrate color-induced melanization occurs when turtles are reared on black substrates versus white substrates (Rowe et al. 2006b, 2014b) and such phenotypic plasticity in melanization is reversible and can be induced in adults (Rowe et al. 2009, 2014a). Substrate-color induced

melanization may not occur in terrestrial emydid species (e.g., Eastern Box Turtles, *Terrapene carolina*) that reside on a mosaic of colors in a terrestrial environment (Rowe et al. 2014b).

The Blanding's Turtle (*Emydoidea blandingii*) is a North American freshwater turtle of the Midwest, Great Lakes, and northeastern United States and adjacent Canada that occupies highly vegetated wetlands (Ernst and Lovich 2009) and appears to prefer aquatic habitats with darkly colored water (Power et al. 1994). *Emydoidea blandingii* frequently travels large overland distances among aquatic habitats (Rowe and Moll 1991; Beaudry et al. 2009; Edge et al. 2010) or travels relatively short distances to terrestrial habitats for atmospheric basking or aestivating (Rowe and Moll 1991; Joyal et al. 2001). The ground color of the carapace of *Emydoidea* is dark gray to black and can be flecked with light cream to yellow spots or streaked with elongate stripes. The hinged plastron has a cream or yellowish ground color with black lateral blotches. Perhaps the most distinctive feature of *E. blandingii* is its elongate neck with its strongly demarcated, immaculate yellow-orange ventrum that extends from the lower jaw to the plastron (Ernst and Lovich 2009). The function of the yellow throat is unclear but males may use it to signal females during courtship (Baker and Gillingham 1983). Males have dark upper beak that may be used for sexual recognition during courtship or combat between males (Rowe 1992). Although females and males differ in body size and shell proportions (Rowe 1992), it is not known if color quality, including ultra violet (UV) reflectance, varies between sexes in *E. blandingii* or whether substrate color-induced melanization occurs.

We studied substrate color-induced melanization in hatchling *E. blandingii* reared on either black or white substrates for 210 d and spectral reflectance of wild caught adults. Using spectrophotometry, we evaluated color quality in terms of percentage reflectance across the UV and visual spectra and intensity, a dimensionless measure of brightness (Cooper and Greenberg 1992). We predicted that, because *E. blandingii* inhabits highly vegetated aquatic habitats with dark-colored water rather than open water muck or sand bottomed habitats, the dark, dorsal ground color of the head and carapace would show little, to no, capacity for color change. If substrate color-induced melanization occurs, then the dorsal integuments of turtles that are reared on a black substrate should remain black or darken while those reared on a white substrate would be expected to lighten relative to the initial hatchling color. Because the yellow regions of the throats of *E. blandingii* are likely to lack, or have very few, melanophores (melanin producing cells; Alibardi 2013), we did not anticipate any effect of a dark or a light substrate on color quality over time. Similarly, because ventral coloration is not affected by substrate color over time, at least in emydid species (Rowe et al. 2014b), we did not expect to see differences in plastral pigmentation between turtles reared on black or white substrates. If, in our wild caught adults, the yellow of the throat is a sexually selected trait, then males would be expected to have a brighter color than females and attractiveness could be accentuated by possession of UV reflectance. We also evaluated the likely sexually selected black upper beak of males for the presence of UV reflectance.

Materials and Methods

Collection of turtles and husbandry.—During early June, 2015, we collected three gravid female *E. blandingii* as they crossed roads in Isabella County, Michigan, USA, and we injected them with oxytocin to obtain eggs (Tucker et al. 2007). We incubated eggs in moist vermiculite at 27–30° C and we randomly assigned 33 individuals to either black or white substrate treatments. Following hatching, we clip-marked the marginal scutes of the carapaces of hatchlings for individual recognition using scissors. We randomly assigned using a random numbers table three to four hatchlings, each from a different clutch, to one of 10 translucent plastic rearing bins (51 × 34 × 30 cm) that we painted on the outside with black or white semi-gloss paint to 25 cm on a side. Initially, we maintained water levels in bins at about 5 cm but we increased the levels to about 10 cm as turtles grew. We conditioned water with Prime® (Seachem, Madison, Georgia, USA) and maintained water temperature at 27 ± 1° C by submersible heaters (Visi-Therm Deluxe, 150W; Marineland®, Blacksburg, Virginia, USA) and each bin had a basking brick that we painted either white or black. We positioned a shop light equipped with 34 watt Philips© Alto full spectrum fluorescent lights (Eindhoven, Netherlands) at 25 cm above the top of the rearing bin and set to a 12 h × 12 h dark-light cycle. We fed turtles *ad libitum* an alternating diet of ground beef heart and Reptomin® pellets (Spectrum Brands, Inc., Cincinnati, Ohio, USA) 6 d per week. Each bin received two submersible filters (Duetto 100, Marineland®) that we cleaned and we replaced water as needed following conditioning with Prime (Seachem Laboratories Inc., Madison, Georgia, USA). We measured spectral reflectances of the dorsal head skin, gular region, third vertebral scute, and the medial and lateral regions of abdominal scutes at 30-d increments between days 0 and 210. We thoroughly dried the shell and skin prior to obtaining spectral readings.

We also collected three female and three male adult *E. blandingii* from a farm pond in Isabella County, Michigan, USA, using baited hoopnets. We determined the sex of captured turtles by the presence of a concave

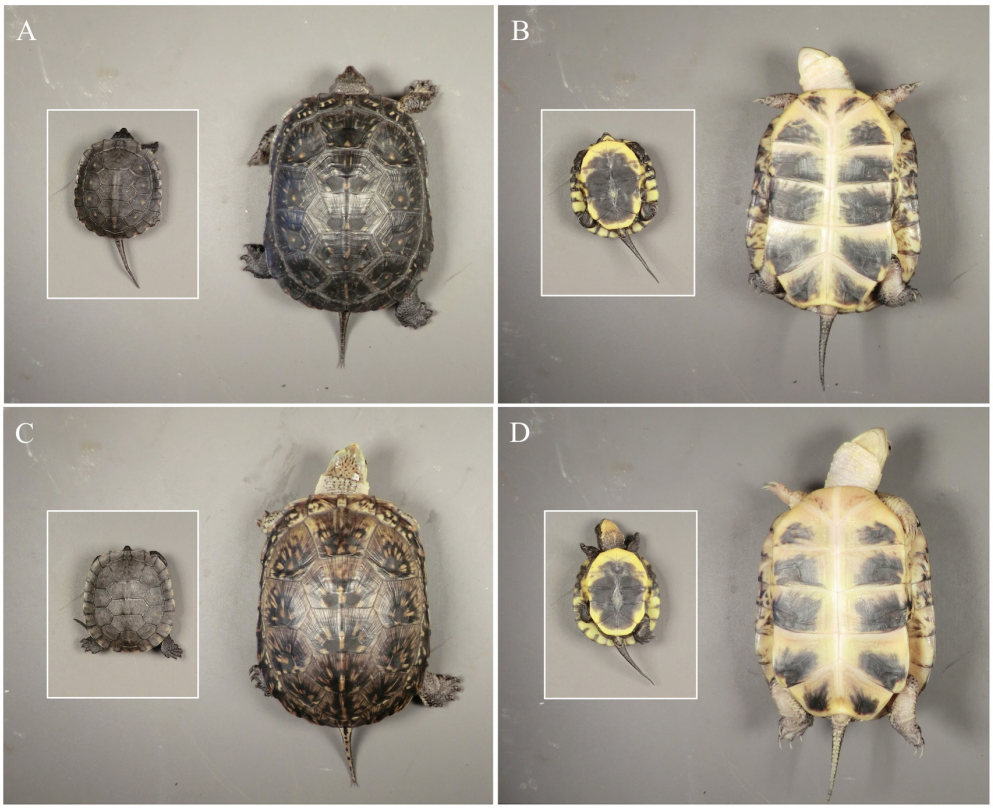

Figure 1. Dorsal and ventral views of two individual Blanding's Turtles (*Emydoidea blandingii*) after rearing on a black substrate (A and B) or on a white substrate (C and D) for 120 d and at hatching (insets). Both individuals were from the same clutch collected in central Michigan, USA. (Photographed by John Rowe).

plastron and relatively long pre-anal tail length in males (Ernst and Lovich 2009) and we notched the marginal scutes for individual recognition using a hacksaw blade. We restrained handheld turtles and we measured spectral reflectance on the dorsal head skin, the gular region, the carapace, the upper beak, and the third vertebral scute of the carapace.

Spectrometry.—To obtain reflectance measurements, we used a reflectance probe (R-400) connected to a deuterium-halogen lamp of an USB 2000 portable spectrometer, and a notebook computer running Spectasuite software (all from Ocean Optics, Dunedin, Florida, USA). We took measurements at a fixed distance of 2 mm from the integument surface and sampled at 45° from the perpendicular. To calibrate the reflectance measurements, we scanned a white standard (Labsphere Spectral WS-1; Labsphere, Inc., North Sutton, New Hampshire, USA) before we took a measurement. Our data was comprised of wavelengths that included the visible and ultraviolet spectra (300–700 nm) in 1 nm increments. We measured intensity, a dimensionless measure of darkness (Cooper and Greenberg 1992), as the total area under the spectral curve between 300–700 nm. We obtained three measurements per sample location per turtle and then averaged them per individual.

Statistical analyses.—Intensity data were normally distributed ($P > 0.05$ in all Shapiro-Wilk W tests) and so we analyzed variation in intensity of the dorsal head skin, gular region, third vertebral scute, and lateral and medial regions of the abdominal scute by general linear models (GLMs) with substrate color (black or white) and day-of-measurement as main effects, and with clutch origin and individual hatchling identification number included as random variations to account for autocorrelation. Least square means multiple *t*-tests (JMP Software, SAS Institute, Cary, North Carolina, USA) were used for *post hoc* comparisons of means adjusted for all main effects and interactions (hereafter referred to as adjusted means) when an GLM term was deemed significant at the 95% significance level.

Results

Effects of substrate color on melanization.—Substrate color-induced melanization occurred in dorsal, but not ventral, soft skin and shell components (Table 1; Figs. 1–3). Intensities of dorsal head skin (DHS) and

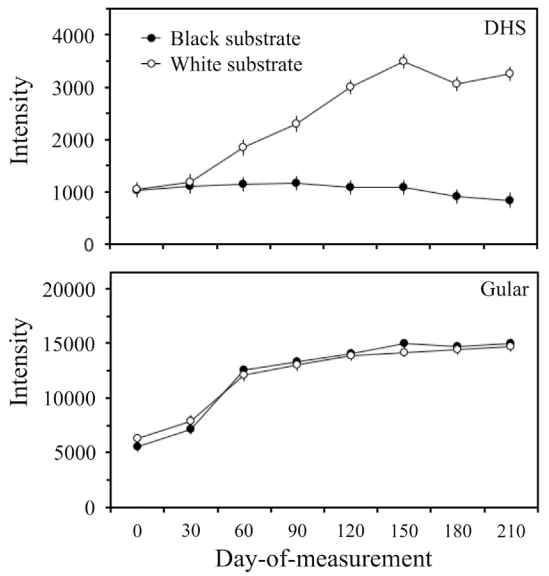

Figure 2. Adjusted mean intensity (± SE) of the dorsal head skin (DHS) and the gular area in Blanding's Turtles (*Emydoidea blandingii*) that were reared on either black or white substrates for 210 d.

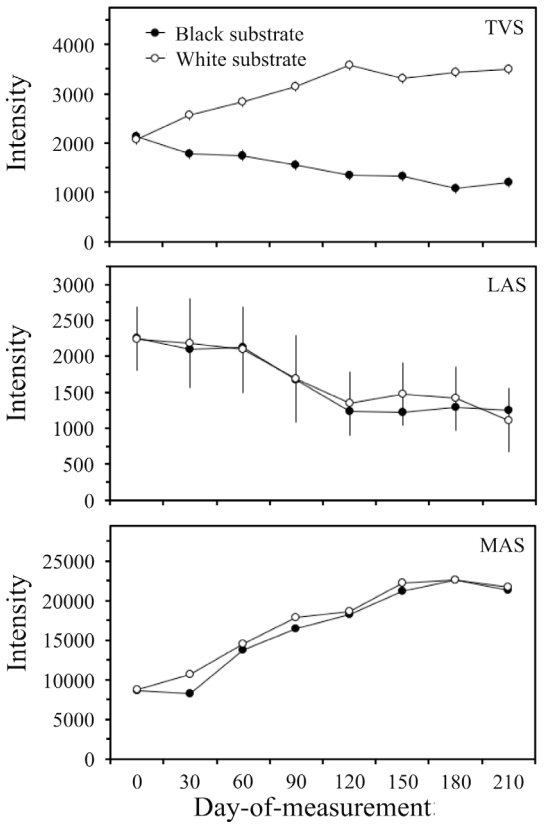

Figure 3. Adjusted mean intensity (± SE) of the third vertebral scute (TVS) and the lateral abdominal scute (LAS) and the medial abdominal scute (MAS) regions in Blanding's Turtles (*Emydoidea blandingii*) that were reared on either black or white substrates for 210 days.

the third vertebral scute (TVS) differed significantly by substrate color, day-of-measurement, and the interaction effects (Table 1). For the intensity of the gular area and lateral and medial regions of the abdominal scute (LAS and MAS, respectively), only the day-of-measurement effect was significant (Table 1). Adjusted mean *post hoc t*-tests indicated that the DHS of turtles reared on a black substrate was initially relatively dark and adjusted mean values remained constant throughout the duration of the study (Fig. 2). Adjusted mean intensity of the DHS in individuals reared on a white substrate, however, diverged from values of those reared on a black substrate by day 60 and significantly lightened (intensity increased) through day 120, remaining unchanged thereafter (Fig. 2). Regardless of substrate color, the skin of the gular region increased in intensity through day 60 and then remained approximately constant through day 210 (Fig. 2). The intensity of the TVS significantly lightened in turtles reared on a white substrate and darkened in turtles reared on a black substrate by day 60 and remained constant in intensity after day 120. The heavily pigmented region of the LAS remained constant in value between days 0 and 60, declined significantly between days 60 and 120 but attained constant values between days 120 and 210 (Fig. 3). Adjusted mean intensity of the relatively light MAS increased significantly between most 30-d increments through day 150 after which no change in intensity was observed (Fig. 3).

Spectral reflectance.—Comparisons of spectral reflectance curves indicated that adults tended to

Table 1. General linear model of intensity for soft skin regions that included dorsal head skin (DHS) and the gular area and shell that included the third vertebral scute (TVS) of the carapace and the lateral and medial areas of abdominal scute (LAS and MAS respectively) in Blanding's Turtle (*Emydoidea blandingii*) reared on either black or white substrates. Intensity measurements were made at 30 d increments over a 210 d period.

		DHS		Gular		TVS		LAS		MAS	
Source	DF	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	1,238	140.0	< 0.001	0.040	0.844	636.4	< 0.001	0.601	0.445	3.357	0.079
Day	7,238	25.86	< 0.001	226.5	< 0.001	2.369	0.024	25.91	< 0.001	113.6	< 0.001
Treatment × Day	7,238	30.39	< 0.001	1.421	0.202	37.61	< 0.001	0.477	0.851	0.574	0.780

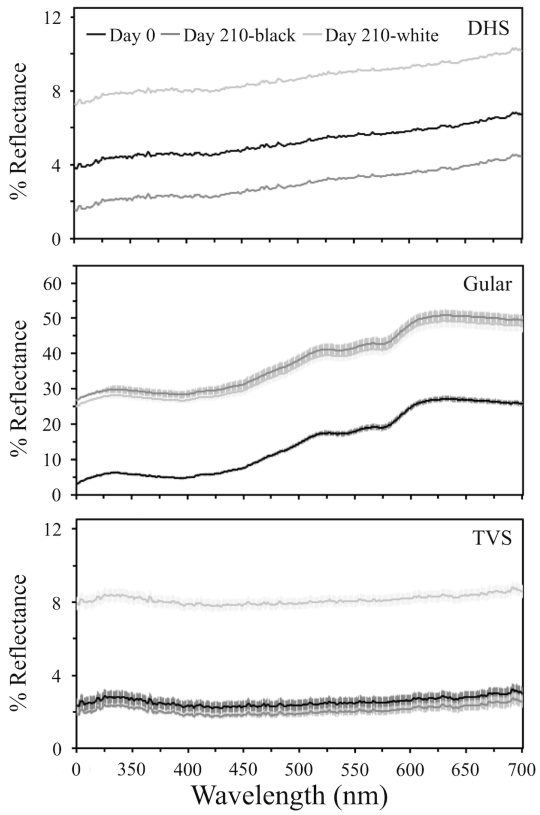

Figure 4. Spectral reflectance (mean ± SE at 1 nm increments) of the dorsal head skin (DHS), gular region, and third vertebral scute (TVS) of the carapace of hatchling Blanding's Turtles (*Emydoidea blandingii*) at hatching (Day 0) and after rearing for 210 d on a black or white substrate.

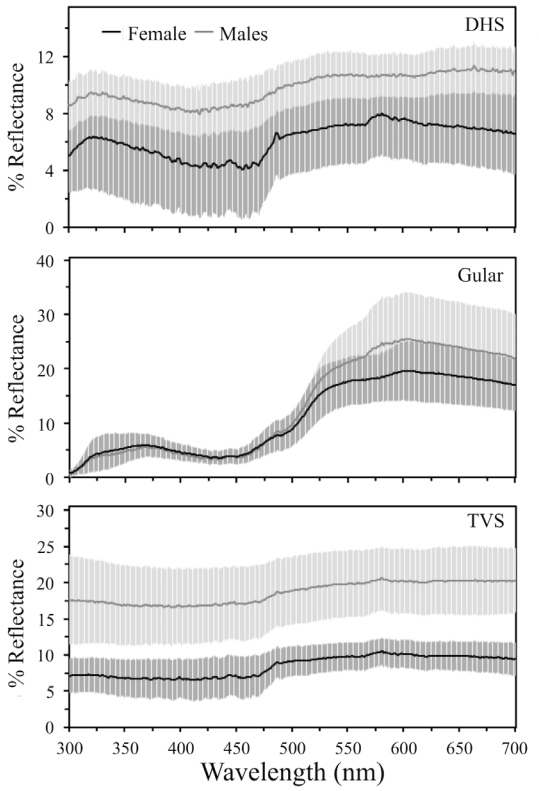

Figure 5. Spectral reflectance (mean ± SE at 1 nm increments) of the dorsal head skin (DHS), gular region, and third vertebral scute (TVS) of three adult female and three adult male, wild caught Blanding's Turtles (*Emydoidea blandingii*).

be brighter than hatchlings, and we found potential intersexual differences in brightness of adults. In hatchlings and in turtles reared on black or white substrates for 210 d, spectral reflectance curves indicated a low reflectance across the UV and visible wavelength ranges in both the DHS and TVS indicating that the ground colors were essentially black (Fig. 4). Spectral reflectance of the gular region showed high reflectance in the 500–600 nm range, but especially the 600–700 nm range, indicating that it was light yellow-orange in color. Based on the elevations of lines, the reflectance spectra of the DHS of wild caught adults were intermediate between hatchlings and in turtles that were reared on a white substrate (Fig. 5). The TVS of wild caught turtles was slightly lighter than both hatchlings and turtles reared on a black substrate but the TVS of adult females was lighter than hatchlings or turtles reared on black or white substrates. The upper beak of males was nearly black and darker than that of females. The spectral reflectance lines of gular regions of adults had relatively high elevations and a greater percentage reflectance in the yellow range of the visible spectra when compared to hatchlings. In adults, reflectance in the 320–400 nm range was < 7%, indicating very little UV reflectance in the carapace and head region (Fig. 5).

Discussion

In contrast to our expectations, we demonstrated substrate color-induced melanization under laboratory conditions in *E. blandingii*, which seems typical for a number of other North American species studied to date (Rowe et al. 2014b). Substrate color-induced color change in emydid and chelydrid turtles involves the production of melanosomes (melanin-filled vesicles) within dermal melanophores that either accumulate (Lindgren et al. 2015) or are transferred to, and accumulate in, the keratinocytes of the epidermis (Alibardi 2013; Rowe et al. 2013b). Differential dispersion of melanosomes within melanophores (Woolley 1957) and variation in melanosome size (Barsh 2005) may also play a role during the melanization. That some species develop a very dark black pigmentation when reared on a black substrate (Rowe et al. 2014b) suggests that eumelanin (a gray-black pigment), as opposed

to pheomelanin (a red-brown pigment), is employed during melanization in freshwater turtles. Although the degree of melanization between days 0 and 210 in *E. blandingii* reared on a black substrate is less than in some previously studied species (Rowe et al. 2014b), we presume that eumelanin production is involved with melanization. The melanization process in reptiles likely involves variation in both melanocortin secretion as well as variations in the activity of its receptor, Mc1R, that result from coding sequence mutations (Rosenblum et al. 2004; Hoekstra 2006; Lindgren et al. 2015).

Some differences in the control of melanization in response to different substrate colors appear to exist among species and within individuals (Rowe et al. 2014b). For instance, some species (e.g., Spiny Softshell Turtles, *Apalone spinifera*, Smooth Softshell Turtles, *A. mutica*, Common Snapping Turtles, *Chelydra serpentina*, and Midland Painted Turtles, *C. picta marginata*) are initially heavily pigmented as hatchlings and remain dark, or slightly darken, when reared on a black substrate but lighten when reared on a white substrate. In contrast, other species (e.g., Northern Map Turtles, *Graptemys geographica*, Common Musk Turtles, *Sternotherus odoratus*) are initially dark but become lighter over time with individuals reared on white substrates becoming lighter than those reared on black substrates (Rowe et al. 2014b). Still other species, such as Red-eared Sliders, *Trachemys scripta elegans*, have intermediate pigmentation levels as hatchlings and either lighten or darken on white or black substrates respectively. Interestingly, in *E. blandingii* reared on a black substrate, the initially dark dorsal head skin remained constant in intensity while the somewhat initially lighter carapace became significantly darker over time. The light and dark blotches of the plastral scutes were unaffected by substrate color but both darkened over time and it is possible that cells of each region darkened through the use of pheomelanin and eumelanin, respectively. The relatively light regions of Eastern Hermann's Tortoise (*Eurotestudo boettgeri*) shells contain pheomelanin as a pigment (Roulin et al. 2013) and it is possible that pheomelanin is also used by other turtle species as well. That different tissues (carapace, dorsal head skin, and dark abdominal scute regions) responded differently to a black substrate indicates regional tissue-level differences in response to melanocortin secretion. Such differences among tissues would probably involve regional differences in plasma membrane receptor responses and variations in second messengers that respond to melanocortin secretion (Aspengren et al. 2008).

Comparisons of laboratory reared *E. blandingii* and wild caught adults indicated possible ontogenetic changes in color or environmental influences on color. The ground color of our hatchlings and of laboratory reared *E. blandingii* was slightly darker than in adults suggesting that wild caught turtles probably lightened with age and perhaps that adult turtles experienced natural substrates that were on average lighter than the entirely black substrate of our rearing bins. Although our relatively small sample sizes for wild caught turtles preclude definitive conclusions, the relatively dark ground colors of males when compared to females would be consistent with sex-specific melanism observed in other emydid turtles (Lovich et al. 1990).

Whereas we had no wild caught juveniles with whom to compare, the gular regions of wild caught adults were clearly more intense yellow-orange than were hatchlings or laboratory-reared turtles. We assume that our hatchlings from laboratory-incubated eggs would be similar in color to hatchlings from naturally incubated eggs and so an ontogenetic development of intense yellow in adults seems likely. In turtles, yellow-orange colored pigment is produced by xanthophores and lipophores (Alibardi 2013), presumably through the use of pteridines or dietary carotenes (Olsson et al. 2013). Therefore, the lack of yellow pigment in our laboratory-reared turtles at day 210 may have been age related to, or resulted from, insufficient dietary carotene. Interestingly, the reflectance curve of the adult male gular region peaked slightly higher than that of adult females and could indicate that the bright yellow throat of males is a sexually selected trait; although, we recognize that our sample size for each sex was relatively small. A thorough evaluation of intersexual variation in the yellow throat *E. blandingii*, with expanded sample sizes, could be important because the expression of carotene based traits, such as a yellow-orange throat, might be an indicator of immune system or antioxidant functions and thereby be an important condition-dependent intersexual signaling trait in males (Polo-Cavia et al. 2012; Ibáñez et al. 2013; Olsson et al. 2013). Indeed, the yellow throats of males are aggressively waved within the visual fields of females during mating (Baker and Gillingham 1983). The restriction of black upper beak to males would certainly suggest a role in intra- or intersexual communication (Rowe 1992) that could contrast sharply with the yellow of the gular region. Ultraviolet reflectance may also be used in visual communication (see Olsson et al. 2013 for a review in lizards). Although UV sensitive cones have been shown to exist in the retinas of *Trachemys scripta elegans* (Loew and Govardovskii 2001), little UV reflectance in the integuments of adult freshwater turtle has been shown to occur in species studied to date (Spotted Turtles, *Clemmys guttata*, Rowe et al. 2013a; *Chrysemys picta*, Rowe et al. 2014a; and *E. blandingii*, this study). Lipetz and MacNichol (1982) studied the

visual system of *E. blandingii*, which seems similar to that of *Trachemys scripta elegans*, although they did not assess cones within the UV spectrum of reflectance.

That melanization occurred in the integuments of the dorsal surfaces of the turtle, but not on the plastron or gular region, suggests a potential background matching function. When predators view turtles from above, a dark carapace, head and extremities would converge with the color of a dark substrate (Rowe et al. 2006a; Rowland et al. 2009). In a light-bottomed environment, turtles with a light-colored dorsum might be less conspicuous to predators than would a dark-colored turtle. When viewed from below by a predator in the water column, the light-colored regions of the plastral scutes may lighten a backlit plastron while the lateral dark patches of the plastral scutes could obscure the outline of the turtle, particularly when floating vegetation is present (Rowe et al. 2014a). If so, the large range of turtle species with dark carapaces, light plastrons, and patterned plastral and marginal scutes would suggest that such potential countershading and obliterative patterning (Rowland et al. 2009) is likely to be common in freshwater turtles (Rowe et al. 2014a). Under natural conditions, dark and light-colored turtles that reside in dark and light-bottomed substrates respectively have been demonstrated for very few species (*Chrysemys picta marginata*, Rowe et al. 2006a, *Apalone spinifer atra*, McGaugh 2008) and reduction of predation rates through background matching has yet to be demonstrated for any turtle species. However, the Asian Four-eyed Turtle (*Sacalia quadriocellata*) attains the highest densities in habitats in which it most closely matches its background, thus implying a survival advantage to background matching (Xiao et al. 2016). Dorsal color patterns that presumably facilitate background matching are pervasive in reptiles (Norris and Lowe 1964; Hamilton et al. 2008; Isaac and Gregory 2013) and the risk of predation through background matching has been demonstrated in the field (Stuart-Fox et al. 2003; Cuthill et al. 2005; Vignieri et al. 2010). However, because the carapaces of *E. blandingii* do not lighten to the extent that they do in some other species, relatively dark carapaces may not afford crypsis to individuals in a light-bottomed habitat. Furthermore, *E. blandingii* inhabit heavily vegetated aquatic habitats with characteristically dark benthos and might only rarely encounter light-bottomed habitats such as open sandy lakes or rivers. It is possible that species within the *Actinemys-Clemmys-Emydoidea-Emys-Terrapene* clade (Fritz et al. 2011) have limited, or even vestigial, capabilities for substrate color-induced melanization given their propensities to be terrestrial or to occupy shallow, more densely vegetated habitats (Ernst and Lovich 2009).

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