BACKGROUND COLOR-MATCHING IN THE TEXAS HORNED LIZARD (*PHRYNOSOMA CORNUTUM*)

by

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journey to re-carpet the Texas landscape with these beloved icons of the Old West. Robert Reed McClure at Sul Ross State University likewise provided some much-needed color-calibrated photos of lizards from the Big Bend. The International Herpetological Symposium, East Texas Herpetological Society, and the Horned Lizard Conservation Society all provided me with generous grant monies for graduate school research. Jeff Adams (of SnakeDays), Bob Hansen (editor of *The Herpetological Review*), John Karges, Chris McMartin, Gerry Salmon (Southwestern Center for Herpetological Research), Craig Trumbower, and many others in the herping community all provided much-needed heckling and support in various ways. I would like to thank Dr. Dean Williams for being a great advisor with whom I got along famously—no easy task. TCU GIS instructor Tamie Morgan helped me with a difficult semester. In fact, I have never considered myself a "good" student, ever. My main saving grace is that I am just stubborn enough and enthusiastic about the subjects I have studied and feel enamored by the romance of science—i.e. the "cosmic perspective", but I have needed a slew of patient professors and teachers to help me through personal obstacles to my formal education throughout my life. Stevie Mirkin was invaluable as a labmate, fieldmate, sounding board, and frequent meal companion. Rachel Alenius provided good conversation, a welcoming horned lizard pin, "Hobbit" music, feedback, and wheels on long trips. Thanks to my brilliant committee members, Dr. Sarah Hill, Dr. John Horner, and Dr. Laura Luque for their valuable suggestions, expertise, probing questions, and support. Finally, how can I adequately express my love and admiration for my Colton? His love, goodness, and encouragement are the most important things I have.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

Introduction

As a general rule, animals tend to blend in, rather than stand out, with their colors and patterns matching the overall colors and patterns of their background (Diamond and Bond 2013). The signal of background color matching is strongest in species that are more sedentary (Thayer 1909; Egan et al. 2016), in species that have high reproductive isolation per unit area (Rosenblum 2008; Robertson and Rosenblum 2009; Sobel et al. 2010; Rosenblum and Harmon 2011), and in areas where the background surroundings are most uniform in color (Hoekstra 2010). These phenotype-to-environment color adaptations have important implications for conservation and the maintenance of biodiversity, as organisms adapted to match their respective environmental background colors have been shown to decline in number when the matching background environment becomes rare or changes into a different hue (Majerus 2009).

Historically, quantitative background-color matching studies of wild fauna have been largely limited to those researchers who have access to reflectometers (or spectrophotometers), and to organisms and their substrates that can easily be manipulated (captured, procured, and transported back to laboratory facilities) for color analyses (Benson 1933; Dice and Blossom 1937; Hoekstra and Nachman 2003; Rosenblum 2006). These sorts of studies therefore have often excluded organisms that are difficult to maintain in captivity, are not easily captured or restrained (e.g. large, flighted, or venomous species), or are complicated further by phenotypically plastic color changes that occur to individuals when subjected to environmental stressors such as temperature changes or handling (e.g. many fishes, anurans, and

lizards). Finally, while standardized laboratory analyses are useful for controlling lighting conditions, these studies bring with them a number of drawbacks. A relatively recent methodology paper summarized a list of practical and theoretical limitations to these types of colorimetric studies (Samia et al. 2015). An alternative approach to using expensive spectrophotometers in controlled lighting and laboratory settings is with digital photography (Stevens et al. 2007; Troscianko and Stevens 2015). Until recently, however, there has not been a way to use this commonly accessible technology to quantify background-color matching.

Among the most cryptically colored of terrestrial vertebrates in North America are the horned lizards of the genus *Phrynosoma*. Biologically, horned lizards are ecological specialists — of the 96 species of lizards in the American Southwest (as defined by Jones and Lovich 2009), the nine species of horned lizards are perhaps the group that depends most on crypsis and remaining motionless at the approach of a predator (Norris and Lowe 1964; Pianka and Parker 1975). While not all members and populations of the genus are in serious decline, the Texas Horned Lizard (*Phrynosoma cornutum*) is a threatened species in the State of Texas (Price 1990). Most of its populations have been reduced or altogether extirpated from the landscape in the last half century (Price 1990). Culturally, it is an important icon of the American West, and among the more than 200 native reptile species in Texas, it has been officially recognized as the State Reptile.

Phrynosoma cornutum have highly variable dorsal skin coloration and anecdotally appear to covary with geographic location and prevailing substrate type (Price 1990). This alleged concealing coloration, however, has not been tested.

Among the other proposed but untested crypsis adaptations for *P. cornutum* is that the species is adapted to terrestrial habitats with sun-bleached plant stems, as hypothesized by the discrete color pattern of a ubiquitous white vertebral stripe spanning the dorsum (FIG. 1; Sherbrooke 2002). Understanding whether Texas Horned Lizards match their substrate is potentially important because several Texas zoos and Texas Parks & Wildlife are releasing both wild caught and captive-raised babies into areas where Texas Horned Lizards have become extinct. The bulk of the reintroduced individuals are being lost to predation (Nathan Rains, pers. comm.). This reintroduction effort is currently being done without any knowledge of the role that geographic color pattern adaptation and crypsis plays in survival, and so it is possible that the high predation levels might arise from lizards being introduced into areas where they do not closely match the substrate.

Figure 1. An adult *P. cornutum* from Rolling Plains Quail Research Ranch, showing apparent plant-stem mimicry with its dorsal stripe, as hypothesized by Sherbrooke 2002.

In this study, I use a new method for measuring animal-background contrast with digital photographs called the Color Overlapping Index (COI) to test the hypotheses that (a) Texas Horned Lizards' color matches the substrates they are found on and (b) that the vertebral white stripe helps conceal the lizard. Specifically, I ask three questions: 1) do Texas Horned Lizards from specific populations match their own substrates better than substrates from other populations?, 2) do vertebral stripes of Texas Horned Lizards match nearby plant stems better than other nearby objects (i.e. soil and gravel) in their environment?, and 3) do the vertebral stripes of Texas Horned Lizards from specific populations match their own habitat's plant stems better than the plant stems from other Texas Horned Lizard populations?

Materials and methods

Data acquisition

Photos taken in the field. In situ photographs of lizards were taken in the field from May 20th - August 31st, 2018 in populations with grey-tan-hued lizards (Karnes County, Texas) and reddish-hued lizards (Rolling Plains Quail Research Ranch [RPQRR] in Northwest Texas and the Chaparral Wildlife Management Area in South Texas). Lizards included in the analyses were not handled prior to photographing in order to not influence stress-induced color change which has been documented in this and other lizard taxa (Parker 1938). Digital photographs were taken at ~1m and 2m directly above lizards in RAW format with a Canon EOS Digital Rebel XSI SLR camera with Canon EF-S 18-55mm lens. To make photographs standardized, higher quality, and with less noise, all images were shot outdoors, *in situ* (precisely where lizards were found) with ISO-200 at aperture f/14 with a shutter speed of 1/400 s—these are optimal settings chosen for shooting in variable amounts of sunlight, with higher depth of field (for background comparisons), and for freezing the occasional moving object, respectively. Flash was not used in order to mitigate: any unnatural shadows, modifications to the habitat's natural lighting spectrum, and color metamerism—i.e. a phenomenon that renders dissimilar wavelengths of color similar to the observer, or vice versa (Fairchild and Heckaman 2016; Qiu et al. 2017; Samia et al. 2015; Stevens et al. 2007). White balance and correct color values were achieved in the field by placing a 24-patch color standard, the X-Rite ColorChecker Passport (Photo version), within ~10cm of each individual lizard in the photo frame, and using the ColorChecker Camera Calibration software

version 1.0.0 (X-Rite Inc. Grand Rapids, MI 49512, USA) and the X-Rite ColorChecker plugin in Adobe Lightroom to create DNG profiles and adjust field photographs to their true color values (see Appendices A & B; Myers 2009; Sakai et al. 2013; Varghese et al. 2014).

Photos sampled from citizen scientists. In situ photographs of non-handled lizards were sampled from citizen scientists via the iNaturalist repository of data for this taxon. Only vouchered photos that were vetted as "research grade" were sampled to supplement photos taken by the author in order to accumulate at least 10 photos of individual lizards per locale (see Appendix C for lists of specimens). Additionally, several RAW photographs of *in situ* specimens (n=9) were taken with the ColorChecker Passport (Photo version) included near the vicinity of Alpine, Texas by Robert Reed McClure (Biology Dept., Sul Ross State University) that we also used in the calibrated vs uncalibrated test. The camera and settings used were a Canon EOS Rebel T4i with ISO-100, aperture f/11, shutter speed 1/320 s, with no flash at ~1m above the lizards.

Sampling the color information of the photographs. Color data for images were first extracted by using the rectangular selector tool in ImageJ to select both an area of each lizard's body and an equiareal portion of the substrate it was found on (Fig.2). In some analyses comparing color-calibrated photos to the same set of uncalibrated photos, the freehand selector tool in ImageJ was used to select the entire body of the lizard and the entire soil sample, and the XY coordinates of each selection were saved as .TXT files and reimported for the comparison so that the exact same selections were being compared and contrasted. The substrate portion

was picked randomly by using the Grid plugin for Image J (see Appendix A) with the "random offset" on, with the cells of the grid sized to the same square pixel area as the lizard portion. All cells immediately adjacent to the lizard with less than ~15% non-soil debris (and the remainder, soil) and in the same lighting as the lizard selection were considered eligible for color analysis. All eligible cells were then assigned a number and picked using a random number generator to mitigate sampling bias. Then, color frequency (i.e. LUT) tables were obtained for the selected portions of the images using the histogram function of the Color Inspector 3D v. 2.3, a plugin of ImageJ. All measurements of the present study were performed with a color interval of 30 (the default of the Color Inspector 3D v. 2.3 program).

Figure 2. *In situ* photo of unmanipulated lizard with typical ColorChecker placement and rectangular selection of lizard and adjacent substrate with ImageJ photo-editing software.

Quantifying animal-background matching. Color tables from the lizards and corresponding substrates were then pasted in Excel spreadsheets, and the percentage of background color matching was quantified by using the Color Overlapping Index (COI) in RStudio (Samia et al. 2015). The COI is an index that calculates the percent overlap between color classes in two or more images. COI is defined quantitatively as the sum of the lowest relative frequencies among the color classes shared by animal and substrate (Samia et al. 2015). It is calculated as:

$$COI = \Sigma \min(p_{1i}, p_{2i})*100$$

where, p_{1i} and p_{2i} are the relative frequencies of the i*th* color class of the animal (p1) and the substrate (p2). COI scores range from 0 to 1. Scores near 0 display high contrast between lizard and substrate and very little color overlapping, while higher scores indicate more extensive color matching. More precisely, the COI is a proxy for measuring the percent overlap in color-pattern, since the frequencies of individual color classes (counted in numbers of pixels) is one method for quantifying color-pattern.

COIs of calibrated vs uncalibrated photographs. In order to ascertain the accuracy and suitability of using uncalibrated photos for analyses, COIs were taken from the same selections between calibrated and uncalibrated versions of the same photos of 36 individual adult *P. cornutum*. Within-habitat COIs were also calculated from calibrated and uncalibrated photos (n = 10 lizards) from a single population in Karnes County, TX. In these comparisons, the entire bodies of lizards were freehand selected (instead of the usual smaller rectangular selections) and stored as XY

coordinates (in .TXT files) and imported for use in both color-calibrated and uncalibrated sets. This test was devised to ensure that photographic lighting conditions could not account for any significant trend or discrepancy in COI scores between soil substrates in its broader habitat.

COI scores within own habitat and between different habitats. Four populations of *P. cornutum* were chosen for this analysis due to their differences in soil substrate colors: 1) Alpine, TX (brown soil), 2) Freer, TX (tan soil), 3) Karnes City, TX (gray soil), and 4) RPQRR, TX (red soil) (FIG. 3). A sample of 10 lizards from each locale (total n=40) were scored for their COIs both within their respective habitats and between the other three habitats. This test was devised to verify if *P. cornutum* in a population really do match their own habitat substrate colors better than the colors of substrates from other regions.

Lizard-to-lizard COI scores to test for variance differences between two populations. This test was developed because a significant difference in "*between-habitat*" COIs was detected. A sample of n=10 lizards each from Karnes City, TX and RPQRR, TX (total n=20) were freehand selected around their entire bodies in ImageJ. Each lizard's color data was then compared via COI's "fuzzy mode" to the other nine lizards in its own respective habitat, and the total number of colors of each lizard's dorsum was also recorded.

COI lizard-to-substrate scores of 100 individual P. cornutum across the species' range in the United States and Mexico. To get an estimate for the overall trend in background color-matching for *P. cornutum*, COI was calculated for 100 adult lizards and their individual *in situ* substrates sampled across the species' range in North

Texas, South Texas, West Texas, southwestern New Mexico, and Chihuahua, Mexico (see Appendix C for the list of individuals).

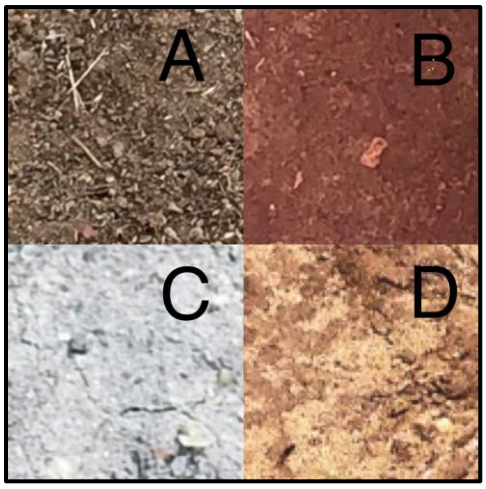

Figure 3. Soil samples representing different colored substrate habitats in (A) Alpine, TX (brown), (B) RPQRR, TX (red), (C) Karnes City, TX (gray), and (D) Freer, TX (tan).

Testing the hypothesis of vertebral stripe mimicry of sun-bleached grass stems. A sample of n_1=9, n_2=7, and n_3=7 *P. cornutum* from all three major biogeographic clades (as in Williams et al. 2019)—Northern, Western, and Southern, respectively—was analyzed to test (a) whether vertebral stripes of *P. cornutum* match nearby plant stems better than other nearby objects (i.e. soil and gravel) in their environment and (b) whether the vertebral stripes of *P. cornutum* from each biogeographic clade match their own group's plant stems better than the plant stems from the other *P. cornutum* populations. High-resolution *in situ* images of lizards were chosen and the vertebral stripe of each lizard was freehand selected in ImageJ, along with 2 – 5 nearest (to the lizard) bleached grass stems, and as much soil and gravel substrate as possible (i.e. as much as was in the frame). The COI scores of each lizard's vertebral stripe to the grass stems and the soil/gravel

substrate were then calculated. Then, COI scores of each lizard's vertebral stripe to the grass stems of the other lizards within its own clade, and between the other two clades were calculated.

Statistics.

I used non-parametric Mann–Whitney and Kruskal-Wallis tests for most COI comparisons. I used a linear regression model to compare COI values before and after color calibration and t-tests assuming unequal variances for all other comparisons if they met test assumptions. Means ($\tilde{y}$) are presented as +/- standard error (SE).

Results

Calibrated vs Uncalibrated Photographs

There was no significant difference in COI scores of lizards and the soil in their pictures (n = 36 lizards) using color-calibrated or uncalibrated photos. The linear regression between calibrated and uncalibrated photos had an R^2 of 0.99 and a slope which did not differ significantly from 1 ($y = 1.004x - 0.21$, $F_{1,34} = 2{,}602.8$, $P = 1 \times 10^{-33}$, $P = 0.43$ for a slope $\neq 1$) (FIG. 4).

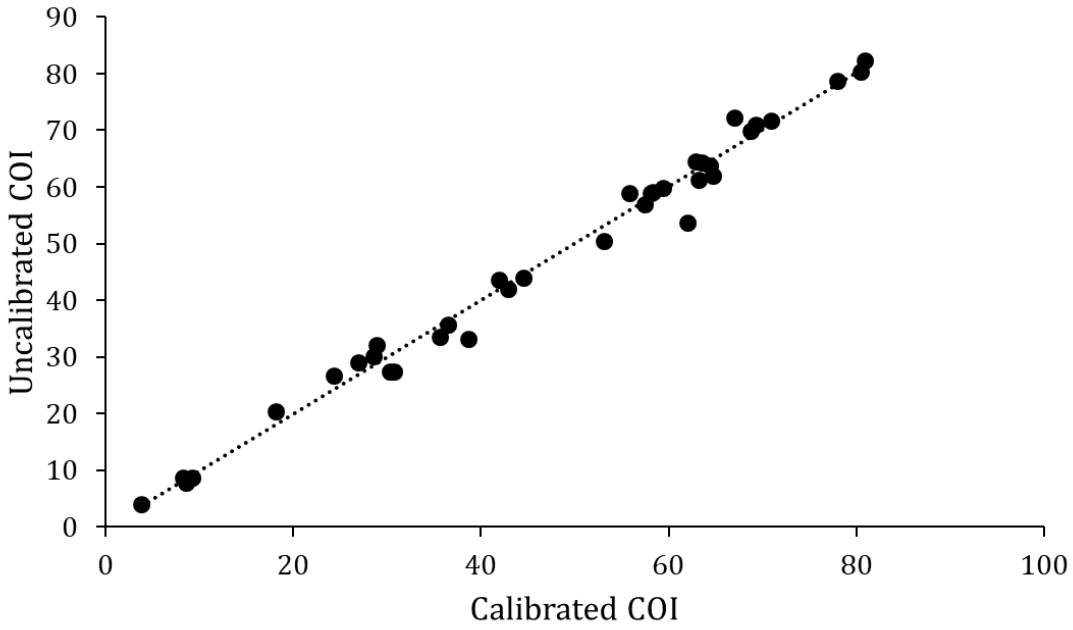

Figure 4. Linear regression model showing no significant difference between using color-calibrated photos or uncalibrated photos to calculate COI.

I then compared the COI values calculated between pictures that were calibrated with those that were uncalibrated using 10 individuals from Karnes City. The average COI difference between calibrated and uncalibrated was low ($\bar{y}$ = -1.25; range: -1.88 – 3.90) and median COI values were almost identical between calibrated and uncalibrated comparisons (Median COI = 56.96 in calibrated and 56.52 in uncalibrated comparisons; Mann–Whitney W = 100, $n_1 = n_2 = 10$, P = 0.73). The average number of colors each lizard had on its dorsal aspect was also not different between calibrated (89.8 ± 7.66 SE colors) and uncalibrated photos (85.3 ± 6.68) (t-test: t = 2.10, df = 18, P = 0.66). These analyses suggest that color calibration of photos does not appreciably change the calculated COI values and so the rest of the analyses have been performed on non-calibrated photos.

COI scores within own habitat and between different habitats.

The COI of a lizard was higher ($\bar{y}$ = 62.64 ± 2.37, median = 65.57) to the soil within its own picture than to the average for its habitat ($\bar{y}$ = 45.14 ± 1.54, median = 45.97) (Mann-Whitney, W = 1095, P = 4.5 X 10^{-7}) (FIG. 5). The average within habitat COI was higher than the average between habitat COI indicating that lizards more closely matched the soil in their own habitat than in other habitats (Mann–Whitney W = 744, $n_1 = n_2 = 40$, $P < 0.001$) (FIG. 6).

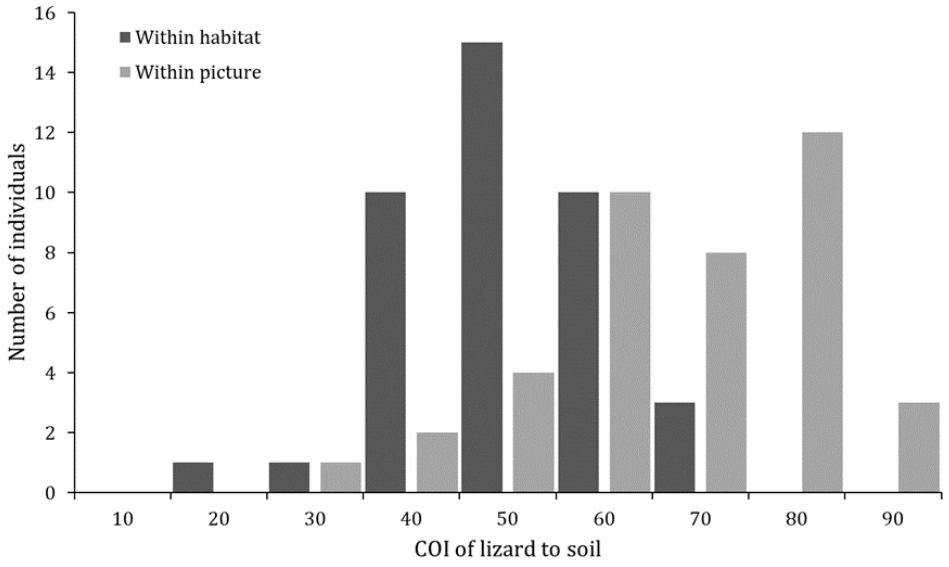

Figure 5. The COI of a lizard to its own substrate, i.e. within its own picture, averaged higher than the COI of the lizard to all the sampled substrates within its habitat.

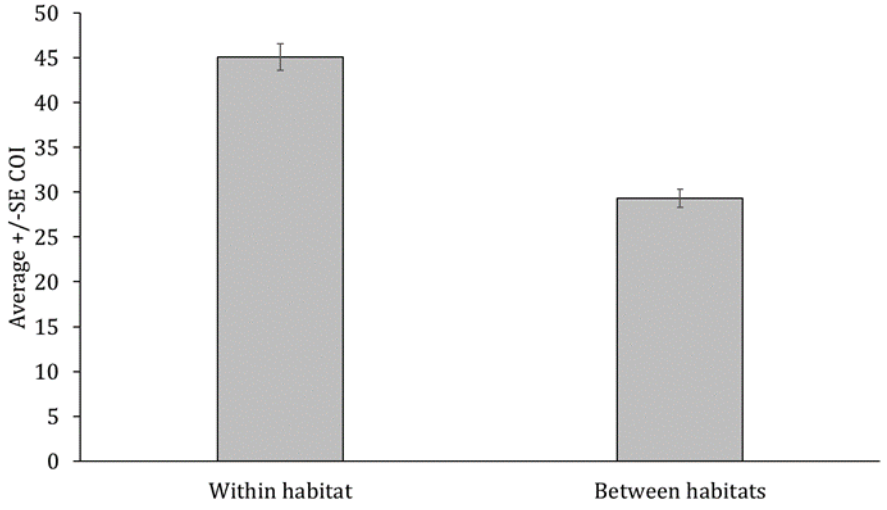

Figure 6. The average "within habitat" COI was higher than the average "between habitats" COI (n=40 lizards).

The four sites differed significantly in their within habitat (Kruskal-Wallis, H = 22.36, df = 3, P = 5.5 X 10^{-5}) and between habitat COIs (Kruskal-Wallis, H = 13.23, df = 3, P = 0.004) (FIG. 7). Lizards from Karnes City most closely matched their own soils and had the lowest COI between habitats compared to the other sites. Within habitat COIs were lower for Freer than Alpine and RPQRR and between habitat COIs were similar between these three sites. I then asked if lizards in Karnes City might be more variable in their colors than lizards at RPQRR which might account for the lower between habitat COIs. Average lizard-to-lizard COI variances in Karnes City were higher than in RPQRR (Median = 271.41 and 135.4 respectively; Mann–Whitney W = 132, $n_1 = n_2 = 10$, P = 0.045). Karnes City lizards also had more colors ($\bar{y}$ = 85.3 ± 6.68) on their dorsal skin than did RPQRR lizards ($\bar{y}$ = 68.6 ± 3.16) (t-test: t = 2.26, df = 13, P = 0.04).

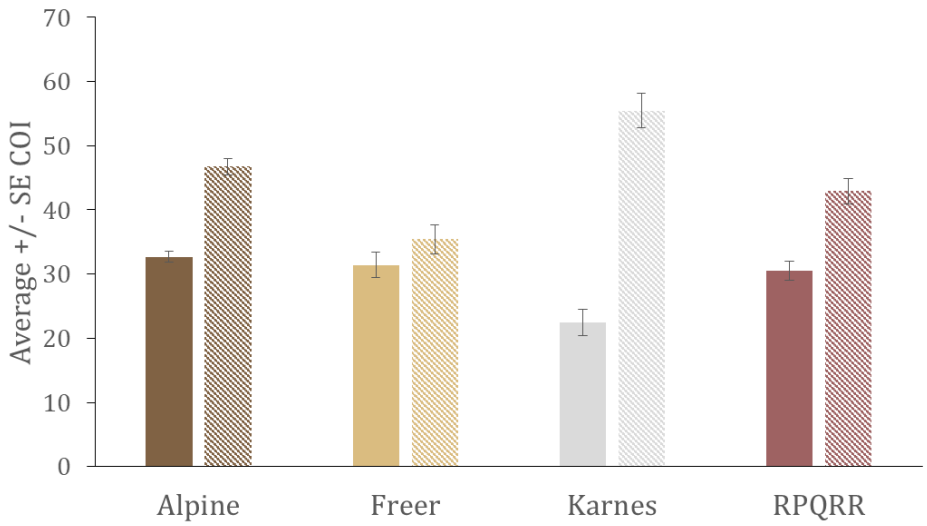

Figure 7. Average COIs between lizards and soils. Colors match the most common soil color from each site. Solid colors are between habitat comparisons and hatched colors are within habitat comparisons (n = 10 lizards per site).

I then compared 100 lizards to the soil in their pictures using 25 pictures I took and 75 from iNaturalist. The average COI score for a lizard to its own soil substrate across the species' range was $\bar{y}$ = 60.92 ± 1.37 and ranged from 26.7 – 86.5, with 81% of the comparisons at a COI of 50 and above (FIG. 8).

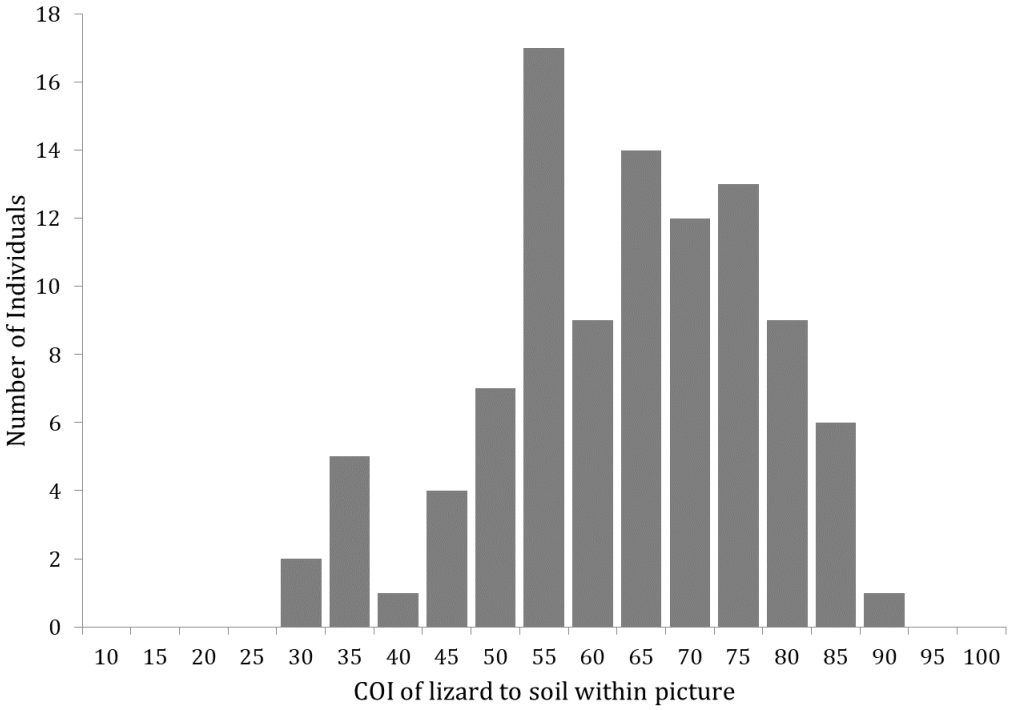

Figure 8. The distribution of COI scores for 100 lizards to their own substrates across the *P. cornutum* range.

Testing the hypothesis of vertebral stripe mimicry of sun-bleached grass stems.

Mean stripe-to-stem COI scores were higher than the mean stripe-to-soil/gravel COI scores (Mann-Whitney W = 280, P = 1.1×10^{-8}) (FIG. 9). Similar to the results for soil, individual lizards matched the stems in their own picture more closely ($\bar{y}$ = 46.88 ± 3.14 SE) than to others within their cluster ($\bar{y}$ = 28.05 ± 2.14) (Mann-Whitney W = 353, P = 5×10^{-5}). The "between biogeographic clusters" stripe-to-stem COI values ($\bar{y}$ = 25.62 ± 1.34), however, were comparable in magnitude to the within

biogeographic clusters stripe-to-stem COI values ($\bar{y}$ = 28.05 ± 2.14) (Mann-Whitney W = 858, P = 0.50). A closer look at the data reveals that the "within Western clade" stripe-to-stem COI scores are significantly higher than both the Northern clade (Mann-Whitney W = 53, P = 0.02) and the Southern clade of lizards (Mann-Whitney W = 70, P = 0.03) (Fig. 10). "Within cluster" comparisons between the Northern and Southern clades of lizards are similar (Mann-Whitney W = 87, P = 0.29) (Fig. 10).

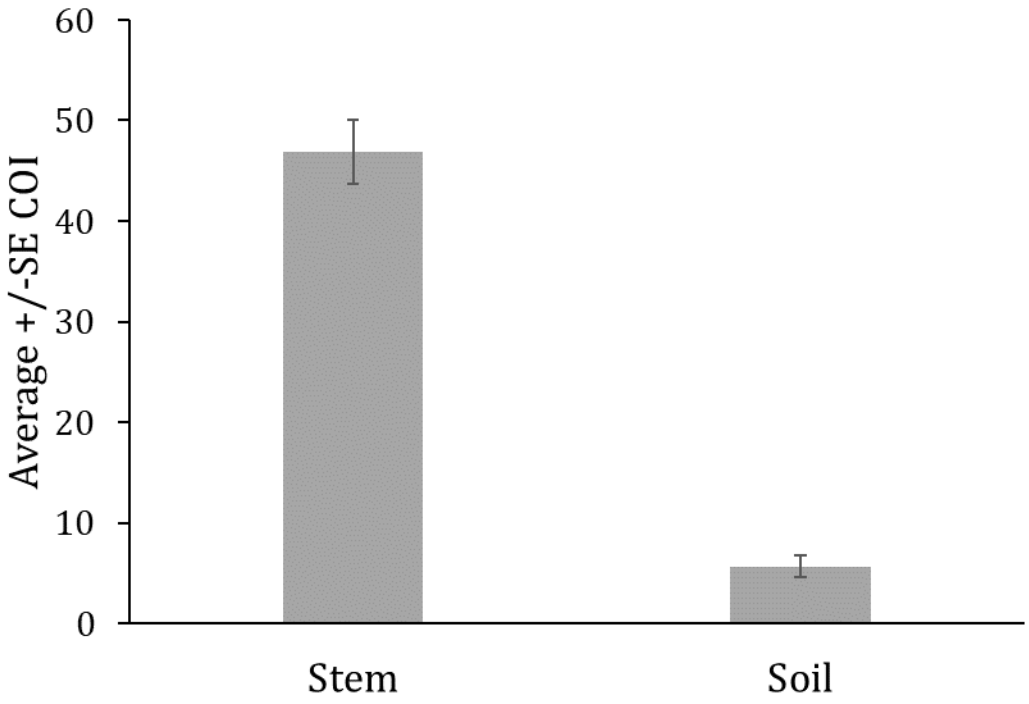

Figure 9. Mean stripe-to-stem COI scores were higher than the mean stripe-to-soil/gravel COI scores (n = 23 lizards across all biogeographic clades).

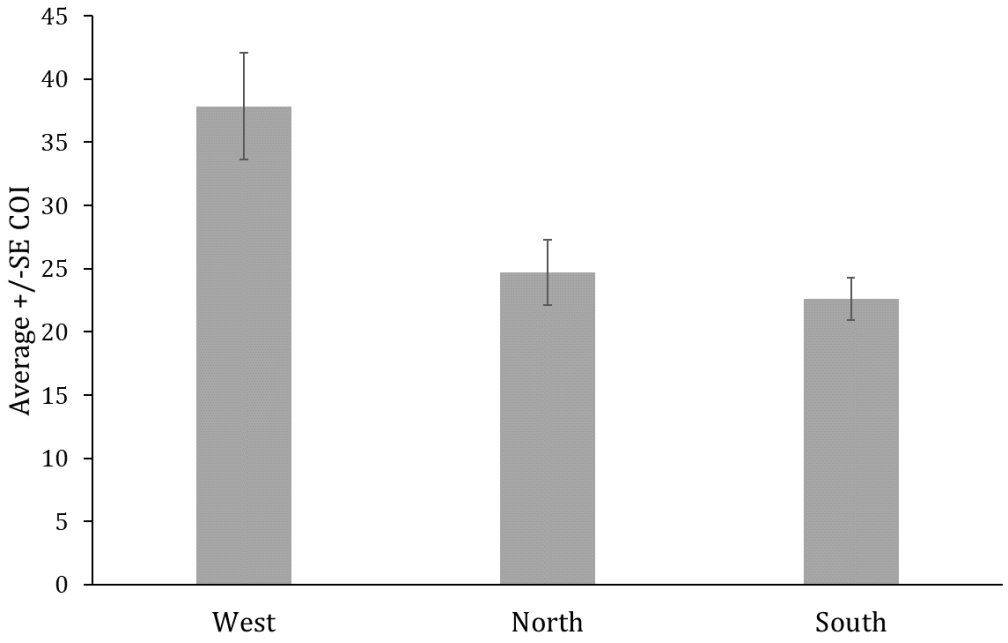

Figure 10. The "within biogeographic cluster" stripe-to-stem comparisons were significantly higher for Western clade lizards than for Northern or Southern clade lizards ($n_{1\text{-}3}$= 7 western lizards, 7 southern, 9 northern).

Discussion

This study uses a newer method for measuring animal-background contrast with digital photographs—instead of the classic use of a reflectometer—to test the broad hypotheses that (a) Texas Horned Lizards' body color matches the variably colored soil substrates the lizards are found on throughout their geographic range (Price 1990) and (b) that their vertebral white stripe helps conceal the lizards by mimicking nearby sun-bleached grass stems (Sherbrooke 2002). The results of this study support these hypotheses.

Horned lizards are slow-moving and largely remain motionless at the approach of would-be predators (Pianka and Parker 1975; Sherbrooke 2003). One noteworthy exception is that Texas Horned Lizards flee from slow-moving

rattlesnakes (*Crotalus* spp.) which hunt predominantly by scent but do not flee when approached by swift-moving coachwhip snakes (*Masticophis* spp.) that hunt predominantly by sight (Sherbrooke 2008).

Horned lizard antipredator defenses occur in a sequence: (1) evading visual detection via immobility and crypsis, (2) escaping via locomotion, and (3) employing behavioral resistance via posturing, puffing their lungs full of air, poking skins of predators with sharp cranial horns, and squirting blood from a sinus behind their ocular cavity (Middendorf and Sherbrooke 1992; Sherbrooke 2003; Young et al. 2004). While the relative contributions to survival of these three levels of defenses remain unknown, many aspects of their anatomy, color-pattern, and behavior suggest that the first level—escaping the detection of visual predators—has had a profound, multifaceted influence on their evolution (Norris and Lowe 1964; Sherbrooke 2002). For instance, one of the three species that occurs in Texas, the Roundtailed Horned Lizard (*Phrynosoma modestum)*, is known to mimic small pebbles and stones by the smooth, hornless shape of its dorsum, background color-matching to local geology (Bundy and Neess 1958)—and, more precisely, to nearby stones (DDR and K. Larson, unpublished data using this same COI method)—and behaviorally tucking its legs underneath its body and lowering its head flush with the ground while arching its back to appear pebble-like (Cooper, and Sherbrooke 2010; Cooper and Sherbrooke 2012; Sherbrooke and Montanucci 1988).

There are also two examples of apparent plant-stem litter mimicry and plant-stem shadow mimicry in the genus. Of the fourteen or so species of *Phrynosoma* (Sherbrooke 2003), only two consistently have vertebral stripes. One species, the

Flat-tailed Horned Lizard (*P. mcallii*), has a very restricted distribution centered around sand dunes in the Lower Colorado River subdivision of the Sonoran Desert near the Colorado River's delta into the Gulf of California (Sherbrooke 2002). This taxon bears a thin, black dorsal stripe. There is minimal plant litter in its arid environment and during the hottest parts of the day and when avoiding predators, it takes refuge under the skirts of shrubs, where its dark vertebral stripe resembles plant-stem shadows on the sand (Sherbrooke 2002). In contrast, the Texas Horned Lizard has a white vertebral stripe and is the easternmost taxon within the genus, and its macrohabitats include all of the intact major grasslands of the southwestern deserts and the southcentral Great Plains, choosing microhabitats below shrub-skirts or at the bases of grass clumps (Sherbrooke 2002). Unlike the dunes habitat of *P. mcallii*, Texas Horned Lizard microhabitats tend to accumulate much litter in the form of windblown grass- and shrub-stems, usually of reflective tan or grayish-white color, in varying stages of decomposition and bleaching by solar radiation (Sherbrooke 2002). However, like *P. mcallii*, Texas Horned Lizards also spend much of the day underneath the skirts of bunch grasses and shrubs (Whitford and Bryant 1979), and our survey team in Karnes City observed this repeatedly in the field (Ackel 2016). While there is of yet little experimental evidence to support the hypothesis that the vertebral stripes of *P. mcallii* mimic the shadows of plant stems, populations of *P. mcallii* in areas where shadow-casting vegetation is almost nonexistent, such as near Ocotillo Wells (San Diego Co., California), have nearly immaculate mid-dorsa with greatly reduced vertebral stripes in comparison to those found at sites with many shrubs on the Barry M. Goldwater Air Force Range

near Yuma, Arizona (Sherbrooke 2002). These features of plant stems and plant stem shadows are more extreme than any other habitat for the other dozen or so horned lizard species, so if the stem/stem-shadow mimicry hypothesis is true, such observations and habitat features are appropriate predictions.

In our study, Karnes City lizards were better matched to their soil and they had lower between-habitats COI scores than the other three populations. Karnes City lizards were also more variable in their colors and they had more colors than the RPQRR lizards. The Karnes City population of lizards is isolated with low genetic diversity (Wall 2014) compared to more natural areas. Recent work found that predators rarely attack foam lizard models in Karnes City while models in a nearby natural area were heavily attacked by predators suggesting that predation levels may be lower in town (Mirkin 2019). This is a common outcome from a known phenomenon in which many natural predators become rare in urban environments while a number of mesopredators are not rare, but are subsidized by foraging on human refuse, thus relaxing their need to capture prey (Fischer et al. 2012). The observed greater color variance between lizards is consistent with relaxed selection from predators in town, yet Karnes City lizards matched their soils more closely than the more natural areas. Even within Karnes City, lizards have limited movement and are isolated by roads and so perhaps this has also led to more variance in coloration (Wall 2014). Karnes City, the most urbanized of the four populations, may also have a greater diversity of soils and landscaping than natural populations, leading to a greater diversity of colors in the lizards. Perhaps this could be corroborated by GIS analysis of soil layers in future studies.

Western clade lizards have vertebral stripes that color match to nearby blanched grass stem litter significantly better than the Northern and Southern clades match to their respective grass stems (FIG. 10), and overall, all the lizards had a moderately high stripe-to-stem COI average (FIG. 9), suggesting the importance of this feature in the environment. With lower amounts of vegetation in the deserts of the western clade range, it is possible that nature selects for higher levels of crypsis when hiding underneath sparser shrubs and bunch grasses than in the two grassier eastern (i.e. Northern and Southern) clades. However, this is conjecture and warrants further testing.

Lizards had significantly higher COI scores when compared to soils in their immediate surroundings, i.e. in their own photo, than they did when compared to soils where other lizards were found in their population. This same pattern was also observed for stripe-to-stem COIs. Some preliminary non-reviewed data from experiments at Trinity University involving the San Antonio Zoo's captive-bred population suggests that Texas Horned Lizards are not "color-aware" when it comes to selecting a matching microhabitat but imprint on the color of substrate they were raised on, though there are some limitations to these experiments that warrant further investigations (Kira McEntire, pers. comm.). Is it possible that lizards can change color at will to enhance camouflage? This seems somewhat unlikely, given that captive reddish San Antonio Zoo lizards in our study had higher COI scores when compared to remotely taken photographs of their red native soils at Chaparral WMA than they did to the whitish sands they are housed on in their terrariums at the zoo (DDR; unpub. data). It is possible that there is always inherently a higher

COI between two or more objects within a single photograph than for objects in different photographs (e.g., due to nearby objects reflecting light off each other), but theoretically, color-calibration is meant to mitigate if not altogether eliminate any inaccuracies due to lighting conditions in a photograph. This leaves at least another possibility—background color matching imposed by selective forces on a fine scale—but this possibility was beyond the scope of this study.

This study also demonstrated that color-matching (COI) scores do not significantly differ for color-calibrated photos versus uncalibrated photos and are, in fact, nearly identical (FIG. 4). This freed up the ability to use uncalibrated photographs taken by ourselves in the field as well as vouchered *in situ* photographs from other researchers and citizen scientists. Depending on the questions being asked, the implications of being able to use uncalibrated photographs of organisms potentially opens up an enormous resource for organismal color researchers in citizen science projects like iNaturalist and other repositories where scientific naturalists upload field data and photographs.

There are perhaps several conservation implications from this study. First, our results show that Texas Horned Lizards match their immediate surrounding soil at an average COI of ~61% and match their broader habitat's soil at an average COI of ~45%. Therefore, as a conservation strategy, an organization involved in reintroductions or translocations in hopes of reestablishing viable populations of Texas Horned Lizards should aim for making sure their lizards have a COI of at least 45% when comparing those lizards' colors to any prospective site's soils. To stay consistent with methods used in this study, they should also take photographs of

soils on prospective sites at 10 or more different locations within each site and run the COI in fuzzy mode.

Mean stripe-to-stem COI scores were at ~47%. "Within habitat" stripe-to-stem COI averages were at ~23 – 25% for Southern and Northern clade lizards and at ~38% for Western clade lizards. This result implies that having the correct type of sun-lightened (and likely, native) grass stem litter nearby is important for color-matching crypsis, and therefore, for evading predators, at least in the Western population. A conservation strategy involving reintroduction, for instance, should not only make sure such grasses are present in redundancy, but also should aim for at least these COI scores before considering release at a site.

This is the first study that has tested the long-held idea that Texas Horned Lizards are highly cryptic to their soils and to some of their native grasses. Since nearly all literature has historically suggested that crypsis is their first line of defense against predators (Pianka and Parker 1975), any conservation plan concerning this species should make it a priority to include crypsis (with appropriate soils and grasses) in their strategy. The COI method used herein to measure color-pattern matching should convince researchers and citizen scientists the usefulness of capturing photographic data as raw material for investigating color-related questions in ecology, evolution, and conservation.

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Appendices

Appendix A. How to Calculate COI

- Note: these instructions are for a Windows/PC platform, so adjust commands etc accordingly. (Mac installation instructions for the **Color Inspector 3D/Color Histogram plugin for ImageJ** can be found in Appendix B.)
- Download **Adobe DNG Converter** software here: https://supportdownloads.adobe.com/thankyou.jsp?ftpID=6589&fileID=6607
- Download the **ColorChecker Camera Calibration** software here: https://xritephoto.com/ph_product_overview.aspx?ID=1257&Action=Support&SoftwareID=917
- Download & Install **R** and **RStudio** (free online). Go here, for instance: https://courses.edx.org/courses/UTAustinX/UT.7.01x/3T2014/56c5437b88fa43cf828bff5371c6a924/ and follow the instructions for
    - downloading them both. Open RStudio to run it and to make sure the installation worked, **and then close it**.
    - Download coi.R and solution.R files and save them in the same folder (**just for your reference**, but not necessary, taken from here: http://www.faunajournal.com/vol2Issue1/Issue_feb_2015/2-2-35.1.txt; the COI code for R is included at the end of Appendix A)
        - (the actual .R files for this step are already made here: https://drive.google.com/open?id=1BSjuQNn_x1_awEnlC3dPmHbNxSl3EIi8 --- **download these**)
        - Save both of them and the HL17.txt file in a single folder on your desktop called "coi_solution"
    - Open to the coi_solution folder on your desktop and click on each of the two R files and choose to open them in RStudio
        - (then click to allow all .R files to likewise open in RStudio in the future)
    - Copy and paste **source("coi.R")** into the console (i.e. bottom left of RStudio interface) and hit Enter
    - now you need to look/verify that **coi.R** should now be populated/loaded in the Environment functions (i.e. top right window of RStudio interface)
    - Make sure there's a txt file with LUT scores in the same folder (like the HL17.txt example file) and copy the name into this command in the lower left console, for example:
        - In this case, since there are only two objects (e.g. one lizard sample and one soil sample) copy and paste **coi("HL17.txt", "pairs")** into the console

- Note: **coi("HL17.txt", "fuzzy")** can be used when more than 10 columns of data are in the txt file (i.e. more than two lizards/soil etc to compare)
- Hit Enter to get COI scores (if you get a COI output, this tells you that the COI calculating function worked properly)
- (Note: Save all future LUT txt files in this same folder where the .R files are also saved)

- Now, go and download the **3D Color Inspector/Color Histogram plugin** for Image J: https://imagej.nih.gov/ij/plugins/color-inspector.html
- Download **Grid plugin** for Image J: https://imagej.net/Grid
- Then finally Download **Image J** for your Mac or PC here: https://imagej.nih.gov/ij/download.html **but don't run it yet**
    - Show both 3D Color Inspector/Color Histogram and Grid plugin files in Finder or download folder and transfer (click, drag, and copy) them to the Plugins subfolder of Image J files
    - Then finally "Extract all" in Image J and then run the program
        - (Image J and these two plugins should now be installed and working)

- Open an original image file (i.e. any picture you have saved) in Image J
    - Use rectangular selector tool or Freehand tool to crop image of lizard
        - Resize crop rectangle by clicking Edit, then Selection, then Specify
        - Hit Shift-X, then Shift-D to finish crop
            - Save as JPEG with specimen number and "lizard" and pixel dimensions in cropped image name
        - For Freehand tool, simply trace animal and then click Plugins at the top of the interface and select Color Inspector 3D
    - Reopen same original image as before
        - Click Plugins and then click "Grid plugin"
            - Specify area of grid line cells and check the box to "random offset"
            - Choose cell closest to lizard snippet sample with less than 15% vegetation and rocks.
                - Use random number generator (e.g. random.org) if more than one suitable cell
            - Use rectangular selector and Edit, Selection, and Specify to match the correct dimensions as the Grid
            - Click on Grid plugin to hit cancel and thus remove Grid cells/lines **before** saving image crop/snippet
            - Shift-X, then Shift-D, then save JPEG with specimen and "soil" and pixel dimensions in its name
    - From Image J, select Plugins drop arrow, then click on/open **3D Color Inspector**

- Open lizard snippet/sample and make sure the Color Space is in “RGB”, the Display Mode in “Histogram”, and “Number of color cells” remains at the default setting
    - Click LUT button to open a lookup table/color table for the image
    - Copy and paste LUT table contents (Control-A, Control-C) to the first five columns of an Excel spreadsheet
    - Repeat the above bullet for the soil/substrate/background snippet sample and paste all further LUT tables into subsequent columns (one more set of five columns for “pairs” mode; and X sets of columns for “fuzzy” mode)
    - Save spreadsheet as a txt file with specimen, snippets pixel dimensions, and, if necessary, the type of analysis (e.g. overall COI) in its name and save in the same folder as the coi.R and solution.R files (the folder on the desktop, “coi_solution”)
    - Finally, open RStudio and run the txt file name, e.g. **coi("HL17.txt", "pairs")** to get COI scores

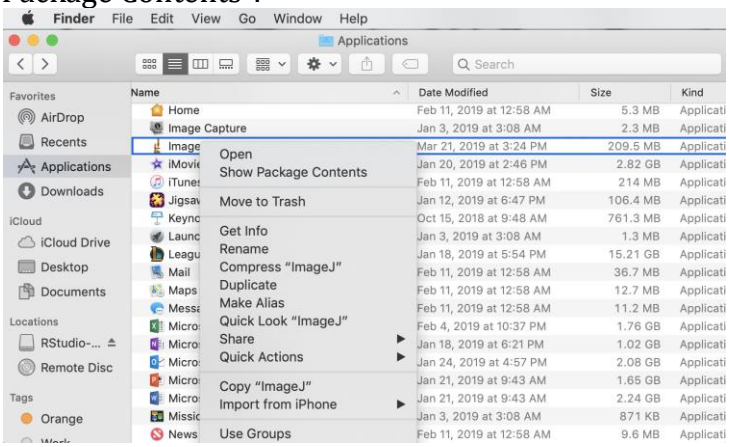
- COI code for R

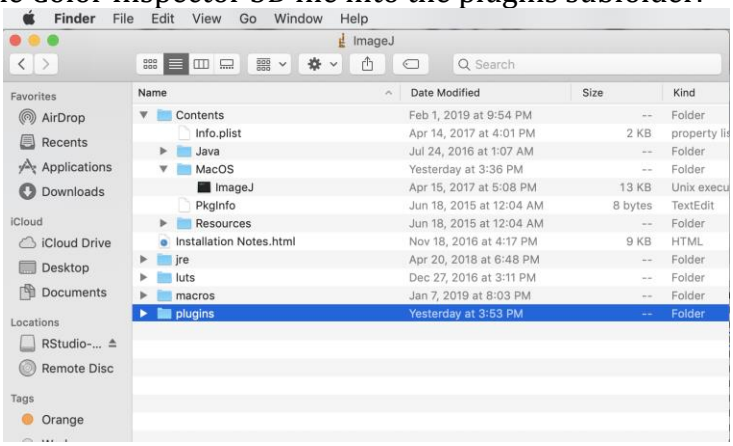
```
#-------------------------------------------------------------------
-----------------------------------
#An affordable method to measure animal-background contrast using
digital images
#authors: Diogo S. M. Samia and Ronaldo B. Francini
#corresponding author: diogosamia@gmail.com

#The function has two arguments: data and comparison mode
#data => a data frame object containing your data set. You should
either save it as a data frame object before
#run the code (using "read.table" or "read.csv" command), or use the
full name of the file as the argument of
#the function (e.g. "anolis.csv" or "anolis.txt).
#comparison mode => "pairs" or "fuzzy". See paper for details.
#To run the COI function, write in the workspace: source("COI.R")
#After, write the command line as the example: coi(mydata, "pairs")
#The R code and your data files should be both in the same directory
as R.
#-------------------------------------------------------------------
-----------------------------------

coi<-function(file, method='pairs'){

ext<-strsplit(file,"\\.")[[1]][2]
if(ext=="txt"){
data<-read.table(file,sep="\t")}
else{data<-read.csv(file,sep=",")}

data[is.na(data)]<-0
data
rows<-nrow(data)
col<-ncol(data)
```

```
matrix.coi<-matrix(0,rows,col/10)
matrix.coi2<-matrix(0,rows,(col/5)-1)
resu<-numeric(0)
y=1

            if(method=='pairs'){
            for (k in seq(1,col,10)){
            limit<-
ifelse(any(data[,k+3]==0),which(data[,k+3]==0),rows)
            for (i in 1:limit){

            find1<- ifelse(data[i,k]==data[,k+5] &
data[i,k+1]==data[,k+6] &  data[i,k+2]==data[,k+7] & data[i,k+3]>0 &
data[,k+8]>0 , which(data[i,k]==data[,k+5] &
data[i,k+1]==data[,k+6] &  data[i,k+2]==data[,k+7]),0 )
            find2<-sum(find1)
            matrix.coi[i,y]<-
ifelse(find2>0,min(data[i,k+4],data[find2,k+9]) ,matrix.coi[i,y]+0)
            }
          y<-y+1
            }

            resu<-colSums(matrix.coi)
            n<-col/10
            min<- min(resu)
            max<- max(resu)

    #output screen
            output1<-list('method'='PAIRS MODE','N'=n, 'COI
index'=resu,'min'=min, 'max'=max)

            #output csv
            output1b = as.matrix(output1)
            nome = strsplit(file,"\\.")[[1]][1]
            write.csv(output1b, paste(nome,"output.csv"))


return(output1)
}


            if(method=='fuzzy'){
            for (k in seq(6,col,5)){
            limit<-ifelse(any(data[,4]==0),which(data[,4]==0),rows)
            for (i in 1:limit){

            find1<- ifelse(data[i,1]==data[,k] &
data[i,2]==data[,k+1] &  data[i,3]==data[,k+2] & data[i,4]>0 &
data[,k+3]>0 , which(data[i,1]==data[,k] &  data[i,2]==data[,k+1] &
data[i,3]==data[,k+2]),0 )
            find2<-sum(find1)
            matrix.coi2[i,y]<-
ifelse(find2>0,min(data[i,5],data[find2,k+4]) ,matrix.coi2[i,y]+0)
            }
          y<-y+1
            }
```

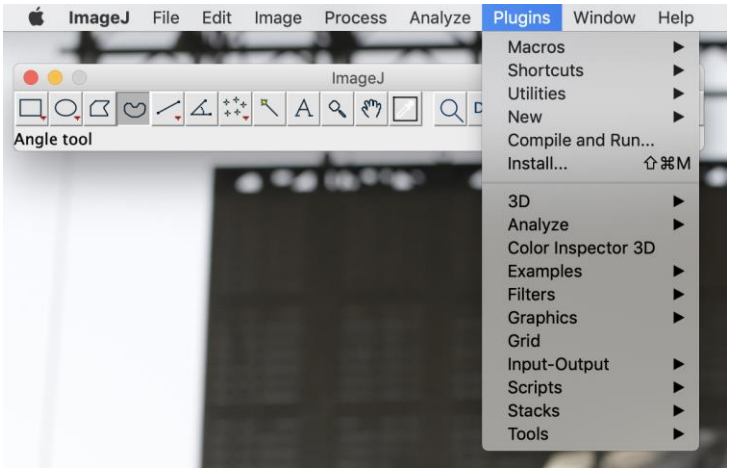
```
            resu<-colSums(matrix.coi2)
    mean<-mean(resu)
            sd<-sd(resu)
            min<- min(resu)
            max<- max(resu)
            n<-(col/5)-1

    #output screen
    output2<-list('method'='FUZZY MODE','N'=n, 'COI
index'=resu,'mean'=mean,'standard deviation'=sd,'min'=min,
'max'=max)

    #output csv
            output2b = as.matrix(output2)
    nome = strsplit(file,"\\.")[[1]][1]
            write.csv(output2b, paste(nome,"output.csv"))


return(output2)
}
}
```

Appendix B. Color Inspector 3D plugin Downloading Instructions for iOS

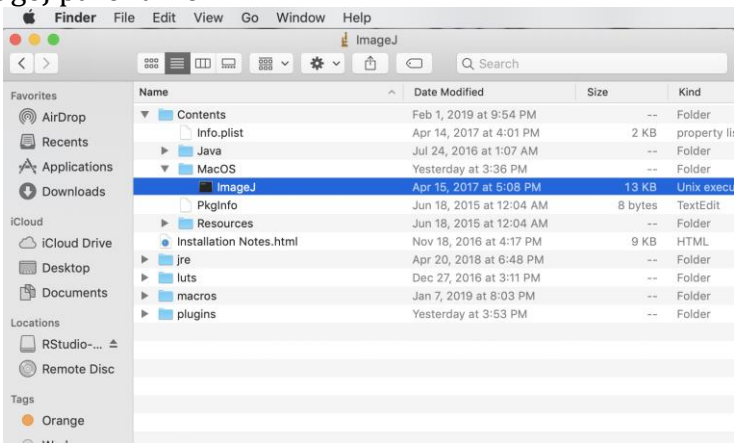
- Follow the downloading instructions for ImageJ and Color Inspector 3D, as above.
- Open two separate windows for Finder. On one window open your downloads or recent folder to locate the Color Inspector 3D file. On the other, right click on the ImageJ file which should be under Applications and click "Show Package Contents".
- 

- Drag the Color Inspector 3D file into the plugins subfolder.
- 

- Run the ImageJ program and check on the plugins menu if the Color Inspector 3D plugin is there.



- If this does not work, open the package contents for ImageJ again and locate the ImageJ file in the "MacOS" folder under the "Contents" subfolder inside the ImageJ parent file.



- Drag this file out of the ImageJ parent folder. You can place it anywhere, but make sure the file is no longer in the parent file. If after dragging it out, the file is still in the parent folder, you will need to hold down the command button while dragging it out.
- After this file has been removed, drag the file back into the "MacOs" folder. Somehow this corrects the problem if the Color Inspector 3d plugin was not working before.
- If you run the program and the plugin is still not working, check inside the ImageJ plugin subfolder.
    - When I checked here, the Color Inspector 3D folder had disappeared which I think is because I had removed the ImageJ file from the "MacOS" subfolder.
- Re-download the Color Inspector 3D plugin and drag it into the ImageJ plugin folder. Quit and close ImageJ if it is running to "restart" it.
- Run the ImageJ folder again and when you check the plugin menu, the Color Inspector 3D plugin will be there.

*Make sure that every time you are checking if the plugin is working in ImageJ, you are closing and shutting it down completely before. If you make changes to the file and check the app without doing this, it won't "update" any changes you made.

Notes from Kristen Larson regarding installing this 3D plugin on a Mac: her husband used the instructions found at https://imagej.nih.gov/ij/docs/install/osx.html. Here's his typed directions:

there is one step that was crucial to get it working. Once the downloaded ImageJ folder is extracted and moved to the Applications folder, you have to move the ImageJ.app file out of the ImageJ folder and then move it back in. This doesn't sound like it would do anything but there is some weird stuff happening internally in the Mac operating system that is affected by this. I don't think it matters where the ImageJ.app file is moved to temporarily, as long as it is moved out of the ImageJ parent folder and then moved back.

Appendix C. iNaturalist Specimens Used in this Study

Specimens used in lizard-to-soil analyses:
https://www.inaturalist.org/observations/16396340
https://www.inaturalist.org/observations/13569356
https://www.inaturalist.org/observations/12944203
https://www.inaturalist.org/observations/12942455
https://www.inaturalist.org/observations/9659110
https://www.inaturalist.org/observations/5259128
https://www.inaturalist.org/observations/3900061
https://www.inaturalist.org/observations/3564578
https://www.inaturalist.org/observations/2895102
https://www.inaturalist.org/observations/2338502
https://www.inaturalist.org/observations/1974541
https://www.inaturalist.org/observations/3406386
https://www.inaturalist.org/observations/3896381
https://www.inaturalist.org/observations/6779194
https://www.inaturalist.org/observations/7228213
https://www.inaturalist.org/observations/7253409
https://www.inaturalist.org/observations/7313611
https://www.inaturalist.org/observations/7313619
https://www.inaturalist.org/observations/11278761
https://www.inaturalist.org/observations/15986750
https://www.inaturalist.org/observations/350476
https://www.inaturalist.org/observations/350479
https://www.inaturalist.org/observations/765216
https://www.inaturalist.org/observations/791437
https://www.inaturalist.org/observations/791450
https://www.inaturalist.org/observations/791506
https://www.inaturalist.org/observations/791517
https://www.inaturalist.org/observations/1549716
https://www.inaturalist.org/observations/1924716
https://www.inaturalist.org/observations/13479903
https://www.inaturalist.org/observations/14995339
https://www.inaturalist.org/observations/5753434
https://www.inaturalist.org/observations/3432646
https://www.inaturalist.org/observations/3407483
https://www.inaturalist.org/observations/5898590

https://www.inaturalist.org/observations/1627112
https://www.inaturalist.org/observations/3448335
https://www.inaturalist.org/observations/766408
https://www.inaturalist.org/observations/365432
https://www.inaturalist.org/observations/4753246
https://www.inaturalist.org/observations/4753244
https://www.inaturalist.org/observations/18741090
https://www.inaturalist.org/observations/18697631
https://www.inaturalist.org/observations/13904219
https://www.inaturalist.org/observations/7253014
https://www.inaturalist.org/observations/7250528
https://www.inaturalist.org/observations/6348138
https://www.inaturalist.org/observations/6114958
https://www.inaturalist.org/observations/3427230
https://www.inaturalist.org/observations/3085368
https://www.inaturalist.org/observations/2906615
https://www.inaturalist.org/observations/1441061
https://www.inaturalist.org/observations/16438908
https://www.inaturalist.org/observations/13377622
https://www.inaturalist.org/observations/8844546
https://www.inaturalist.org/observations/16247432
https://www.inaturalist.org/observations/12272477
https://www.inaturalist.org/observations/5866176
https://www.inaturalist.org/observations/5865601
https://www.inaturalist.org/observations/5496155
https://www.inaturalist.org/observations/4541011
https://www.inaturalist.org/observations/1896423
https://www.inaturalist.org/observations/868825
https://www.inaturalist.org/observations/293528
https://www.inaturalist.org/observations/283879
https://www.inaturalist.org/observations/16422948
https://www.inaturalist.org/observations/9256815
https://www.inaturalist.org/observations/9256813
https://www.inaturalist.org/observations/9256809
https://www.inaturalist.org/observations/9256531
https://www.inaturalist.org/observations/7227767
https://www.inaturalist.org/observations/4206375
https://www.inaturalist.org/observations/4206367
https://www.inaturalist.org/observations/4206334

https://www.inaturalist.org/observations/2430961
Specimens used in stripe-to-stem analyses:
https://www.inaturalist.org/observations/2053323
https://www.inaturalist.org/observations/5259128
https://www.inaturalist.org/observations/3564578
https://www.inaturalist.org/observations/29444749
https://www.inaturalist.org/observations/28948178
https://www.inaturalist.org/observations/28845064
https://www.inaturalist.org/observations/2895102
https://www.inaturalist.org/observations/16422948
https://www.inaturalist.org/observations/14972955
https://www.inaturalist.org/observations/14578956
https://www.inaturalist.org/observations/12449539
https://www.inaturalist.org/observations/27180635
https://www.inaturalist.org/observations/24940575
https://www.inaturalist.org/observations/22499649
https://www.inaturalist.org/observations/321872
https://www.inaturalist.org/observations/19855349
https://www.inaturalist.org/observations/10419862
https://www.inaturalist.org/observations/247316
https://www.inaturalist.org/observations/5665578
https://www.inaturalist.org/observations/33781910
https://www.inaturalist.org/observations/25385068
https://www.inaturalist.org/observations/24702675
https://www.inaturalist.org/observations/22042696
https://www.inaturalist.org/observations/27757939

VITA

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Education B.S. Integrative Biology, Brigham Young University, 2009

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Experience Teaching Assistant, Texas Christian University

Selected Publications Rhoads, Dustin D. and Gerard T. Salmon. 2012. A Much-Belated Obituary of an Important American Zoo Collector, with Discussion of the Type Locality for *Bogertophis subocularis* and *Lampropeltis alterna*. *Herpetological Review* 43(2): 270—273.

Rhoads, Dusty. 2008. *The Complete Suboc: A Comprehensive Guide to the Natural History, Care, and Breeding of the Trans-Pecos Ratsnake*. ECO Herpetological Publishing and Distribution, Lansing, MI. 291 pages. (book)

Selected Presentations Rhoads, Dusty and Rachel Pikstein. 2019. *Quantifying Color Pattern Mimicry and Background Color-Matching in Rock Rattlesnakes (Crotalus lepidus) and Gray-banded Kingsnakes (Lampropeltis alterna) Utilizing Digital Photography.* Oral presentation on original research presented on July 13th 2019 at Biology of Pitvipers 3 Conference, Chiricahua Desert Museum, Rodeo, New Mexico.

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Rhoads, Dusty. 2018. *How the H-Snake Lost Its Hs: Mendelian Inheritance and Geographic Distribution of the Blonde phase of the Trans-Pecos Ratsnake (Bogertophis subocularis)*. Oral presentation at the Texas Conservation Symposium, January 12th 2018 at Southwestern University, Georgetown, Texas.

Grants & Awards Horned Lizard Conservation Society Grant, 2018
Finalist 3MT Competition at TCU, Spring 2019
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ABSTRACT

BACKGROUND COLOR-MATCHING IN THE TEXAS HORNED LIZARD (*PHRYNOSOMA CORNUTUM*)

By: Dusty Rhoads, M.Sc., 2019
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Thesis Advisor: Dean Williams, Professor in Biology

Most of the literature on the basic ecology of Texas horned lizards cites "cryptic color pattern" as the first line of defense against predation in this taxon, and yet the degree to which horned lizards color-match their backgrounds has never been quantified. Several zoos and state wildlife agencies are releasing captive-bred and translocated lizards to parts of their former range; however, the new populations are not self-sustaining, with most releases lost to predation. Background color-matching may be important to consider when moving lizards into a new habitat where predation may be higher if they are not closely color-matched to the local soils. I quantify background color-matching in this taxon across its known range in the United States and in Mexico from *in situ* photos taken, as found, in the wild. I also present background color-matching variation and trends both within and between phenotypically and genetically diverse populations and ask whether lizards more closely match their local soil colors and sun-bleached plant stems than soils and stems from other areas. Finally, I suggest a method for zoos and wildlife agencies to score coloration in their captive populations of lizards, thus possibly enabling these institutions to objectively consider color-matching *a priori* as an applied

conservation strategy to potentially increase the survival of reintroduced Texas horned lizards.