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RESEARCH ARTICLE

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Correlates of melanization in multiple high- and low-elevation populations of the lizard, *Sceloporus occidentalis*: Behavior, hormones, and parasites

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Abstract

Hormones mediate the expression of suites of correlated traits and hence may act either to facilitate or constrain adaptive evolution. Selection on one trait within a hormone-mediated suite of traits may lead to a change in the strength of the hormone signal, causing changes in correlated traits. Growing evidence suggests that melanization, which is in part regulated by hormonal signals, is tightly linked to other traits, such as aggression and stress physiology. Here, we examine six populations of *Sceloporus occidentalis* lizards differing in degree of melanization (three dark higher-elevation populations and three less-melanized lower-elevation populations) to investigate potential correlations between behavior, hormones, and parasites. We measured aggression by recording behavioral responses of males to staged territorial intrusions; behavior was summarized by two principal components. Analysis revealed that males in the three darker populations signaled aggression less often and made more physical contact than males in the lighter populations. Analyses of plasma steroid hormones (corticosterone and testosterone) revealed significant population differences, but counter to expectation higher aggression was associated with lower testosterone compared across populations. Finally, the three darker populations had higher mean mite loads than the three lighter populations. Overall, this array of phenotypic correlations does not parallel patterns of within-population differences in melanization found in other vertebrates, suggesting that hormonal correlations do not constrain phenotype variation across populations in this species. Given this contradiction between population- and individual-level variation, we urge more study at both levels of variation in traits potentially associated with melanization in other vertebrates.

1 | INTRODUCTION

The rate at which a trait evolves can depend upon interactions with other traits (Lande and Arnold 1983). When traits under natural selection are completely independent of one another, evolutionary responses may occur freely. However, when traits depend on a common mechanism for their expression, as in the case of hormonal control, they may co-evolve as a unit and hence there may be constraints on the responses to selection (Maynard Smith, Burian, Kauffman, Alberch, Campbell et al., 1985). Most cases likely lie somewhere between these two extremes.

One such type of constraint may be hormonal "pleiotropy" (e.g., Ketterson, Atwell, & McGlothlin, 2009)—for example, the evolution of traits linked to melanocortin hormones (reviewed in Ducrest, Keller, & Roulin, 2008). Suites of traits associated with melanistic color can be affected directly or indirectly by melanin and by the factors that regulate it (Quesada & Senar 2007; Ducrest et al., 2008). The existence of potential pleiotropic effects of the melanocortin system on other traits can be described as the "Melanization Hypothesis" (MH). Accordingly, one would expect consistent associations in melanization in animals, if there is strong linkage among the melanocortin system that affects such suites of associated traits. Alternatively, weaker linkages may occur if environmental conditions, such as thermal environment, also influence individual elements of this suite of traits (e.g., differences in melanization; Fedorka, Lee, & Winterhalter, 2012). Costs associated with specific components within the suite of traits might differ depending on the context, and hence plasticity may be favored. For example, selection might favor a plastic response in the degree of melanization due to spatial or temporal variation in energetic costs (Roulin, Gasparini, Bize, Ritschard, & Richner, 2007). Hence, under strong directional selection, the hormonal control of key traits may not constrain evolution of other traits.

Differences in melanization can be associated with social signals, hormonal profiles, stress reactivity, and immune function. Melanin-based ornaments, which can be regulated by physiological and social mechanisms, commonly function as social signals of aggression or of hormonal status (e.g., Gonzalez, Sorci, & Smith, 2001; McGraw, 2008). Melanocortins, such as α-MSH, can affect aggressiveness via several routes. In mice, aggression is enhanced by a peptide hormone when it binds to a melanocortin receptor, such as MC5R, which is typically expressed in the brain (Morgan, Thomas, & Cone, 2004). However, melanistic coloration is not always associated with higher aggressive behavior (e.g., Berggren, 2008). This peptide can also be synthesized in the skin, and the peripheral production is likely responsible for melanization (Slominski, Tobin, Shibahara, & Wortsman, 2004). Other physiological mechanisms, such as testosterone, have been linked to changes in ornamentation and/or aggression, such as the size and color of melanin-based markings (Haase Ito, & Wakamatsu, 1995; Gonzalez et al., 2001). For example, testosterone-implanted House Sparrows had larger black bib sizes compared with controls (Evans, Goldsmith, & Norris, 2000). There are a variety of relationships between the hypothalamic-pituitary-adrenal (HPA) axis and melanin production (Slominski et al., 2004). The HPA axis consists of corticotropin-releasing hormone stimulating the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary that, among other actions, activates the synthesis of glucocorticoids (Charmandari, Tsigos, & Chrousos, 2005). Importantly, both α-MSH and ACTH in the pituitary are products of the proopiomelanocortin, and upregulation of the synthesis of one requires downregulation of the other. Melanized individuals can show reduced hormonal stress reactivity compared with less melanized individuals (Almasi, Roulin, Korner-Nievergelt, Jenni-Eiermann, & Jenni, 2012; Kittilsen, Johansen, Braastad, & Øverli, 2012). Differences in melanization also can be associated with differences in immune function and parasite exposure (Ducrest et al., 2008). For example, parasite exposure can differ as a function of melanization because of associated life-history traits and behavior (Roulin, 2004), and parasite resistance may covary with melanization due to differences in antibody production (Gasparini, Bize, Piault, Wakamatsu, Blount, Ducrest, & Roulin, 2009) or in innate immunity (Bonato, Evans, Hasselquist, & Cherry, 2009).

Here, we present a study measuring potential correlations between melanization, aggression, hormones, and parasite loads. We assessed relationships among these variables across six populations of the Western Fence Lizard (*Sceloporus occidentalis*). First, we assessed association between melanization and aggression in male lizards by performing staged encounters to measure lizard behavior during male–male encounters in the field. The MH predicts darker individuals will be more aggressive. Second, in two additional groups of males, we measured corticosterone and testosterone levels at either a baseline time point or following 1 hr of confinement, to assess stress reactivity. The MH predicts that melanization will be positively correlated with baseline plasma testosterone (see citations above), negatively correlated with baseline corticosterone, and that stress-reactivity will differ between more- and less-melanized populations. Third, we assessed mite loads to test the prediction that males in darker populations will have lower ectoparasite loads because of enhanced immune function. Overall, the MH predicts that populations differing in melanization will show distinctive suites of correlated traits

Temperature can affect behavior and physiological processes in ectotherms. Therefore, we also assessed field-active body temperatures of the males we studied, to evaluate and potentially statistically control for possible temperature effects on these variables.

2 | MATERIALS AND METHODS

We examined associations between melanization, aggression, hormones, and parasites in males from six California populations of the Western Fence Lizard. Elevational pigmentation differences among *S. occidentalis* populations of Yosemite National Park (YNP) and surrounding areas of California have been documented (Camp, 1916; Leaché, Helmer, & Moritz, 2010; Seddon & Hews, 2016). We worked in the summers of 3 years (2013, 2014, and 2015), during the respective breeding seasons of populations at each of six sites. Three high elevation sites contained highly melanized populations and three low elevation sites contained less-melanized populations. Given that body temperature in reptiles can affect behavior and physiology, any population differences in other traits could be related to differences in body temperatures. Hence, we also measured temperature in a subset of males for four of the six population to evaluate the potential for population differences in average field-active body temperatures.

2.1 | Study species and sites

The western fence lizard is an oviparous, diurnal lizard broadly distributed across the western United States with a spring-summer mating season from April to July (Stebbins, 2003). For two of the six populations, we use data from Seddon and Hews (2016), collected in 2013 in YNP in the Sierra Nevada Mountains. A site along the Merced River was the low elevation site (N = 30, 650 m; N 37°43′, W 119°39′), and the population along Tioga Road was the high elevation site (N = 29, elevation 2,000 m; N 37°50′, W 119°28′). We add additional data from four other populations studied in two subsequent years. Specifically, in June 2014, we caught lizards from one low and one high elevation site in YNP: along the Tuolumne River (N = 42, elevation 850 m; N 37°54′, W 119°41′) and at May Lake (N = 40, elevation 2,200 m; N 37°51′, W 119°29′). Similarly, in June 2015, we caught lizards along the shores of Millerton Lake in the western foothills of the Sierra Nevada (N = 11, elevation 500 m, N 37°0′, W 119°42′) and on White Mountain in the Inyo mountains, east of Owens Valley (N = 18, elevation 2,400 m, N 37°35′, W 118°16′). In all years, we controlled for stage of breeding season by working on the low-elevation population first. In each year and at each site, undisturbed individuals were frequently observed to engage in courtship and aggressive behavior, indicating our sampling captured high levels of breeding activities in the populations. Published literature indicates that we captured the breeding behaviors associated with the first egg clutch for each population (Goldberg, 1974; Morey, 1983; Sabo, 2003), thus minimizing seasonal variation in the intensity of male–male competition, which could affect aggressive responses to our staged encounters.

2.2 | General lizard processing

We captured lizards by noosing (Moore & Lindzey, 1992), working between 10:00 and 14:00 hr to minimize effects of potential diel changes in plasma hormone concentrations and/or subsequent behavior (Seddon & Hews, 2016). All procedures in this study were approved by the Institutional Animal Care and Use Committee of Indiana State University (protocol #425155).

2.3 | Body temperature

We measured body temperature in field-active males, and ambient (air) temperatures for four populations (two in 2014 and two 2015). In 2013, our thermal gun failed so we do not have body temperature measurements for that year. To measure body temperature we used a digital thermal gun (IRT0424; Kintrex, Vienna, VA). For all males, immediately upon capture, we held the thermal gun at 2–3 cm from the lizard's dorsum, took readings twice in rapid succession, and recorded the average of those two measures. We then immediately measured ambient air temperature at 1 m above the site of capture with a digital thermometer. We calculated average temperatures (air, body) by combining all measurements taken during the daily activity periods that we worked, into 5- or 6-day bins (depending on how long we worked each site). While we only examined surface body temperature, some studies have shown that surface and body temperature are highly correlated (Dzialowski & O'Connor, 2001).

2.4 | Color measurements

To quantify degree of melanization, we took a digital image of the ventral surface of each lizard with a Canon® PowerShot A640 camera while in the shade in the field. Color images were standardized to the same color standard within each lizard photograph (Hamilton, Gaalema, Laage, & Sullivan, 2005), and by controlling the subject-to-camera lens distance. We assessed the ventral surface because it was the target of analysis by Leaché et al. (2010) and it is revealed during postural displays. We quantified degree of melanization for all males using Adobe Photoshop CS2 (version 3.2.4, 1994) to measure the chest and throat area. The chest region extended from one armpit to the other and up to the neck, following a straight line from each armpit to the neck. The throat area extended from the rostrum to the neck. We outlined the extent of the black pigment using the lasso tool and used the histogram function to calculate average brightness. We calculated average black coloration by subtracting average brightness from 100 (Brightness values vary between 1 and 100). We use the term "more melanized" to indicate lizards with higher values of blackness (less white, more black). Because throat melanization and chest melanization were correlated ($r^2 > 0.67$, in each population), we present statistical results for both throat and chest melanization, but only show figures for chest melanization.

2.5 | Agonistic behavior

To measure aggression, we ran staged territorial intrusions (STIs) and recorded the behavioral responses of free-ranging focal males. Focal males were observed for several minutes prior to starting a trial. They maintained positions on conspicuous perches and in alert postures, often displaying, suggesting they were not intruders but on their own territories. Males used as the stimulus (intruder male) were captured at the same study site, but at least 100–200 m from the focal male's territory. Each stimulus male was used only once as an intruder. For a STI, we presented a resident male lizard with an intruder male, using a standardized protocol (e.g., Klukowski & Nelson, 1998; Martins, Ossip-Klein, Zúñiga-Vega, Vital García, Campos, & Hews, 2015). The intruder lizard was attached to a 2 m pole with a noose of fine line attached anterior to the hind legs, then placed at a distance of 2 m from and in "line of sight" for the focal male. The observer then moved away from the two lizards (mean = 4 m, range = 3–6 m) to record behaviors performed by the focal male (see below). We continued the STI trial until either the resident attacked the intruder (biting) or 5 min had passed. At the end of the trial, we captured the resident male, took a post-STI blood sample (see "Hormone Measures," below), measured morphology and took digital images of the ventral surface. To control for effects of body size on aggression, we discarded seven trials so as to only analyzed data from STIs in which the snout-vent-length of resident and intruder male differed by less than 3 mm (e.g., Quinn & Hews, 2000).

We recorded focal male behavior in several categories. Movements included retreat, approach, and charge (a rapid move toward the stimulus male). We originally recorded any movement away from the stimulus male in two subcategories, "repositioning" (focal male moved away from stimulus male a short distance of up to 1 m before returning to the stimulus male) and "retreating" (focal male ran more than two body lengths away and did not return to the stimulus male). However, we observed few instances of "repositioning" in a total of 75 trials, with only one or two instances of this behavioral act per population, and hence dropped analysis of it. Social displays recorded included "head bob" (a broadcast display done when moving and also in a variety of social contexts) and "full show" (a highly aggressive display done in escalated male-male encounters, usually when males are in close proximity; Martins et al., 2015). A "head bob" was scored as a sequence of rapid raising and lowering of the head, and included displays using modifiers to elevate and lower the whole body (either bending two or four limbs) but not using dorsolateral flattening. "Full show" was defined as a head bob that included dorsolateral flattening and arched back, exposing ventral coloration. Physical contact was recorded and included biting and wrestling. Finally, chemosensory behaviors were recorded and included tongue-flicks, licks, and gapes. Chemosensory behaviors directed toward the substrate are often exploratory and can be positively associated with movement through the environment (e.g., Bissinger & Simon, 1981)

2.6 | Hormone measurements

To assess adrenal function, we measured baseline and post-handling corticosterone. We used the same blood samples taken from the undisturbed males that were captured immediately for the testosterone and corticosterone measures, and then additional males were captured and held for 1 hr to measure hormonal stress reactivity. This is a standard stress-handling paradigm (1 hr of confinement in a cloth

bag) for small birds and reptiles (Hews & Abell Baniki 2013). Every day, we alternated between capturing and sampling the second (immediate bleed) and third (1-hr confinement) sets of males. We did not use repeated measures on the same individuals to assess hormone levels and stress reactivity because repeated blood sampling may affect hormone concentrations (Harris, Benson, Gilardi, Poppenga, Work, Dutton, & Mazet, 2011), and because of potential hemodilution effects in a small lizard.

We sampled blood from the orbital sinus using heparinized microcapillary tubes (e.g., Baird & Hews, 2007; Seddon & Klukowski, 2012) within 2.9 min (range: 1.1–3.4 min) after capture or after the 1-hr confinement. After blood sampling, we counted the total number of mites, took digital images of lizard ventral surfaces, and recorded the time of day of capture. To avoid resampling, we temporarily marked lizards by applying a small spot of non-toxic white paint to the dorsum.

We collected approximately 100 μl of blood from each lizard. Whole blood samples were held on wet ice or Blue Ice® blocks for several hours. After the cell fraction settled, we pipetted the overlying plasma into clean microcentrifuge vials with a Hamilton syringe. Following Goymann, Schwabl, Trappschuh, and Hau (2007), we stored Plasma samples in known volumes of ethanol (10:1, ethanol:plasma) in screw-top vials; in the laboratory, we stored sample vials at −20°C until assay (see below).

2.7 | Hormone assays

Plasma testosterone and corticosterone concentrations were determined via ELISA (Enzo Life Sciences, Inc. Correlated-EIA™ Testosterone Kit 900-065, and Correlated-EIA™ Corticosterone kit 900-097, Farmingdale, NY). We first optimized use of each kit with *S. occidentalis* plasma following protocols from Wada, Hahn, and Breuner (2007). Then, for either hormone assay, we dried 40 μl of the ethanol and plasma solution under a nitrogen gas stream. To reconstitute the mixture, we added 4 μl of assay buffer along with an equal volume of steroid displacement reagent (2%). We then added assay buffer to dilute the samples to a 1:80 ratio (determined in our optimization procedures for each hormone), and samples were split into three aliquots to assay in triplicate. Hormone concentration was found by competitive binding between endogenous steroid hormone and alkaline phosphatase-labeled steroid hormone for sheep antibody binding sites for 60 min (for the corticosterone kit, endogenous corticosterone was allowed to bind to antibody binding sites for 2 hr prior to the addition of alkaline phosphatase-labeled corticosterone as per kit instructions). The unbound reagents were washed away and p-nitrophenyl phosphate substrate was added. The plate was incubated for 60 min (testosterone at 37°C; for corticosterone at RT, as per kit instructions), a stop solution added, and the plate read. Sample hormone concentrations were determined through comparison with a standard curve, run in triplicate. Values on each standard curve ranged from 0.008 to 2 ng/ml for testosterone and 0.032 to 20 ng/ml for corticosterone. Sample concentrations for each hormone were determined with a 4-parameter logistic curve-fitting program (Softmax Pro *5.2*™). Intra-assay variation, calculated as the coefficient of variation of values obtained from replicates of samples, averaged 5.9% (range: 2.4–19.2%) for testosterone and 6.2% (range: 1.2–21.1%) for corticosterone, which is similar to other studies (e.g., Klukowski, 2011). For each hormone, all plasma samples from both populations were run on a single plate. The testosterone antibody in the kit has low (7.2%) cross-reactivity with androstenedione and negligible (<1%) cross-reactivity with dihydrotestosterone, dehydroepiandrosterone, β-estradiol, progesterone, and corticosterone. The corticosterone antibody in the kit has moderate (28.6%) cross-reactivity with deoxycorticosterone, low (1.7%) cross-reactivity with progesterone, and negligible (<1%) cross-reactivity with testosterone, tetrahydrocorticosterone, aldosterone, cortisol, and 17β-estradiol.

2.8 | Statistical analysis

All statistical tests were run on R i386 v3.3.1 (The R Foundation, 2016), and *P* values smaller than 0.05 were considered significant. Because of the large number of tests we conducted, we were conservative and used two-tailed tests, in spite of having directional predictions for some comparisons. We assessed data for normality and log-transformed (base 10) hormone values prior to analysis to correct for non-normal distributions. We used ANOVA to compare the degree of melanization (chest and throat) among lizards in the six populations. We generated composite variables to summarize aggressive behavioral responses from our STIs using a principal component analysis (PCA), with Varimax rotation. For factors retained (based on eigenvalue greater than 1.0, the break point of the scree plot, and the percentage of total variation explained), we examined their associations with other traits.

To assess relationships with behavior scores, we used a general linear model using the PCA scores with population, chest melanization, throat melanization, post-STI testosterone, and elevation as factors, with time to capture-and-bleed, time of day, and date as covariates. Relationships between steroid hormone concentrations and melanization were assessed using a multivariate analysis with stress treatment (immediate bleed or 1-hr confinement), population, population × treatment interaction term, and mite load as factors, with time to capture-and-bleed, time of day, and date as covariates. Then we performed an ANOVA to assess population differences in mite loads using population and melanization (chest and throat) as factors.

We also examined within-population variation in relationships between melanization and other traits, which had been done for the two 2013 populations (Seddon & Hews, 2016). Thus, we examined correlations between melanization and aggression, testosterone, corticosterone, and mite loads for a total of four populations studied in 2014 and 2015.

3 | RESULTS

3.1 | Body temperature

Male *S. occidentalis* were consistent and relatively precise in their behavioral thermoregulation. At four different sites in 2 years (2014, 2015), we found that average body surface temperatures were maintained between 31 and 34°C. Body temperature differed significantly

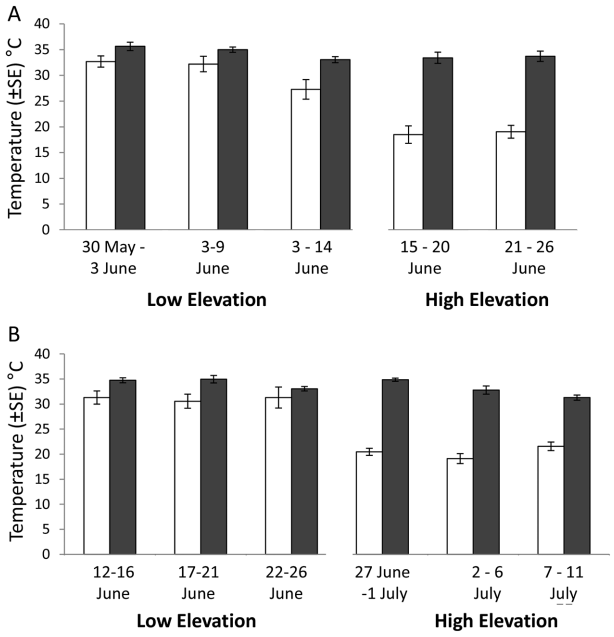

FIGURE 1 Near-weekly average air and body temperatures of adult male *S. occidentalis*. (A) Two populations (one high and one low) studied in 2014. (B) Two populations (one high and one low) were studied in □ Air Temperature ■ Body Temperature 2015. Sample sizes ranged from 5 to 12 males per site and per date bin

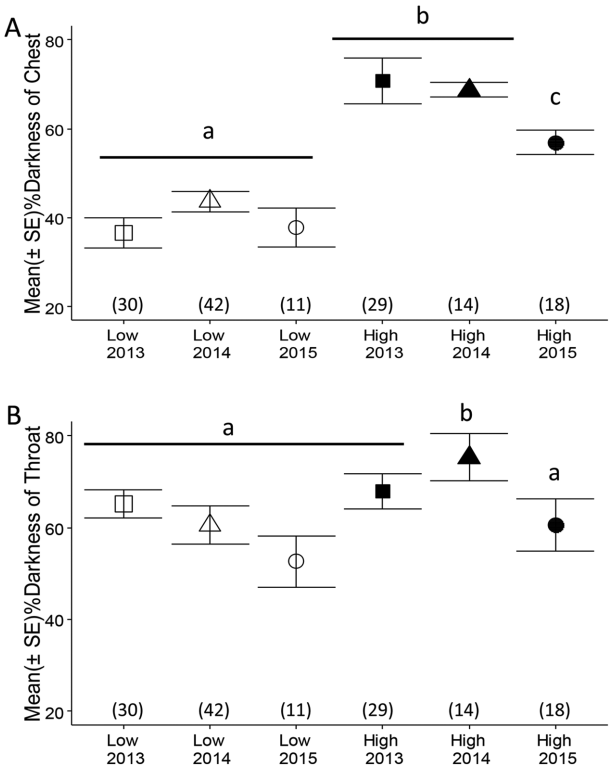

FIGURE 2 Percent darkness of (A) chest (degree of melanization) and (B) throat, in six populations of adult male *S. occidentalis*. Both population average (±SE) of melanization are shown. Different high- and low-elevation populations were sampled each year. Mean chest blackness was lowest in the lower elevation populations and highest in the high-elevation populations. Means not sharing a letter differ by $P < 0.05$. Sample sizes in parentheses

from air temperatures, but not between sites sampled within each year in spite of significant site differences in air temperature (Figure 1). We calculated average temperatures (air, body) by combining all measurements taken during the trials (1000–1400 hr each day) into 5- or 6-day bins (depending on how long we worked each site). In both years, air temperatures were colder at high-elevation sites (averaging 16–18°C) than at low-elevation sites (averaging 27–33°C), and these differences were statistically significant (2014, ANOVA $F_{4,68} = 14.96$, $P < 0.001$; 2015, ANOVA $F_{4,32} = 21.76$, $P < 0.001$). In contrast, average body temperature in each of the bins did not differ between populations in either year (2014, ANOVA $F_{4,68} = 0.917$, $P = 0.459$; 2015, ANOVA $F_{4,32} = 0.885$, $P = 0.505$).

3.2 | Coloration

The degree of melanization differed among lizards from the six populations sampled. As expected, we found significant population differences for chest ($F_{5,200} = 40.03$, $P < 0.001$; Figure 2A) with the three high elevation populations being more melanized than the low elevation populations. Post-hoc analysis showed that all three low elevation populations were similar ($P > 0.50$). Likewise, two of the three high elevation populations (the Sierra Nevada sites: High 2013 and High 2014 populations) were not significantly different ($P = 0.27$), but the High 2015 population was significantly higher than all other populations ($P < 0.01$ for all). Like chest melanization, the throat melanization also differed with elevation ($F_{5,200} = 4.96$, $P < 0.001$; Figure 2B), but post-hoc analyses revealed that only the High 2014 population (high elevation, 2014) significantly differed from all other populations (all P values < 0.01). Because of this, and because all subsequent statistical results involving throat melanization reported below were non-significant, we present only the test results and not the data graphs for this trait.

3.3 | Agonistic behavior

PCA on behavior rates from the STI (aggression) trials yielded two axes with eigenvalues greater than 1 (eigenvalue = 1.64 and 1.46, respectively; Table 1 and Figure 3) explaining 33.7% and 27.4% of the observed variation in behavior, respectively, and showing a break point in the scree plot. The first axis, PC1, which we termed Approach-Aggressive Signaling, described variation in moving toward the stimulus male and performing the highly-aggressive full show display, which is typically performed in escalated contests while in two males are in close proximity. The second axis, PC2, which we termed Physical Contact/Retreat-Explore, described an inverse relationship between the physical contact of biting and wrestling (negative loadings) and exploratory behaviors including chemosensory behavior, back-off behavior and head bob (high positive loadings). Head bob is a broadcast display used in a variety of social contexts and when moving through a territory; similarly chemosensory behaviors are often exploratory and in *Sceloporus*, are performed after moving to a new position (Bissinger & Simon 1981; Martins et al., 2015). Hence these combinations of variables for PC1 and PC2 are biologically consistent and interpretable.

TABLE 1 Varimax-rotated component matrix for the principal component analysis, showing the contribution of each behavior toward the respective component

	Principal component (Eigenvalue)	
% Variation explained	PC1 (1.64) 27.4%	PC2 (1.46) 24.3%
Behavior	"Approach -Aggressive Signaling"	"Physical contact/ Retreat-explore"
Chemosensory	0.04	**0.505**
Back off	−0.198	**0.631**
Move toward	**0.858**	−0.247
Headbob	0.186	**0.718**
Fullshow	**0.912**	0.17
Physical contact	0.110	**−0.493**

The behavior variables are per minute rates performed by focal male Western Fence Lizards, *Sceloporus occidentalis*, in staged territorial intrusions of stimuli males. Combined results for all six populations are shown below. See *Materials and Methods* section for description of behavior patterns.

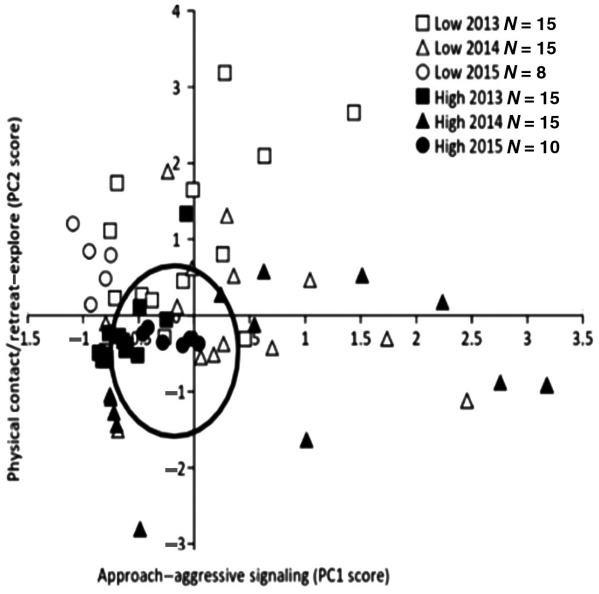

FIGURE 3 Individual behavior scores from a principal components analysis on the per minute rates of behavior. Behavior was performed by free-ranging focal males towards intruder males in six populations recorded during staged territorial intrusions. (A) PC1 (Approach-Aggressive Signaling) was defined by move towards and fullshow display. (B) Physical Contact/Retreat-Explore (PC 2) was defined by positive loadings for chemosensory behaviors, back off, headbob display, and negative loadings for physical contact. The ellipse highlights a cluster of values that show a relationship between low rates of signaling with higher rates of physical aggression

We assessed associations of the STI behavior (PC1 and PC2) with degree of melanization (chest, throat), population, and post-STI testosterone, with time to start of trail to completing the capture and bleed as a covariate in the MANOVA. The only variable that explained variation in both PC1 and PC2 behavior scores was population (PC1: $F_{5,57} = 5.45$, $P = 0.023$; PC2: $F_{5,57} = 5.80$, $P = 0.019$; Figure 4). Performing post-hoc analysis for PC1 (Approach-Aggressive Signaling), we found a mixed result with some low elevation populations having higher mean scores (i.e., higher rates of aggressive signaling and approaches) that some of the mean PC1 scores for high-elevation population. The Low 2014 population had lower mean PC1 scores compared to the Low 2013 and Low 2015 populations (both $P < 0.015$). The High 2014 population had a higher mean PC1 score compared with the other high elevation populations (2013, 2015; $P < 0.01$). Comparing scores for PC2 (Physical Contact/Retreat-Explore), we found that all three high-elevation populations had lower mean PC2 scores (high rates of physical contact) than the low-elevation populations ($P < 0.05$). The High 2014 population had the higher mean PC2 score (lower rates of physical contact, higher rates of exploration) than the other two high elevation populations (both $P < 0.05$). Finally, the mean PC2 behavior score for Low 2014 population was significantly lower than other two low-elevation populations (both $P < 0.01$).

This MANOVA also evaluated associations between melanization and PC1 and PC2 behavior scores. Independent of population, chest melanization was a significant predictor of behavior, but only for PC2 ($F_{1,57} = 22.42$, $P < 0.001$); melanization was negatively associated with signaling behavior and positively associate with approach. None of the other predictors (throat melanization, population, and post-STI testosterone) were significant (all P values > 0.2). Finally, we found no within-population associations between either PC1 or PC2 and melanization, for any population (all $P > 0.15$, data not shown).

3.4 | Hormone levels

We examined variables that could contribute to explaining variation in testosterone and corticosterone in a second set of males that we captured for this hormone analysis. We ran a MANOVA that included population, chest melanin, throat melanin, stress treatment (baseline, 1-hr post capture) and ectoparasite load as factors, and time-to-capture-and-bleed as a covariate. Neither time-to-capture-and-bleed (testosterone: $F_{1,75} = 0.04$, $P = 0.84$; corticosterone: $F_{1,75} = 0.56$, $P = 0.56$), nor time of day (testosterone: $F_{1,75} = 0.51$, $P = 0.48$; corticosterone: $F_{1,75} = 3.9$, $P = 0.053$), nor date (testosterone: $F_{1,75} = 0.71$, $P = 0.40$; corticosterone: $F_{1,75} = 0.027$, $P = 0.87$) were significant and they were removed from the model.

In this MANOVA, variation in testosterone (Figures 5A and B) was only explained by population ($F_{1,88} = 13.47$, $P < 0.001$). Mean testosterone was higher in two (2013 and 2014) low-elevation populations than in the other four populations ($P < 0.03$ for all values). Variation in plasma testosterone was not explained by melanization (chest, throat), stress treatment, ectoparasites, or the population-by-stress treatment interaction term ($P > 0.1$ for all variables) in this model. In spite of a significant among-population association between melanization and testosterone, we found no significant within-population associations between testosterone and melanization of chest or throat, for any of the six populations (all $P > 0.45$).

The MANOVA revealed that variation in corticosterone (Figure 6A and B) was explained by population ($F_{1,88} = 5.38$, $P < 0.001$) and, as expected, by stress treatment ($F_{1,88l} = 10.1$, $P = 0.016$). Mean baseline corticosterone levels were higher for low elevation populations compared with the higher elevation populations. A significant treatment term indicates that all populations showed elevated corticosterone

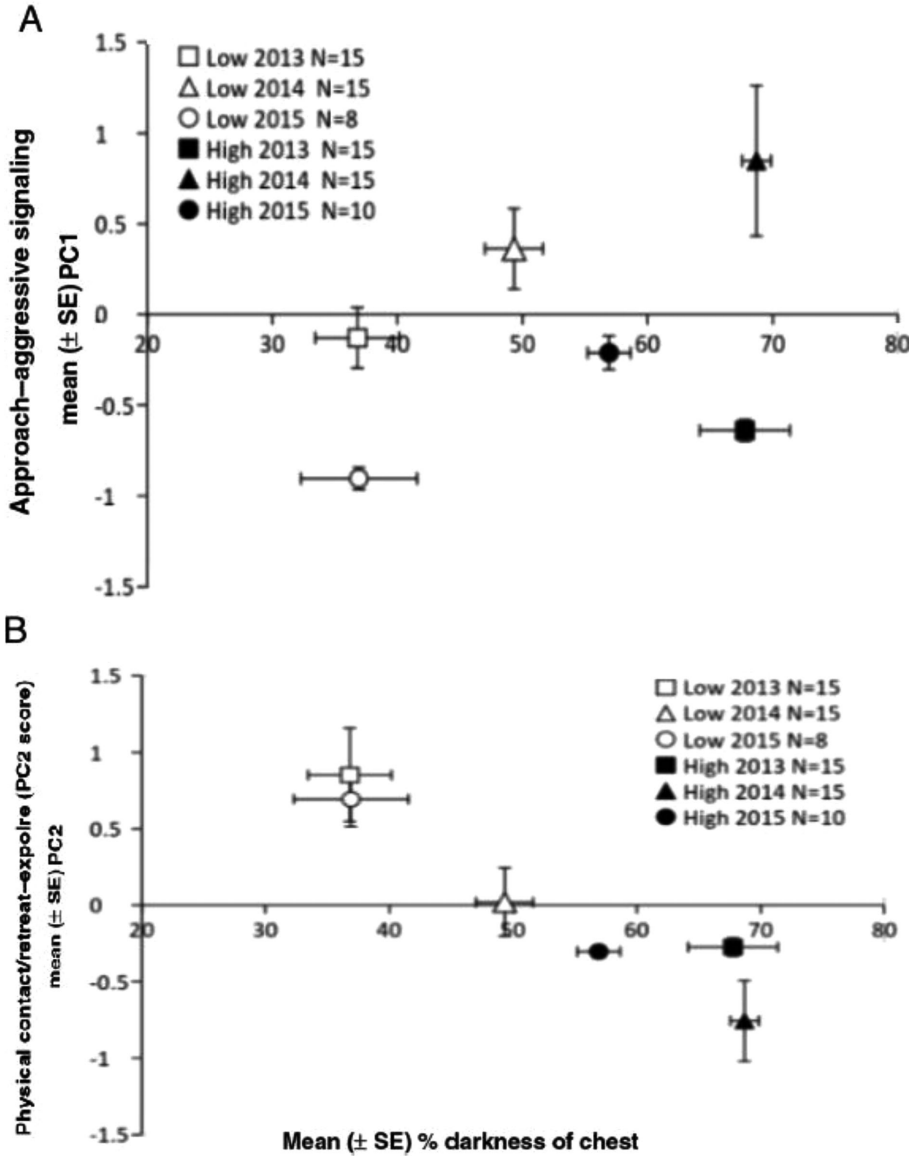

FIGURE 4 Relationships between mean melanization (darkness of chest) and the mean principal component scores of behaviors performed by free-ranging focal males during staged male-male interactions from six populations. At the population level, chest melanization was not significantly associated with (A) Approach-Aggressive Signaling (PC1) as defined by move towards and fullshow display ($P > 0.01$) or with (B) Physical Contact/Retreat-Explore (PC2) as defined by head bob display, chemosensory behavior, back off, and physical contact ($P > 0.01$)

following the captivity treatment (stress-reactivity), as assessed by comparing these cross-sectional measures (i.e., baseline and 1-hr bleed groups were different individuals).

Populations differed in the mean change in corticosterone following the captivity stressor (mean 1-hr confinement minus mean baseline), as revealed by a significant population effect ($F_{1,42} = 22.52$, $P < 0.001$, Figure 6C). The Low 2013 and Low 2014 populations had significantly greater stress reactivity (change in corticosterone concentration) than the other populations ($P < 0.001$ for all comparisons).

The MANOVA also revealed that chest melanin had a significant negative effect explaining variation in corticosterone, independent of population. Despite this result, we found no significant within-population associations between melanization and any hormone (all P values > 0.35).

The MANOVA, which also assessed associations between throat melanization (instead of chest), population, stress treatment, and hormone levels (testosterone, corticosterone), revealed no significant associations between melanization and either hormone ($P > 0.3$; data not shown).

3.5 | Ectoparasite loads

Using data from all males that we captured (focal and stimulus males; stress hormone study males), mean mite loads were higher for high-elevation versus low-elevation populations ($F_{5,150} = 24.42$, $P < 0.001$; Figure 7). Post-hoc tests revealed that High 2013 and High 2014 populations both had significantly higher mean mite loads than all other populations (all values $P < 0.01$). Again, we found no within-population

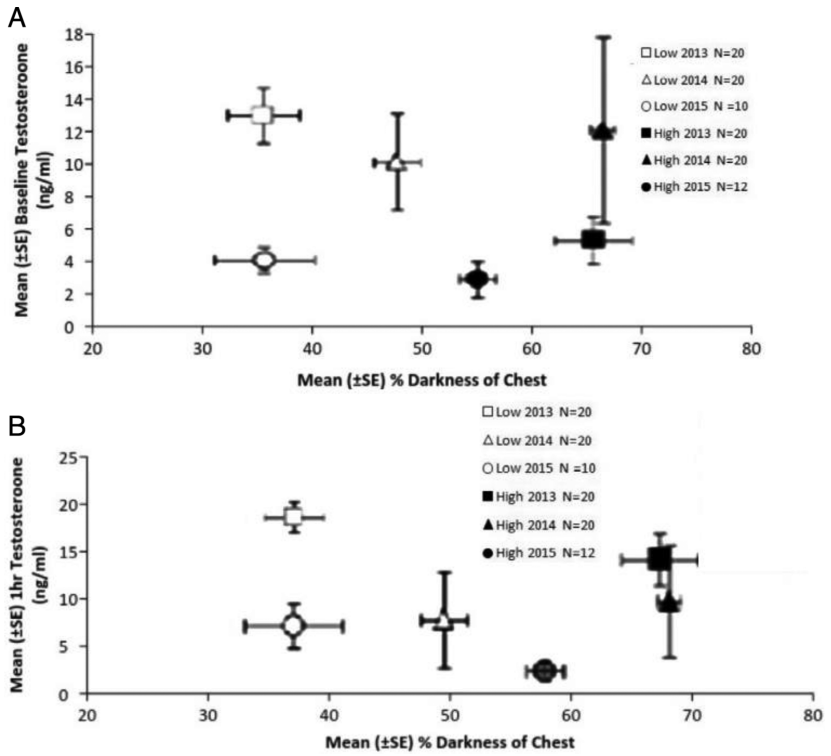

FIGURE 5 Relationships between degree of chest melanization (darkness of chest) and plasma testosterone in male *S. occidentalis*. (A) Baseline and (B) 1-hr confined testosterone were both significantly and negatively associated with mean chest melanization, when analyzed across populations

association between melanization and mites in any population, even with this larger sample of males ($P > 0.45$ all populations).

4 | DISCUSSION

We confirmed that this set of high-elevation populations were, on average, more melanized (lower brightness scores in chest) than low-elevation populations, although one high elevation populations (2015) had a significantly lower degree of melanization than the other high elevation populations. The elevational difference supports earlier work on several Sierra Nevada populations of this species (Camp, 1916; Leaché et al., 2010; Seddon & Hews, 2016). Since our melanization measurement used amount of black, which is produced by melanin, the melanin content of the skin in these lizards likely differed. Melanin and black skin has been documented in other *Sceloporus* (Morrison, Rand, & Frost-Mason, 1995; Quinn & Hews, 2003).

Aggression and melanization were positively associated when comparing across populations, as predicted by the MH. Two of the three more melanized, high elevation populations showed higher rates of approaching and aggressive signaling (high PC1 scores) and all three high elevation, more melanized populations showed higher rates of physical contact (low PC2 scores). However, we failed to detect significant within-population associations of melanization with either PC1 or PC2 behavior scores. This contrasts with other studies examining within-population variation, which find that more melanized individuals can be more aggressive (e.g., Raia, Guarino, Turano, Polese, Rippa, Carotenuto, Monti, Cardi, & Fulgione, 2010; van den Brink, Henry, Wakamatsu, & Roulin, 2012). The lack of these within-population associations between aggression and melanization may be due to a variety of factors. For example, melanin deposition may arise from multiple factors, including individual differences in circulating androgens and/or target-tissue differences in androgen sensitivity. If so, between-population differences in melanin could be explained by our observed between-population differences in plasma androgens, but the within-population variation in melanin could involve individual differences in sensitivity to androgens. Such individual differences in sensitivity can arise from a variety of mechanisms, including genetic variation. Some but not all within-population studies focus on "color morphs" (e.g., that may represent genetic variants; Roulin, Ducret, Ravussin, & Altwegg, 2003; Roulin, 2004). It is not clear if genetic differences have been identified that could explain the more continuously distributed variation in melanization within populations, as we observed. For example, differences in expression of tyrosinase, the rate-limiting enzyme in melanin synthesis (Slominski et al., 2004), could arise from genetic variation and could generate more continuous variation in melanization. Finally, our single point measure of baseline plasma testosterone does not reflect the longer-term average testosterone levels that would potentially be responsible for longer-term deposition of this pigment. There also could be a minimum threshold value for increasing melanization, and this testosterone threshold could vary between populations. This potential variation predicts population differences in sensitivity to androgens in targets (e.g., Ketterson et al., 2009).

While plasma testosterone differed significantly among populations, the between-population differences in mean baseline plasma

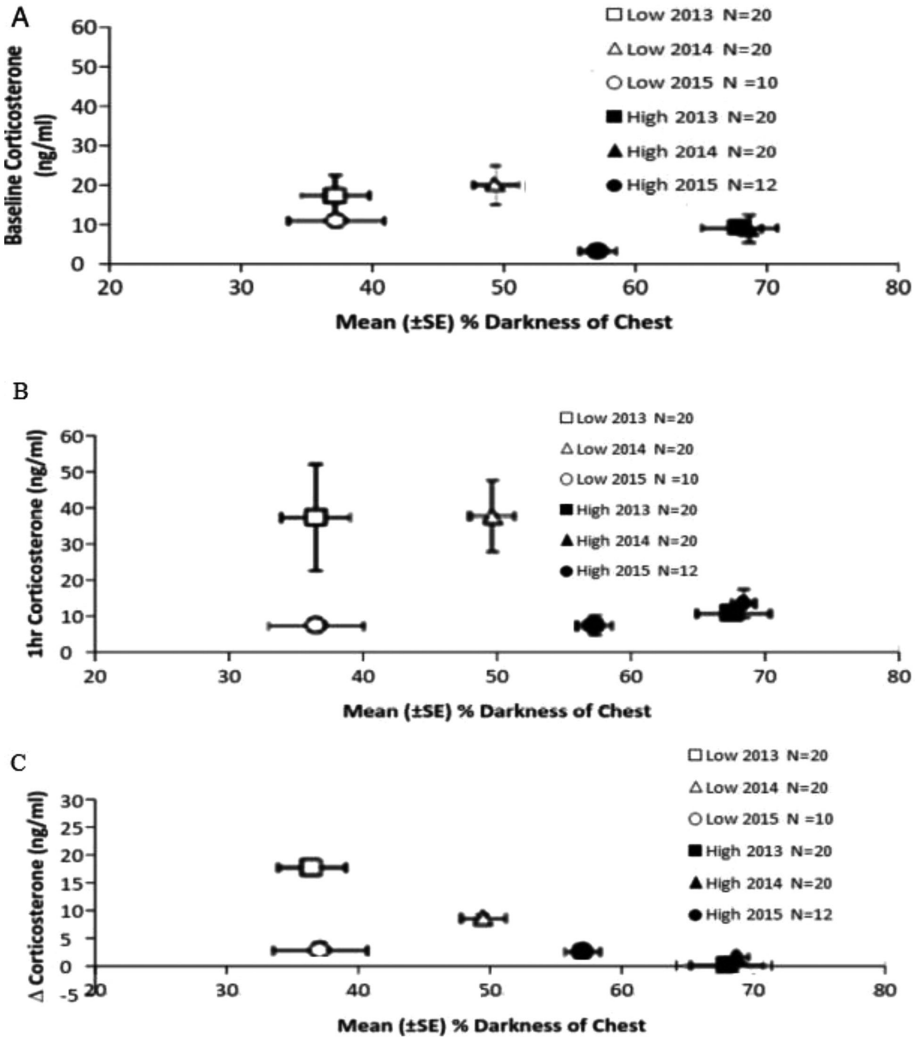

FIGURE 6 Relationships of mean melanization (darkness of chest) with (A) baseline corticosterone (B) 1 hr-confined corticosterone or (C) change in corticosterone and mean melanization of male *S. occidentalis* in the stress-handling study. Only the 2013 and 2014 low-elevation populations differed from other populations, and both were significantly higher for all three hormone measures ($P < 0.03$ for all)

testosterone levels were in an unexpected direction. The low elevation, less melanized populations had mean PC1 and PC2 scores that were interpreted as lower rates of approach, aggressive signaling and physical contact. Yet these populations had higher mean baseline testosterone. This does not support the MH, nor the typical expectation of a testosterone-aggression relationship (e.g., Wingfield, Hegner, Dufty, & Ball, 1990). There is a growing literature showing various relationships between baseline level of testosterone and aggression (Wingfield, 2005; Montoya, Terburg, Bos, & van Honk, 2012; Husak & Lovern, 2014). Our unexpected population differences in testosterone are unlikely to be due to seasonal effects; we sampled all populations during a comparable stage of the breeding season and male *Sceloporus* maintain elevated plasma testosterone throughout the breeding season (e.g., Moore & Lindzey, 1992). Population-specific factors could contribute to the unexpected result. For example, other behaviors such as movement and territorial displays are mediated by circulating testosterone (Marler & Moore, 1988; Wikelski, Steiger, Gall, & Nelson, 2004), and these may differ between our populations because of features of the habitat that affect visibility and connectivity. Rates of encounter and courtship with females could be higher in the low elevation populations if population densities differed, and male–female interactions may elevate plasma testosterone in *Sceloporus* (Smith & John-Alder, 1999). Clearly, additional behavioral studies, such as determining baseline behavior rates, are needed.

The six populations differed significantly in both baseline corticosterone and corticosterone responses to a short-term captivity stressor. As the MH predicts, the more melanized (higher elevation) populations had lower mean baseline corticosterone levels and lower mean post-confinement levels (stress reactivity). All populations exhibited higher mean corticosterone after 1-hr confinement stress (compared to each population's mean baseline). This suggests a hormonal stress response occurred to the handling and confinement treatment, and rapid rises in corticosterone are commonly seen as an adaptation to stressful situation such as confinement (Dunlap & Wingfield 1995; Tyrrell & Cree 1998; Ketterson et al., 2009). The inverse relationship with corticosterone was expected, given α-MSH modulates the HPA stress response via enzymatic processes, such as for the prohormone convertases enzyme (Helwig et al., 2006). Formation of α-MSH requires

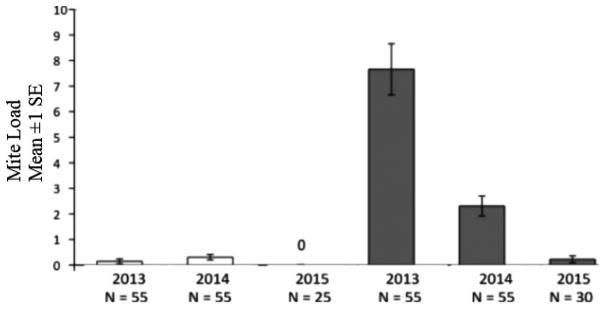

FIGURE 7 Relationships between external mite loads (counts) and melanization levels in males from six populations of *Sceloporus occidentalis*. Melanization (chest) and mite loads were not significantly correlated across populations. There were no mites found on lizards in the low elevation population in 2015

cleavage of this peptide from ACTH, which could result in lower ACTH concentrations in the blood, and thus less adrenal cortex activation, lowering glucocorticoid production. In addition to population differences in melanization, other factors may be associated with high versus low elevation that could favor difference in adrenal function. For example, baseline corticosterone is lower in high elevation populations of several *Sceloporus* species (Hews & Abell Baniki, 2013). The breeding season duration hypothesis proposes that individuals may have a lower stress reactivity in shorter breeding seasons, which may be the case for high elevation populations, compared to those with longer breeding seasons; this was not supported by Hews and Abell Baniki (2013). Another factor that might differ among high and low elevation sites is predation, where populations under more intense predation can show muted stress responses (Mateo, 2007).

Mite loads also differed among populations and higher elevation populations on average had higher mean mite loads than the lower populations, combining data for all captured males (Figure 7). However, mean population mite loads were not related to the population differences in mean melanization. Again, melanin-independent factors could contribute to population differences in mite loads. During all years of this study, California experienced a multi-year drought with low moisture content at the lower elevations (National Weather Service), which might have contributed to the low parasite loads observed at low elevation. While these results differ from most studies examining the relationship between parasites and elevation (e.g., Hasegawa, Ito, & Kitayama, 2006; Fischer & Schatz, 2013), they are partially consistent with some studies revealing a positive relationship between melanin and parasite loads (e.g., Zippel, Powell, Parmerlee, Monks, Lathrop, & Smith, 1996; O'Connor, Dudaniec, & Kleindorfer, 2010). Finally, behavioral differences between the populations could have contributed to differences in parasite loads. Males from the more aggressive, high elevation populations could have performed more patrolling, exposing them to more parasites as seen in males of other *Sceloporus* (Klukowski, 2004).

Across two of our three categories of blood sampling (baseline, 1-hr confinement, post-STI), we found no relationship between mean steroid hormone levels and mean mite loads for between-population comparisons, nor did we find within-population associations. In many species, animals with elevated plasma testosterone levels also have increased parasite loads (see also Greives, McGlothlin, Jawor, Demas, & Ketterson, 2006; Pollock, Vredevoe, & Taylor, 2012), including in some lizards (Olsson, Wapstra, Madsen, & Silverin, 2000). While those studies give support to the immunocompetence handicap hypothesis (Folstad & Karter, 1992), immunosuppression is not always found (e.g., Evans et al., 2000). Other parasite loads, such as blood and intestinal parasites, should be assessed in our populations to examine the generality of our results.

Higher melanization in higher elevation populations suggests possible selection favoring darker skin to confer thermal benefits and/or UV protection. Our thermal data on skin temperatures suggests that the high elevation populations can maintain relatively high levels of homeothermy during the diurnal activity period, supporting the hypothesis that increased melanization could be favored to enhance warming (Clusella Trullas, van Wyk, & Spotila, 2007). Possible strong selection favoring this single trait is one explanation for why we failed to find significant associations of melanization and the other traits within each population. If these population differences in melanization have a major genetic component, our results suggest that hormonal pleiotropy did not constrain this evolutionary variation. It is possible that strong selection on melanism could reduce within-population variation in melanization, perhaps explaining our failure to find significant within-population relationships between melanization and other variables. However, within-population variation in melanization does not appear to be limited (c.f. Figure 4). Finally, while there are known genetic differences among our study populations (Leaché et al., 2010), the relative contributions of genetic and environmental contributions to trait variation is unknown in general (reviewed in Clusella Trullas et al., 2007) and specifically for these populations. Further research, such as common garden experiments, would help assess this. While the scientific literature studying melanism is growing, more studies comparing both within and across-populations are warranted.

5 | SUMMARY

Relative to the MH and to other studies (see Introduction), we found a mixed set of associations between melanization and our measured variables. Compared to the three lower elevation less dark populations, our three higher elevation, darker populations had higher mean physical aggression, lower mean plasma testosterone, lower baseline plasma corticosterone, lower stress reactivity (for two of three high elevation populations), and higher ectoparasite (mite) loads. Hormonal "pleiotropy," would predict a consistent suite of correlated traits across populations. Alternatively, varying selective pressure could result in hormonally mediated suites not showing consistent correlations. Our results suggest that trait associations are not rigidly linked with melanization. On average, high-elevation populations had darker, more aggressive males than their less melanized, lower elevation counterparts, but there was no within-population association between melanin and aggression. We found an unexpected negative relationship between mean melanization and mean baseline testosterone. In addition, reduced stress reactivity was correlated with mean melanization across populations, but mean ectoparasite loads were

not. While other parasites and other aspects of immune function should be assessed in these populations, our results suggest hormonal 'pleiotropy' may not constrain evolution.

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