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Phenotypic correlates of melanization in two *Sceloporus occidentalis* (Phrynosomatidae) populations: Behavior, androgens, stress reactivity, and ectoparasites

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HIGHLIGHTS

- Factors mediating skin pigmentation could affect aggression
- Baseline corticosterone and stress reactivity were not correlated with melanization
- Testosterone levels do not match trait differences between high and low elevation

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ABSTRACT

Mechanisms underlying production of animal coloration can affect key traits besides coloration. Melanin, and molecules regulating melanin, can directly and indirectly affect other phenotypic traits including aggression, stress-reactivity, and immune function. We studied correlation of melanization with these other traits, comparing within- and between-population differences of adult male western fence lizards, *Sceloporus occidentalis*. We compared one high- and one low-elevation population in California where individuals are increasingly darker at higher elevations, working during comparable periods of the breeding season at each site (first egg clutch). We measured agonistic behaviors of free-ranging males in response to staged territorial intrusions (STIs). In other sets of males we measured baseline testosterone and corticosterone levels, and hormonal-reactivity to a stress handling paradigm. We counted ectoparasite loads for all males. There were no significant associations between individual variation in melanization and individual variation in any of the variables measured. However, analysis of behavior from the STIs revealed that males in the darker high-elevation population responded with more aggressive behavior compared to males in the lighter low-elevation population. Males in the low-elevation population had significantly higher mean baseline testosterone, but the two populations did not differ in adrenal function (baseline corticosterone or corticosterone after 1-h confinement stress). Males in the darker high-elevation population had higher mean mite loads compared to males in the lighter population. This array of phenotypic differences between the two populations, and the absence of trait associations when assessing individual variation, do not parallel the patterns in other vertebrates. We describe potential differences in selective regimes that could produce these different patterns across vertebrates. These data suggest that hormonal pleiotropy does not constrain phenotypic variation.

1. Introduction

Hormonal "pleiotropy", a term borrowed from genetics, involves the concept that suites of traits may be mediated by elements (signals, receptors, enzymes) within a hormonal system, such as the melanocortin system, and that the endocrine system can generate phenotypic correlations among suites of traits, with or without underlying genetic variation. Hormonal pleiotropy might constrain or facilitate the evolution of adaptive suites of traits ([58]; *cf.*, [98,102]). A related area in behavioral ecology is the study of how mechanisms underlying production of animal coloration affect more than just body coloration [19,70,81]. For example, suites of traits associated with melanistic coloration can be affected directly or indirectly by melanin [19]. Melanin-based coloration can function in social communication [50] and can covary with many other physiological, morphological, and behavioral traits (*e.g.*, [19,85]). Studies of such covariation with melanin-based coloration are mostly with birds and mammals [19] and very little has been done with reptiles ([59,103]). We examined potential melanin correlates in the western fence lizard, *Sceloporus*

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occidentalis, whose populations are increasingly melanized with increasing elevation in parts of western North America [49].

While ornament expression can be mediated by physiological, genetic, and social mechanisms [42,55,81], hormonal pleiotropy can be important in expression of social signals and correlated effects on a variety of traits [19,81]. Melanin-based ornaments commonly are signals of aggression and/or hormonal status [57,78], and darker individuals can be more aggressive (*e.g.*, [70,97]). The size and color of melanin-based ornaments have been linked to plasma hormone levels, such as testosterone [29,33,38]. Melanocortins, such as α-MSH, can affect aggressiveness, *via* direct binding to melanocortin receptors [61], or indirect affects if it interacts with androgens and melanization (*e.g.*, [15]). Melanocortins also are part of the hypothalamic-pituitary-adrenal (HPA) "stress axis" [11], and thus other HPA axis elements (*cf.*, [69]), including as synthesis of glucocorticoid synthesis may covary with melanization [3]. An equivalent HPA axis also exists in the skin and activates in response to local cutaneous stress response [90]. Several studies link melanin-based coloration with altered HPA reactivity [48,73]. Degree of melanization also can be associated with differences in immune function and in parasite exposure [7,19,27].

There are several possible patterns for how other traits may covary with melanization, when comparing both within and between populations. At the simplest, we might expect similar patterns for both comparisons (within- and between-population). In this scenario, higher levels of melanization would be associated with, for example, higher levels of the other trait (*e.g.*, aggression), within each population and also when comparing population means across populations that differ in average degree of melanization. This could be interpreted as there being some common causal mechanism(s). Alternatively, we could observe parallel population differences in mean melanization and mean aggression levels, but find that within-population variation in these two traits is not correlated. In this second scenario, factors known to contribute to melanization (*e.g.*, plasma α-MSH levels) may or may not contribute to explaining population differences in melanization. A hormone may still be involved in such population differences in trait expression if, for example, there is a threshold effect of the hormone on trait expression (*cf.*, [2,37]). With a threshold effect, within-population variation in melanization must be affected by other factors, such as environmental or genetic factors producing variation in the regulation of rate-limiting enzymes in melanin synthesis. Similarly, other factors may also contribute to within-population variation in the traits. Here, we examine both population differences and within-population patterns of trait associations with melanization. If we detect population differences we are also interested in potential contributing factors, and thus we also compare testosterone in the two populations.

Here report on a study of two populations of the western fence lizard that differ in melanization. We make specific predictions based on empirical and/or theoretical expectations outlined above. Accordingly, darker individuals should be more aggressive, have higher plasma testosterone, altered hormonal stress reactivity and lower ectoparasite loads, assessed by number of mites. Species in this genus are excellent system for studying these questions related to hormonal pleiotropy in social signals, because a great deal is known about coloration in *Sceloporus* lizards [36]. Blue signaling patches in most *Sceloporus* are limited to males [36,38,67] and are exposed during stereotyped displays used for territory defense and/or courtship that include combinations of head-bobs, leg extensions and other body postures (*e.g.*, [36,52]). Abdominal blue patches are due to an underlying layer of melanin that absorbs all wavelengths not refracted back by a platelet layer in overlying iridophores ([13,38,62,63,72]; D Hews, A Leaché, M Shawkey, D Barnes, unpublished). Hormone manipulations reveal that the permanent, sex-limited expression of this melanin layer in the abdominal skin in males is mediated by exposure to androgens early in development [38]. In many vertebrates including reptiles, the perihatching or perinatal periods are also when androgens can masculinize brain regions mediating adult aggression [16] and such early "organizational" hormonal effects are examples of phenotypic linkages that "hormonal pleiotropy" can create.

The elevation-associated pigmentation differences in our study species, the western fence lizard, can include both the dorsum and ventrum [9,49]. We focus on the ventrum, for several reasons. Ventral coloration is the best-understood aspect of signaling in this genus, because the ventral region is revealed during postural displays during social signaling. In addition, variation in ventral melanization may be less likely to result from direct selection arising from thermoregulatory advantages (*e.g.*, females can have very dark dorsum but a white ventrum). We also analyzed variation in throat melanization, because throat coloration can be associated with behavioral variation in lizards (*e.g.*, [40,94]). The role of throat coloration in signaling has, perhaps surprisingly, been less well examined in *Sceloporus*, outside of the yellow-orange-blue variants in two species (*Sceloporus undulatus eythrocheilus*, [75,76]; *Sceloporus grammicus*, [5]). Both sexes may exhibit throat coloration that can vary greatly within and across *Sceloporus* species [92]. Males typically have more black than females in our study species (R Seddon, D Hews, personal observation) and also in many *Sceloporus* (D Hews, personal observation). Although a role of black coloration in aggression has not been examined in *Sceloporus* lizards, such a role for black has been seen in other lizards (*e.g.*, *Podarcis sicula*, [59]).

2. Materials and methods

The western fence lizard (*S. occidentalis*), an oviparous, diurnal lizard broadly distributed across far western United States, has a distinct mating season from April to July [92]. We studied two populations, one at a higher elevation and one at a lower elevation, during comparable phases (first clutch) of their respective breeding seasons [20]. For each trait measured, we assessed mean population differences and also individual associations with melanization. We evaluated male-male aggression using staged-territorial intrusions (STI) to measure responses of free-ranging males. We asked if populations differed in mean levels of post-STI testosterone, and also if individual differences in aggression and post-STI testosterone were associated. In another set of males, we compared both baseline corticosterone and testosterone levels, and hormonal stress responses after one hour of confinement. Finally, we asked if individual variation in ectoparasite loads was associated with degree of melanization and with variation in plasma testosterone or corticosterone, and we compared mean ectoparasite counts between populations for all lizards captured.

2.1. Study sites

We captured western fence lizards from Yosemite National Park in California at two sites that were part of an earlier study on *S. occidentalis* genetic variation and population differentiation [49]. We studied the low-elevation population along the Merced River (elevation 650 m; N37°43′, W-119°39′) from June 2–20, 2013, and the high-elevation population along Tioga Road (elevation 2000 m; N37°50′, W-119°28′) from June 21–25, 2013. This difference in each 2-week sampling period allowed us to sample fairly comparable stages in the breeding season: females were yolking up their first clutch of eggs, undisturbed individuals were frequently observed to engage in courtship and aggressive behavior at both site, and capture and palpation of a sample of females to assess follicles confirmed oviductal eggs were not present, and we could easily see that many females were late-vitellogenic with large follicles. Other studies have shown that higher elevation *S. occidentalis* have a later breeding season then their lower elevation counterparts [28]. Consistent with this interpretation is the fact that the high elevation site was still melting out from the winter snowpack, while the daily temperatures at the low elevation site became consistently adequate for onset of breeding activity. Although sites differed in ambient air temperatures, it is unlikely that any

behavioral differences observed would result from site differences in body temperatures; in several other high- and low-elevation populations of this lizard, we found that field-active males have similar body temperatures (30–35 °C) despite dramatic site differences in ambient air temperatures (R Seddon & D Hews, unpublished data), demonstrating effective behavioral thermoregulation capabilities of this species.

2.2. General lizard processing

All animal procedures were approved by the Institutional Animal Care and Use Committee of Indiana State University (protocol #425155-1 DH). All male lizards were captured, *via* noosing, between 1000 and 1400 h to minimize effects of potential diel changes in hormone concentrations. Immediately on capture, we sampled blood from the orbital sinus using heparinized microcapillary tubes (*e.g.*, [4,87]). Although capturing and blood sample collection was completed on average of only 2.9 min total (range: 1.3–4.4 min), we nevertheless included this time to capture and bleed as a covariate in our analysis. After blood sampling, we measured snout-to-vent length (SVL) to the nearest 1 mm and body mass (to 0.1 g), counted the total number of mites, took digital images of the ventral surfaces and recorded the time of day of capture. To avoid resampling, we temporarily marked lizards on 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 and measured the overlying plasma into clean vials with a Hamilton syringe. Plasma samples were then stored in known volumes of ethanol (10:1, ethanol: plasma) following Goymann et al. [30]) in screw top vials until returned to the laboratory, where vials were stored at −20 °C until assay (see below).

2.3. Color measurements

To quantify amount of melanization, we took a digital photograph of the ventral surface of each subject with a Canon® PowerShot A640 camera in the field. Color images were standardized to the same color standard within each lizard photograph [104], taken in sunlight, and by controlling the subject-to-camera lens distance. We quantified the degree of melanization using Adobe Photoshop CS2 (version 3.2.4, 1994) to measure three ventral regions (throat, chest, abdomen). The throat area extended from the rostrum to the neck. The chest region extended from one armpit to the other and up to then neck following a straight line from each armpit to the neck. The abdominal region excluded the paired blue abdominal patches, but included the medial scales of the ventral region starting at the straight line between the caudal edges of the forelimbs to the cranial edge of the hind limbs on the ventral torso. In each of the three body regions, we first outlined the extent of the black pigment using the lasso tool and then used the histogram function to calculate the average brightness of the area. We then calculated average black coloration by subtracting average brightness from 100. We use the term “melanization” to indicate lizards with higher values of blackness (less white, more black).

2.4. Aggression trials

To measure aggression we ran staged territorial intrusions (STIs) and recorded the behavioral responses of free-ranging focal males. Males used as the stimulus (intruder male) were captured at the same study site but at some distance (100–200 m) from the focal male's territory. While the intruders were likely strangers, it is possible that male lizards might have encountered each other before given that *Sceloporus* lizards are known to have home ranges with dimensions greater than this distance [1], although home range size varies with species and habitat. Each stimulus male was used once as an intruder. For an STI, we presented a resident male lizard with an intruder male, using a standardized protocol (*e.g.*, [43,53]). 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 (average = 4 m, range = 3–6 m) to record occurrences of four categories of behavior performed by the focal male over a 5-min period: (1) movement (move away, move towards, charge); (2) bouts of social displays including ‘head bob’ and ‘full show’; (3) physical contact (bite), and (4) chemosensory (tongue-flick, lick, gape). A ‘head bob’ was scored as a sequence of rapid elevation and lowering of the head, and could include use modifiers of either two or four limbs to elevate and lower the whole body. Full show was defined as a head bob that included arched back and dorsolateral flattening, which exposes the ventral coloration. ‘Moving away’ was divided into two categories: 1) ‘reposition’, when the focal male moved a short distance of up to 1m, often circling or displaying towards the intruder, before returning to the stimulus male; 2) ‘retreat’, when the focal male ran more than two body lengths away and did not return to the stimulus male. ‘Charge’ was a rapid move towards the stimulus male. The STI trial was continued until either the resident attacked the intruder (biting) or five minutes had passed. At the end of the STI, the resident male was captured and we measured morphology and took digital images. We only analyzed data from STIs in which the resident and intruder male were within 2 mm SVL of each other, to control for effects of body size on aggression.

2.5. Plasma testosterone and corticosterone

We captured and took a blood sample (see details above) immediately from a second set of undisturbed males ($N = 10$, each population). This immediate bleed set of males was used to determine baseline levels of testosterone. To assess adrenal function we measured baseline corticosterone from the same blood samples taken from the undisturbed males that were captured immediately for the testosterone measures. We also determined the effects on plasma corticosterone of a standard stress-handling protocol commonly used for small birds and lizards (one hour of confinement in a cloth bag) on a third set of males ($N = 10$, each population). Because changes in corticosterone may affect testosterone, we also measured testosterone in these 1-h confinement blood samples. On any given day, we alternated between capturing and sampling the second (immediate bleed) and third (1 h confinement) sets of males. An independent samples design was chosen to assess hormone levels, rather than a paired-design, because repeated blood sampling can affect hormone concentrations [17], and because of potential hemodilution effects in a small lizard. Stress-handling studies in several *Sceloporus* species indicate that plasma corticosterone typically elevates by 20 or 30 min of captivity confinement and remains elevated for over 1 h (see [35]).

2.6. Hormone assays

Plasma testosterone and corticosterone concentrations were determined *via* ELISA (Enzo Life Sciences, Inc., Correlated-EIA™ Testosterone Kit 900-065, and Correlate-EIA™ Corticosterone kit 900-097, Farmingdale, NY). We first optimized use of each kit with *S. occidentalis* plasma following protocols from [105]. Then, for either hormone assay, 40 μL of the ethanol and plasma solution was dried *via* nitrogen. 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 h 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 was added, and the plate was read. Sample hormone concentrations were determined through comparison with a standard curve, run in triplicate. Values on each standard curve ranged from 0.008 ng/ml to 2 ng/ml for testosterone and 0.032 ng/mL 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, ranged from 5.4 to 18.1% for testosterone and from 3.2 to 16.7% for corticosterone, which is similar to other studies (*e.g.*, [45]). For each hormone, all plasma samples from both populations were run on a single plate. The testosterone antibody has low (7.2%) cross-reactivity with androstenedione and negligible (<1%) cross-reactivity with dihydrotestosterone, dehydroepiandrosterone, β-estradiol, progesterone and corticosterone. The corticosterone antibody 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 β-estradiol.

2.7. Statistical analyses

We first assessed all data to determine if assumptions for parametric analysis were met. Hormone values were log-transformed (base 10) because of non-normal distributions. Simple population differences in melanization of three body regions (throat, chest, belly) were compared with Student *t*-tests for each region. We also present mean rates of individual behaviors from the STI trials, and compare populations with t-tests. We used a t-test to compare mean ectoparasite loads between the populations.

We then examined both population differences and with-population patterns of trait associations with melanization. We generated composite variables to summarize behavioral responses using a principal component analysis (PCA), with Varimax rotation. For factors with eigenvalues >1.0, we examined associations of these measures of behavior with other traits. Relationships between hormone concentrations and chest and throat melanization were assessed using MANOVA and including handling treatment (immediate bleed or 1 h confinement), population, population x treatment interaction terms, and the time to capture and bleed as a covariate. To assess relationships between behavior scores and post-trial steroid concentrations, we also used a multivariate analysis using the PCA scores with population as a factor and associated covariates. We performed ANOVAs to assess contributions of throat and chest melanization, population, and residual body mass (from a regression of body mass onto body length, SVL) in explaining mite load variation. A second analysis assessed if mite load variation was explained by baseline steroid hormone levels and population. All statistical tests were run on SPSS Version 19.0 (IBM Company, 2010). Tests with *P* values smaller than 0.05 were considered significant and all tests were two-tailed.

3. Results

3.1. Coloration

We first verified that melanization differed between the two populations. As expected, males in the high-elevation population expressed higher mean melanization for each of the three body regions analyzed (Fig. 1A) and significantly so for two regions (chest, $t = 4.87$, $df = 69$, $P < 0.001$; abdomen, $t = 4.92$, $df = 69$, $P < 0.001$) but not for the third (throat, $t = 0.61$, $df = 69$, $P = 0.541$). Melanization in the chest and abdominal regions were highly correlated ($P < 0.001$, $r = 0.7062$; Fig. 1B). Because of this, we used chest melanization for all further analyses, in addition to throat melanization given the potential signaling role of throat coloration in other lizard species.

3.2. Aggressive behavior

For the trials analyzed (after excluding trials in which focal and stimulus males differed in SVL by >2 mm), average trial duration was 2.76 min (range 0.5–5.0 min) at the high-elevation site ($N = 15$ trials) and 4.57 min (1.93–5.0 min) at the low-elevation site ($N = 15$ trials). Trials were shorter at the high-elevation site because males there elevated to biting relatively quickly and trials had to be terminated. The high-elevation darker lizards had significantly higher mean rates of most behaviors recorded in the STIs compared to the low-elevation trials (Fig. 2) including chemosensory behavior ($t = -2.08$, $P = 0.047$), reposition ($t = -3.221$, $P = 0.003$), head-bob bouts ($t = -2.50$, $P = 0.019$), fullshow bouts ($t = -2.42$, $P = 0.022$), and charge ($t = 2.213$, $P = 0.035$). Move away consisted of 40 incidents of 'reposition" (moving a short distance and reengaging) and only 1 incident of 'retreat'. Differences in physical aggression (biting) neared statistical significance ($t = -2.04$, $P = 0.051$) with a higher mean in the high-elevation population. Mean rates of move towards were very low and did not differ significantly between populations ($t = -0.42$, $P = 0.681$).

To compare individual variation in melanization and behavior we used a principal component analysis (PCA) to summarize the behavior rate data from the STI trials yielded two significant Varimax rotated axes (Table 1, Fig. 3, eigenvalues = 2.70 and 1.64), explaining 45.0%

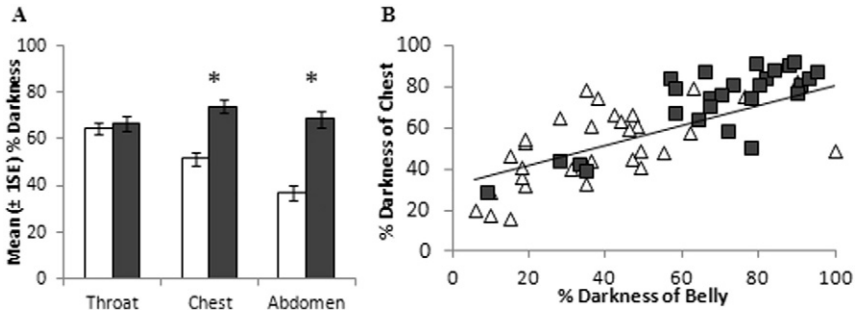

Fig. 1. Degree of melanization in each body region analyzed. A. Average percent blackness of male *S. occidentalis* by body region (Low-elevation, $N = 35$; High-elevation, $N = 36$). See Method section for definitions of body regions. For each of the three body regions, mean blackness was higher in the higher elevation population, and significantly so in the Chest ($P < 0.001$) and the abdomen ($P < 0.001$), but not throat blackness ($P = 0.541$, Low elevation □, $N = 33$; High-elevation ■, $N = 35$). B. Relationships between belly and chest regions of lizards for both populations, and the trend line are for combined populations (low-elevation, Δ; high-elevation, ■). The blackness of both the Abdomen and Chest were significantly correlated (low-elevation site, $N = 35$; high-elevation site, $N = 36$, $P < 0.001$, $r = 0.71$). Samples sizes differ between regions because of excluding images not meeting our scoring criteria (see Methods). *$P < 0.05$.

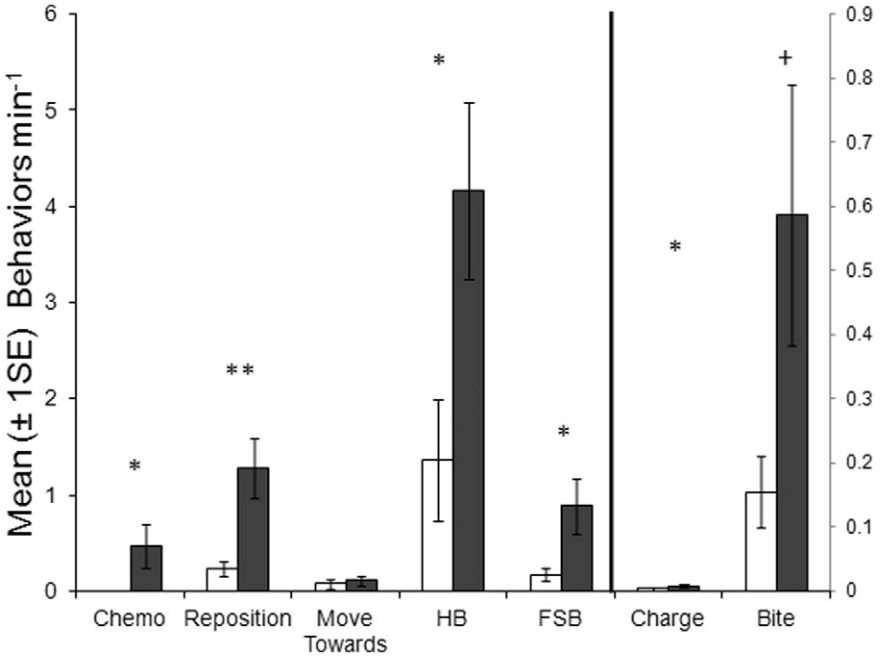

Fig. 2. Comparison of mean rates min^{-1} of behavior by focal males from a high-elevation melanized population (solid bars) and a low-elevation, less melanized population (white bars) of *Sceloporus occidentalis* ($N = 15$, per population) during 5-min staged territorial intrusions, in which focal and stimulus males were size-matched, *a posteriori*, to within 2 mm snout-vent length. See text for definitions of behaviors, which are arranged along the horizontal axis from less aggressive (left side) to more aggressive (right side) responses. Abbreviations: Chemo = Chemosensory; FSB = Fullshow bouts; HB = Head-bob bouts, + $P = 0.051$, $^{*}P < 0.05$, $^{**}P < 0.01$.

and 27.3%, respectively, of the observed variation in behavior. The first axis had higher loading for rates of head-bob, fullshow, reposition, and bite and we refer to PC1 as an "aggression axis" because these behaviors are seen exclusively in aggressive encounters except for head-bob. Head-bob can be seen in other social contexts, but the relative rate of display is elevated in aggressive encounters [53]. The second axis described variation in chemosensory behavior (high negative loading), moving towards and fullshow (both high positive loadings), and we refer to PC2 as "chemosensory and fullshow axis" with the two behaviors showing a strong inverse relationship. Fullshow is a highly aggressive display seen in escalated male-male encounters.

We assessed association between behavior, melanization and population with a MANOVA model, including terms for chest melanin, throat melanin, focal SVL, and population (Fig. 4). We included focal size because although males were size-matched in the STIs, pairs of larger males might differ in aggression compared to pairs of smaller adult males. Only population significantly explained variation in STI behavior and only for PC1 ($F_{1,19} = 5.\ 4.34$, $P = 0.031$), consistent with the analysis of specific behaviors. Neither chest nor throat blackness explained variation in either behavior axis (all P values >0.34), and SVL also was not significant ($P > 0.18$ for PC1, $P > 0.34$ for PC2).

Table 1
Varimax-rotated component matrix for the Principle Component Analysis, showing the contribution of each behavior towards the respective component. The behavior variables are rates performed by focal males in staged territorial intrusions ($N = 15$ trials per population).

Behavior	Component (eigenvalue)	
	PCA1 (2.70)	PCA2 (1.64)
Chemosensory	0.103	−0.842
Back off	0.719	0.338
Move towards	0.796	0.309
Headbob	0.387	0.019
Fullshow	0.369	0.556
Physical contact	0.791	−0.284

3.3. Testosterone levels

For the immediate bleed males ($n = 10$ per population), neither the time to capture and bleed ($F_{1,32} = 0.339$, $P = 0.564$) nor the time of day ($F_{1,32} = 0.728$, $P = 0.40$) were significant covariates explaining plasma testosterone in a two way ANOVA analyzing both steroid hormones. Hence they were removed from the model and the two-way ANOVA was rerun. The mean baseline plasma testosterone was significantly higher in the low-elevation population compared to the high-elevation population (Fig. 5A: $F_{1,34} = 4.833$, $P = 0.035$). Stress-handling treatment (immediate bleed *versus* 1-h confinement) also had a significant effect on plasma testosterone ($F_{1,34} = 5.173$, $P = 0.023$). The stress treatment by population interaction term was not significant ($F_{1,34} = 0.868$, $P = 0.983$) hence males in both populations

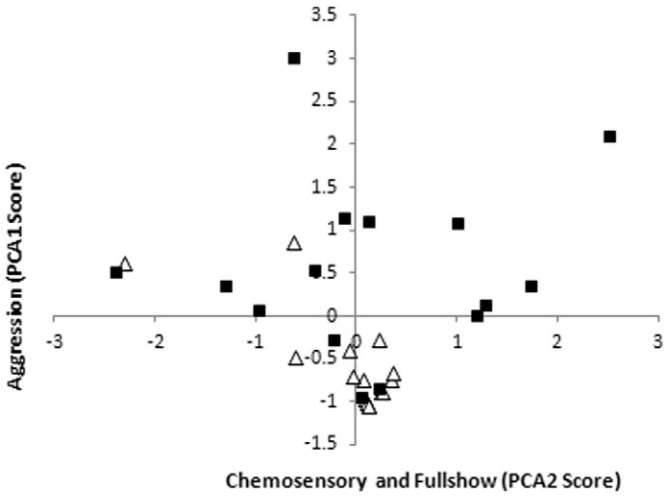

Fig. 3. Behavior scores from a principle components analysis on the rates of behavior performed by focal males in the staged territorial intrusions (high-elevation, ■; low-elevation, Δ). See Table 1 and text for description of the two components.

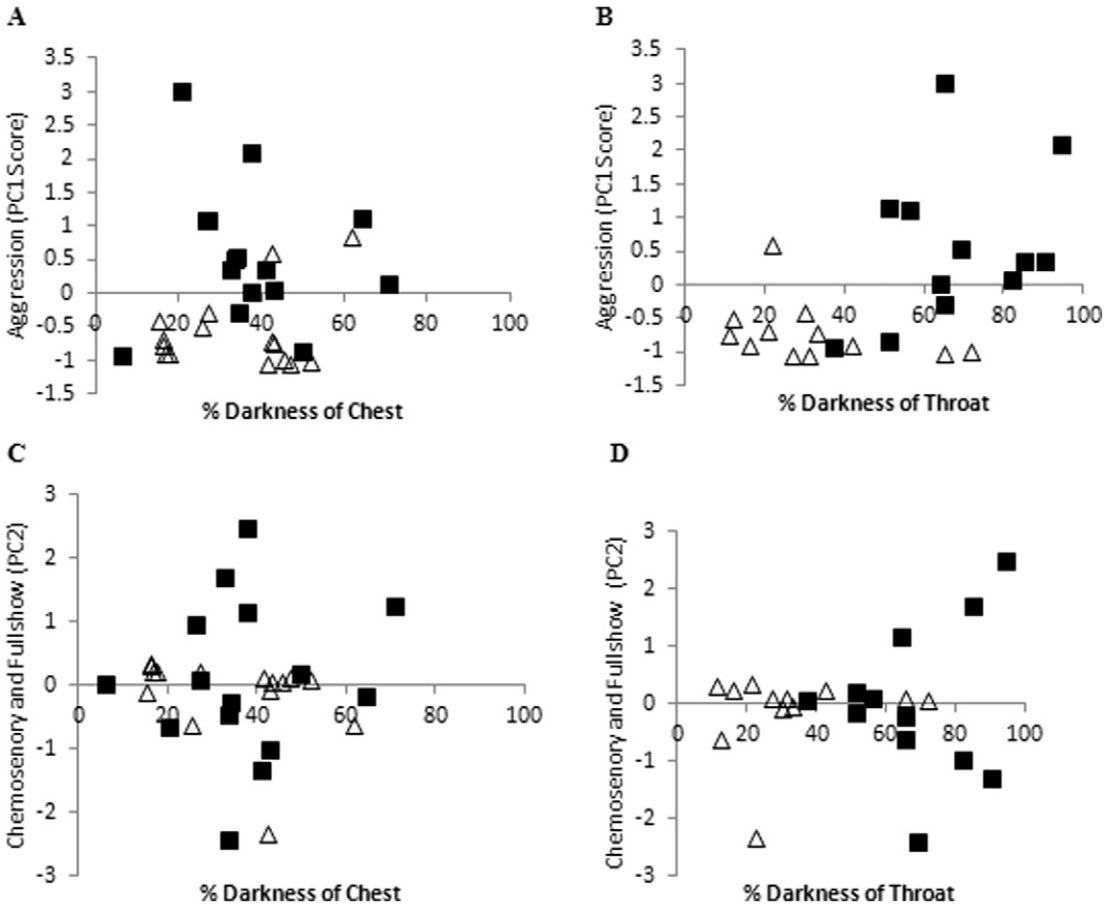

Fig. 4. Relationships in male *Sceloporus occidentalis* in two populations (low-elevation, △; high-elevation, ■) between aggression score (PC1) and A) chest melanin B) throat melanin, and chemosensory-fullshow scores (PC2) and C) chest melanin and D) throat melanin. Individual variation in either of the melanization measures did not significantly contribute to explaining variation in either behavior score.

had significantly lower plasma testosterone following 1-h confinement stress (Fig. 5A).

Examining variables that might explain individual variation in testosterone (and corticosterone, see below), we included chest melanin, throat melanin, population, and stress treatment as factors, with time-to-capture-and-bleed as a covariate. This MANOVA indicated no variable significantly explained variation, though treatment effect was close to significant for testosterone ($F_{1,33} = 3.715$, $P = 0.064$). After removing the nonsignificant time to capture and bleed covariate ($P > 0.517$), the analysis was rerun and only significant variable was the effect of treatment ($F_{1,31} = 2.203$, $P = 0.029$, Fig. 6). While there was a trend for populations to differ ($F_{1,31} = 3.141$, $P = 0.086$), all other terms did not explain variation in testosterone (all $P > 0.4$).

3.4. Corticosterone levels

Populations did not differ in our two measures of adrenal function (baseline and 1 h confinement corticosterone, Fig. 5B). Neither covariate was significant (time to capture and bleed, $F_{1,32} = 0.015$, $P = 0.902$; time of day, $F_{1,32} = 0.034$, $P = 0.854$). These terms were removed from the model and the two-way ANOVA was rerun. Although there was the expected trend for higher mean corticosterone in the 1-h

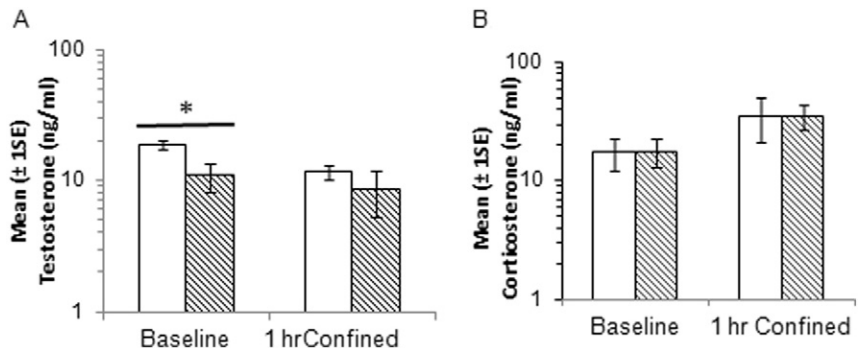

Fig. 5. Mean (±1 SE) plasma steroid hormone levels for male *S. occidentalis* in two populations (low-elevation, □; high-elevation, ■) that were either captured and bled immediately ($N = 10$ each population) or bled after 1 h of confinement ($N = 10$ each population). A. Mean baseline testosterone levels were higher in the low-elevation population (*$P < 0.05$), and there was a significant treatment effect. B. Mean corticosterone levels did not differ between populations, nor was there a significant treatment effect.

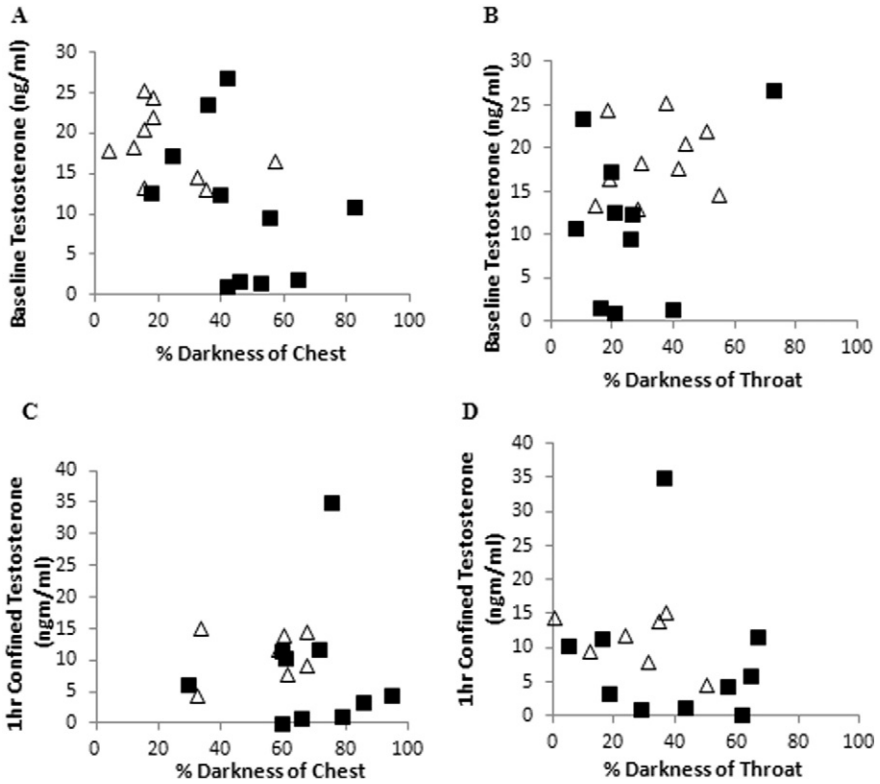

Fig. 6. Melanization and testosterone in male *Sceloporus occidentalis* in two populations (high-elevation, ■; low-elevation, Δ). No significant associations were found for individual variation in baseline testosterone and A) chest melanization or B) throat melanization. Similarly, no significant associations were found for individual variation in 1 h confined testosterone in C) chest melanization or D) throat melanization. The removal of potential testosterone outlier in the 1-h confinement group (C and D) had no effect.

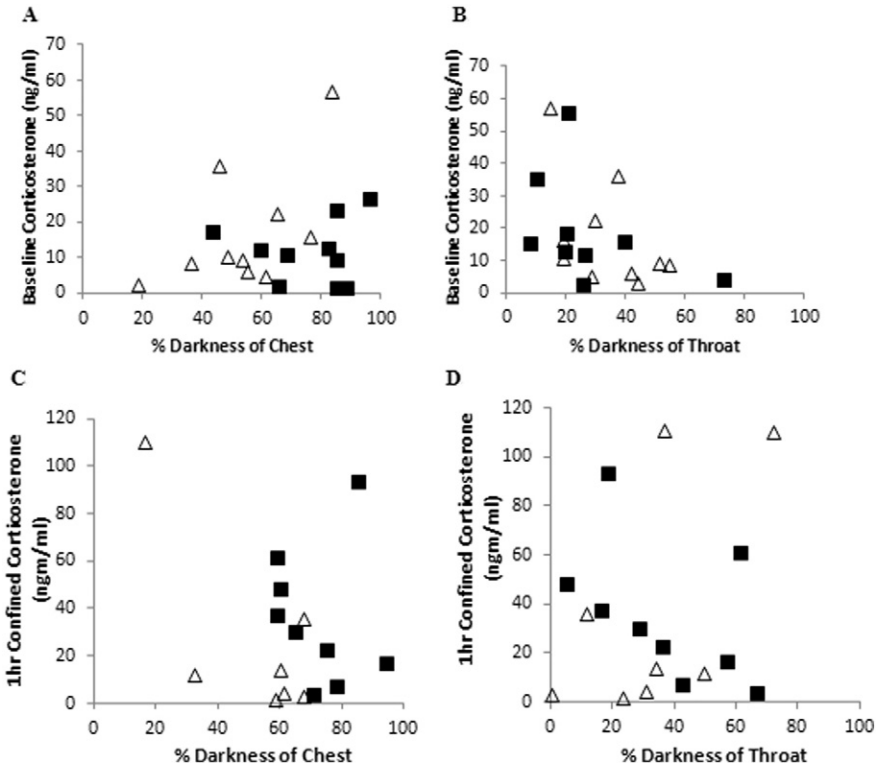

Fig. 7. Melanization and corticosterone in males of two *Sceloporus occidentalis* populations (high-elevation, ■; low-elevation, Δ). No significant associations were found between baseline corticosterone and A) chest melanin or B) throat melanin. Similarly, no significant associations were found between individual variations in 1 h confined corticosterone levels and C) chest melanin or D) throat melanin.

confinement males compared to the immediate bleed males, for each population (Fig. 5B), none of the terms were significant in the ANOVA model (population by stress-handling treatment interaction term, $F_{1,34} = 0.00$, $P = 0.983$; population, $F_{1,34} = 0.01$, $P = 0.971$; stress-handling treatment, $F_{1,34} = 3.83$, $P = 0.059$). Hence, contrary to expectation, hormonal stress-reactivity (which was not detected) of the two populations did not differ.

In the overall MANOVA described above that examined variation in both hormones, no factors significantly explained individual variation in corticosterone (Fig. 7) including chest melanin, throat melanin, population, stress-handling treatment (immediate bleed *versus* 1 h confinement), population × treatment interaction, and the time-to-capture-and-bleed covariate (all P values >0.2). Removing the non-significant time to capture and bleed term and the interaction term did not alter this outcome.

3.5. Mite load

Comparing all males captured, the high-elevation (dark) population had a significantly higher mean mite load than males in the low-elevation (pale) population ($t_{97,\ 0.05} = 8.20$, $P < 0.001$). Variation in mite load was not significantly associated with variation in melanin (chest, $F_{1,36} = 2.203$, $P = 0.146$; throat, $F_{1,36} = 0.725$, $P = 0.866$, Fig. 8), variation in body condition (as measured by residuals from a regression of body mass on to SVL, $F_{1,36} = 0.587$, $P = 0.448$), or population ($F_{1,36} = 0.183$, $P = 0.671$).

Similarly, there were no significant associations between variation in mite load and in baseline steroid hormone concentrations (Fig. 9) after removing the non-significant time-to-capture-and-bleed term from the model (testosterone, $F_{1,33} = 3.847$, $P = 0.058$; corticosterone, $F_{1,33} = 0.724$, $P = 0.401$) or nor did we detect a significant effect of population in this model ($F_{1,33} = 0.023$, $P = 0.880$).

4. Discussion

The image analysis results confirmed that our sample of males from a high-elevation population had more melanized ventral skin (lower brightness scores in chest and abdomen) compared to males from our low-elevation population. Chest and belly melanization were highly correlated in each population. Our measure of the amount of black, which is produced by melanin, hence it is likely that differences we quantified are due to melanin content of the skin [89], including in *Sceloporus* [63,72].

At the population level, mean rates of behaviors recorded in the staged territorial interactions mirrored population differences in mean melanization. Males in the darker population had higher mean rates of behavioral responses to intruders for most behaviors measured, compared to males in the less melanized low-elevation population. We failed, however, to detect significant associations between the degree of melanization and individual variation in aggression or in any variable. This contrasts with other studies examining within-population variation that have found more melanized individuals can be more aggressive (*e.g.*, [8,74]), have fewer parasites [83,84], increased

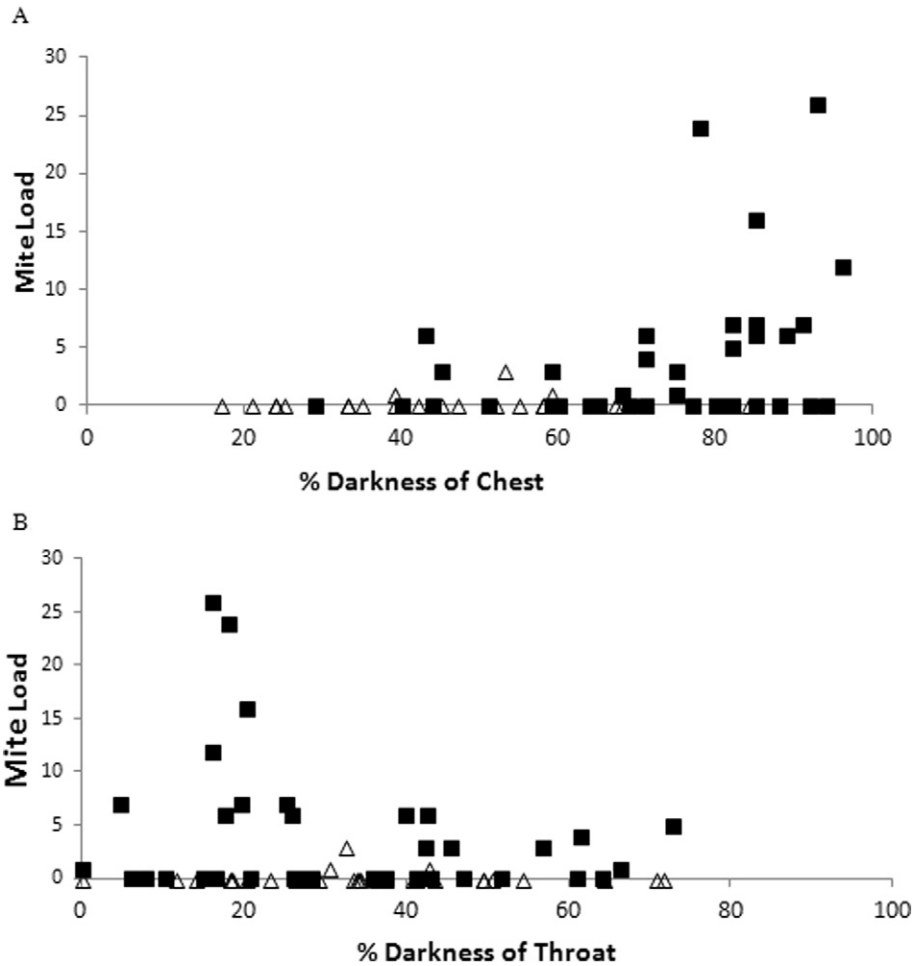

Fig. 8. External mite loads and melanization in males of two *Sceloporus occidentalis* populations (high-elevation, ■; low-elevation, Δ). Although mean mite loads were significantly higher in the high-elevation population (see text), mite loads were not significantly associated with either A) chest melanin (Low-elevation, $N = 44$; high-elevation, $N = 39$) or B) throat melanin (Low-elevation, $N = 39$; high-elevation, $N = 36$).

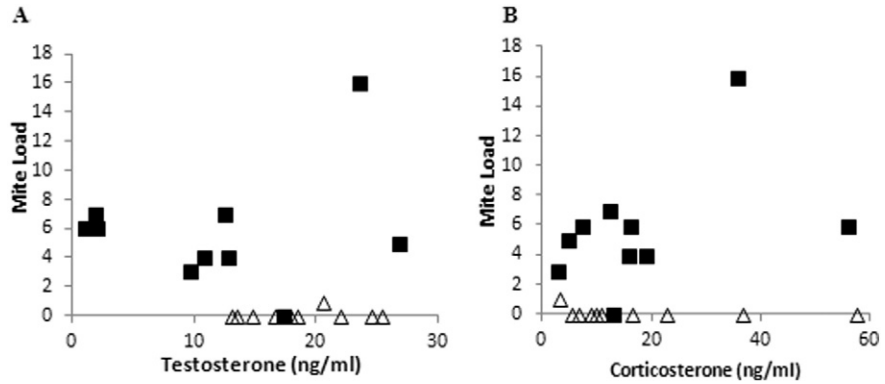

Fig. 9. Relationships between external mite loads and baseline plasma steroid levels in male *Sceloporus occidentalis* in two populations (low-elevation, Δ; high-elevation, ■). Although mean mite loads were significantly higher in the high-elevation population (see text), individual variation in mite load was not significantly associated with variation in A) testosterone or B) corticosterone. The removal of the one potential mite load outlier had no effect.

anti-parasite protection [80], or increased antibody production to an immune challenge [27].

There are several possible interpretations for the finding of no significant associations between individual variation in melanization and aggression, even though populations differed significantly in both of these traits. First, factors contributing to melanization may be unassociated (or become disassociated) from factors that contribute to population-level differences in aggression. Or, as outlined in the introduction, a threshold effect of a hormone (*e.g.*, α-MSH) on aggression may exist and which would differ between populations, and then other factors not associated with this hormone could then generate the observed within-population variation in aggression. Clearly, measures of plasma levels of this melanocortin, and measures of target-tissue sensitivity to this hormone (*e.g.*, receptors) would be a valuable step on delineating the potential roles of this hormone.

We also found significant differences between the two populations in mean baseline plasma testosterone levels, a hormone that often mediates aggressive behavior in adults including lizards. However, the difference was in an unexpected direction: the low-elevation population that was less responsive and less aggressive in the STIs had the higher mean testosterone. We sampled each population at a fairly comparable stage of the breeding season and in *Sceloporus* males maintain elevated testosterone throughout the breeding season (*e.g.*, [60]). A variety of population-specific factors unrelated to melanization could contribute to our unexpected testosterone result, including differences in the intensity of sexual selection, or in population density. For example, baseline rates of patrolling and baseline rates of territorial display behaviors, which are mediated by circulating testosterone [51,99], might be higher in the lower elevation population. Such difference might occur for a number of reasons, such as habitat structure which did appear to be more complex at the lower-elevation site. Furthermore, population differences in aggression could be independent of adult plasma testosterone, if organizational effects of earlier differences in plasma testosterone are strong contributors to adult differences in male behavior (but see [39]).

Adrenal function, as measured by either baseline corticosterone or corticosterone responses to a short-term captivity stressor, did not differ significantly between the two populations. The baseline corticosterone concentrations we measured are similar to those reported for other lizards [10,31,47], although they are higher than values reported for other *Sceloporus* species [35]. Plasma corticosterone concentrations typically increase in *Sceloporus* lizards during handling stress (reviewed in [35]), including after 1-h confinement in several other populations of our study species *S. occidentalis* [21]. We found a trend for higher plasma corticosterone after 1 h of confinement (low elevation, $P = 0.059$; high elevation, $P = 0.11$) and our relatively smaller sample sizes ($N = 9, 10$; *e.g.* Hews & Abell-Baniki had sample sizes of 13–15 lizards per group) suggests we had low statistical power to detect such an effect, especially given the variability inherent in steroid hormone levels.

Lizards in the more melanized population had higher mean mite loads than the lizards from the less-melanized low-elevation population. Study site effects could contribute to differences in mite loads, independent of differences in melanization. In 2013, California was in a multi-year drought and drought effects may be more severe at lower elevations with smaller or negligible snow pack with lower soil moisture reducing mite populations. Mite breeding season also could differ with elevation. Although most studies show that increasing elevation is negatively correlated with parasite numbers [23,34], some studies find positive associations with elevation [64,101]. Finally, males in the more melanized population, which had greater aggressive responses, could also have engaged in more territorial patrolling, resulting in higher ectoparasite loads due to higher encounter rates [44]. Different colored individuals in other species are thought to exploit different habitats and hence be exposed to different parasite environments [26,59].

We found mixed patterns of relationships between melanization, testosterone and parasites. In many but not all species [77], elevated plasma testosterone levels result in increased parasite loads (see also [32,68]), including in some lizards [66], supporting a key assumption of the immunocompetence handicap hypothesis [24]. But immuosuppressive effects of testosterone are not always found (*e.g.*, [22]). In many animals including *Sceloporus* lizards, elevated testosterone, during development [72] or in adults, is associated with increased melanin deposition (*e.g.*, [6,14,93]). Our two lizard populations differing in melanization did not differ predictably in adult testosterone level, aggression or ectoparasite load: the less melanized population had significantly lower aggression but higher baseline plasma testosterone and lower mite loads. Blood and intestinal parasites and other measures of immune function should be assessed in these populations.

Natural selection might favor more melanization at high elevations, perhaps for thermal benefits or for a UV shield. Strong selection could reduce with-population variation in melanization. Our population comparisons of means may be analogous to phenotype manipulation studies, in which the range of natural variation in traits is expanded, facilitating detection of trait associations that cannot easily be detected within the normal range of variation in a population (*e.g.*, [88]). In contrast to population differences in selection, if selection favors suites of adaptive traits that are associated with within-population alternative life-history strategies, then studies of melanistic variation within a population might detect such correlations that we failed to detect (*e.g.*, [82,96]). More studies of species with melanism, comparing both within and across-populations are warranted, as many studies comparing across populations focus primarily on describing genetic divergence, consequences for gene expression and protein function [41]. Finally,

while genetic differences among populations on this elevational gradient in the Sierra Mountains of California have been identified [49], the relative contributions of environment *versus* genes to degree of melanization in these lizards is unknown. Common garden experiments assessing the range of melanization in each population would be informative.

4.1. Summary

If hormonal "pleiotropy" results in strong correlations among traits then we would expect to see a consistent suite of correlated traits. Alternatively, if there are different selection regimes or different factors producing phenotypic plasticity in these two populations, then these hormonally-mediated suites may become "decoupled". Our results, which are limited as they are only from one pair of populations and sometimes hampered with smaller sample sizes, nonetheless suggest that associations among traits that are typically affected by the melanocortin system may not be rigid. We found that in one high-elevation population, males were darker and significantly more aggressive than their less melanized counterparts in one low-elevation population, but that there was no within-population association between melanin and aggression. However, other traits that correlate with melanization in other vertebrates were not consistently associated with melanization, either when comparing the two populations or when analyzing individual variation. Other parasites and other aspects of immune function should be assessed in these populations, as should the possible role of α-MSH in aggression [61]. Studies on more populations of this variable species are warranted.

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