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Sex and species differences in plasma testosterone and in counts of androgen receptor-positive cells in key brain regions of *Sceloporus* lizard species that differ in aggression

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Abstract

We studied neuroendocrine correlates of aggression differences in adults of two *Sceloporus* lizard species. These species differ in the degree of sex difference in aggressive color signals (belly patches) and in aggression: *S. undulatus* (males blue, high aggression; females white, low aggression) and *S. virgatus* (both sexes white, lower aggression). We measured plasma testosterone and counted cells expressing androgen receptor-like immunoreactivity to the affinity-purified polyclonal AR antibody, PG-21, in three brain regions of breeding season adults. Male *S. undulatus* had the highest mean plasma testosterone and differed significantly from conspecific females. In contrast, there was no sex difference in plasma testosterone concentrations in *S. virgatus*. Male *S. undulatus* also had the highest mean number of AR-positive cells in the preoptic area: the sexes differed in *S. undulatus* but not in *S. virgatus*, and females of the two species did not differ. In the ventral medial hypothalamus, *S. undulatus* males had higher mean AR cell counts compared to females, but again there was no sex difference in *S. virgatus*. In the habenula, a control brain region, the sexes did not differ, and although the sex by species interaction significant was not significant, there was a trend ($p = 0.050$) for *S. virgatus* to have higher mean AR cell counts than *S. undulatus*. Thus hypothalamic AR cell counts paralleled sex and species differences in aggression, as did mean plasma testosterone levels in these breeding-season animals.

Keywords

aggression; androgen receptor; brain; lizard; *Sceloporus*; testosterone; sex difference; species differences; immunohistochemistry

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1. Introduction

Species can vary in the degree to which the sexes differ in social behavior, and differences in brain features are implicated in such species differences [e.g., 4, 16, 22, 24, 41, 47]. Differences in androgen receptor (AR) numbers, distribution, function, and regulation may mediate individual or sex differences in aggression in vertebrates [e.g., 20, 48, 58, 74, 80]. Here we document neuroendocrine correlates of both sex and species differences in aggression in two species of *Sceloporus* lizards that differ in aggression and in aggressive color signals. Among *Sceloporus* lizards, males typically are territorial and highly aggressive [9, 11, 68]. Further, color signals can co-vary with aggressive behavior both within and across species [30]: in *S. undulatus* adult males display permanent blue abdominal patches during aggression, but females lack the patches and exhibit little aggression. By contrast, in *S. virgatus* both sexes lack the large blue patches and show comparatively little territorial aggression [30, 31, 68]. While male *S. virgatus* are territorial [1, 60], rates of aggressive behavior and encounter intensity are lower than in male *S. undulatus* [30, 31, 68]. Male territoriality has been characterized more by mate-guarding behavior rather than by defense of an exclusive-use area [1].

We examined two neuroendocrine correlates of differences in aggression in these two species: adult plasma androgen levels and adult numbers of brain cells expressing androgen receptors, as assessed by immunohistochemistry. As in many vertebrates, in lizards seasonal changes in plasma testosterone can correlate with seasonal changes in aggression [38, 45]. If activational effects of testosterone (TESTO) contribute to sex and/or species differences in aggression, differences in plasma TESTO levels should correlate with differences in aggression. Similarly, differences in sensitivity to steroid hormones can correlate with status, sex, or species differences in behavior [6, 7, 19, 33], including in reptiles [23]. Thus, if sensitivity differences contribute to differences in aggression seen among these *Sceloporus* lizards, then differences in density of brain androgen receptors should correlate with differences in aggression. Regions in the hypothalamus are often involved in mediating sex differences in behavior in vertebrates. The preoptic area (POA) is involved in mediating aggression [e.g., 3, 5], and in lizards, as in other vertebrates, there are sexual and seasonal dimorphisms in the POA [13, 49, 72]. Similarly, the ventromedial hypothalamus (VMH) is important in some female-typical behaviors [3, 5, 53, 71], but may also play roles in aggression. For example, the dorsolateral VMH in male tree lizards may integrate afferent and efferent information within an aggression-control circuit [34]. Forebrain distribution of cells immunoreactive for the androgen receptor has been described for *Sceloporus undulatus* [44], but sex and species differences remain unquantified.

2. Material and methods

The original research reported herein was performed under guidelines established by the Indiana State University Institutional Animal Care and Use Committee under protocols issues to D. Hews.

2.1 Plasma Testosterone

To obtain blood samples we captured lizards by noosing [Moore and Lindzey 1992]. We collected *S. undulatus* (n= 14 females, n= 15 males) from Lake Monroe (Monroe Co., Indiana) between May 30 – June 19, 2002, and *S. virgatus* (n= 7 females, n=12 males) from the Middle Fork of Cave Creek (near Portal, AZ) between May 24 and June 4, 2002. For *S. undulatus* this time is slightly past the peak in mating activity (first clutch of eggs) in most years [38] but males were exhibiting territorial aggression at this time and females were still yolking eggs (assessed via palpation and visual inspection). For *S. virgatus*, we collected plasma during the peak of mating activity [1, 54, 60]. We bled lizards from the retro-orbital

sinus with heparinized microcapillary tubes, sampling within a 4.5 hr time window during the daily peak of activity, to reduce possible circadian variation in hormone levels. Samples remained on ice for several hours, were centrifuged and resulting plasma samples were frozen (-20°C and then -60°C) until being assayed.

To measure circulating testosterone (TESTO) concentrations, plasma samples were subjected to phase-partitioning column chromatography for purification and radioimmunoassay [75] with modifications [8]. Briefly, we equilibrated plasma samples (15–20 μL) overnight with 2,400 cpm of tritiated TESTO for calculating recoveries from individual samples. We purified hormone fractions with diethyl ether extraction and column chromatography using microcolumns made with Sigma Celpure P300 filter agent (Sigma 525243); steroids and neutral interfering lipids were eluted with increasing concentrations of ethyl acetate in isooctane. We radioimmunoassayed duplicate sample aliquots, using testosterone antibody (WLI-T3003, RDI-Fitzgerald Industries Int., Concord MA, USA), tritiated steroid (NEN Life Sciences, testosterone NET 553) and Dextran-coated charcoal. Calculations of steroid concentrations were corrected for individual sample recoveries and plasma volumes. Average individual recovery for TESTO was 79%, and RIA accuracy based on six standard columns in the assay was 96.7%. Because untransformed values for plasma TESTO concentrations violated assumptions for parametric analysis, we subjected log-transformed values two- way ANOVA (factors: sex, species). Within-species comparisons were done using two-sample Student t-tests. Alpha was set at 0.05 for all tests, which were two-tailed. Analyses were done using SYSTAT v10 (SPSS, Evanston IL).

2.2 Androgen Receptors

Lizards used for the androgen receptor study were captured during the breeding season, when aggression is high. We collected *S. undulatus* (*N*, females, males = 5, 5) in the glades habitat of the Missouri Ozarks (MO, USA), in early June 2006, and *S. virgatus* (*N*, females, males = 4, 6) along the middle fork of Cave Creek (near Portal, AZ, USA) on May 24, 2006. Prior to sacrifice all lizards were housed with conspecifics, one male with either one or two females, in terraria for four weeks under summer temperature and light conditions (12:12 light:dark with both UV and incandescent bulbs that create a thermal gradient within the terrarium). Lizards were fed vitamin- and calcium-dusted crickets and given water *ad libitum* in addition to misting one wall of each terrarium daily.

To collect brains, animals were each anesthetized with ice and sacrificed via rapid decapitation. The brain was removed with the head held on ice tray and then immersion-fixed with 4% paraformaldehyde for 20min, embedded in O.C.T. (Tissue-Tek) compound and frozen on dry ice. Brains were each cut by cryostat at 14 μm thickness and mounted on coated slides, and slides were kept in -80°C freezer until we performed immunohistochemistry.

We performed immunohistochemistry using PG21, an affinity-purified polyclonal antibody raised against a synthetic peptide representing the first 21 N-terminal amino acids of the rat and human AR [51]. Lizard steroid hormone receptors, including the AR, show high sequence homology to mammalian AR [56, 79]. Brain sections were fixed with 4% paraformaldehyde for 2 min and washed with 0.1M PBS three times and incubated in 5% normal donkey serum for 1 hour at 4°C . Then they were incubated in PG21 rabbit antibody (Affinity BioReagents, CO 1:1000) 48 hours at 4°C . Sections were washed with PBS and incubated in secondly antibody, Cy3 donkey anti rabbit (Jackson Immuno Research 1:200) for 2 hrs at room temperature, and then washed with PBS and coverslipped with Vector Shield DAPI solution (Vector Laboratories). As has been done previously [44] specificity of PG21 binding was confirmed with several controls. Preadsorbing PG21 with the AR21 peptide (aa 1–21 of the AR) used to generate the PG21 antibody completely abolished

staining, whereas preadsorbing with AR462 (aa 462–478 of the AR), an unrelated peptide, did not affect AR-positive staining. This mammalian PG-21 has been used in a variety of non-mammalian vertebrates [e.g., 7, 17, 56] with similar specificity results.

For each hemisphere and each brain area (POA, VMN, and Habenula, Hb, as control region) we took pictures with a Leica DM RXA2 microscope using 40x objective, Spot III camera and software (Molecular Dynamics). We manually counted PG21-positive cells for analysis.

To delineate the three brain regions studied, we used landmarks as described in previous works on *Sceloporus* [44] and other lizards [25, 34, 59]. We subsampled brain sections for counting cells because tissue damage in some brains prevented us counting cells in entire regions. Thus, we counted AR-staining cells in two digital images, each taken from two non-adjacent sections of the anterior-most third of the preoptic area (POA, which starts anteriorly with the third ventricle and ends at the POA transition into the periventricular nucleus). Similarly, we counted AR cells in images of two non-adjacent sections of the anterior-most third of the ventromedial hypothalamus area (VMH, which begins anteriorly as a ventral cluster of cells immediately posterior to the point where optic chiasm disappears, and ends as a cluster of cells disappearing along the edge of the third ventricle). As the brains of these small-bodied lizards are relatively small, two 24µm-thick sections represent approximately 1/5 of the total number of sections for the POA and approximately 1/4 of the VMH. Our control region, the habenula, is a dark-stained (Nissl) region next to the third ventricle at the dorsal end of the diencephalon. We counted cells in two non-adjacent sections in which this nucleus was present, each taken from the anterior-most third of the nucleus.

For each individual, we averaged the value for left and right hemisphere for each brain area. To determine there were body size effects on cell counts, for each species (combining sexes), we first tested whether there was a significant relationship between body mass (a measure of body size) and the cell counts, for each brain region to determine if body size-corrections to cell counts were necessary. In these two *Sceloporus* species adult females are often larger than adult males. We then subjected the count values to two-way ANOVAs (factors: sex, species), and used Holm-Sidak method for protected multiple pair-wise comparisons. Alpha level was $p = 0.05$.

3. Results

3.1 Plasma Hormone Concentrations

The time for us to capture and complete blood sampling (hereafter, handling time) was relatively short for both species (*S. undulatus*, mean = 161 sec, range 106–209 sec; *S. virgatus* mean = 186 sec, range 101–459 sec). Two *S. virgatus*, a male and a female, had handling times of 367 sec (male) and 469 sec (female), with plasma TESTO values of 1.3 and 1.01 ng/ml, respectively. (Excluding these two individual the mean handling time for this species was 159 sec and maximum value was 228 sec.) Importantly, we detected no significant effect of handling time on plasma testosterone concentrations, for either species individually (*S. undulatus*, $n = 29$, adjusted multiple $R^2 = 0.000$, slope = -0.003 , $p = 0.567$; *S. virgatus*, $n = 19$, adjusted multiple $R^2 = 0.087$, slope = -0.002 , $p = 0.118$) or when the species were combined ($n = 48$, adjusted multiple $R^2 = 0.023$, slope = -0.002 , $p = 0.153$). Because of this lack of a handling effect, this term was not used as a covariate in subsequent analyses.

Comparing among sexes and species, there were significant differences in mean plasmatestosterone concentrations (Fig. 1). In the ANOVA of log-transformed TESTO concentrations there was a significant interaction between sex and species ($F_{1, 44} = 4.56$, $p = 0.038$). Hence we compared means within each main effect separately using separate variance t- tests. Male *S. undulatus* had the highest mean plasma TESTO. In this blue-male

species, mean plasma TESTO was significantly (males = 15; females n=14, $t = -4.906$; $p < 0.001$) higher in males than females (Fig. 1). By contrast, in the white-male species (*S. virgatus*) there was not a significant (males = 15; females n=14, $t = -4.906$; $p < 0.001$) difference in mean plasma TESTO (Fig. 1). Thus, the two species differed in whether there was a sex difference in circulating TESTO levels, explaining the significant sex*species interaction term in the ANOVA. Males of the two species differed significantly ($t = 2.461$, $p = 0.022$) in mean plasma TESTO, with the white-male species having the lower mean (Fig. 1). Mean plasma TESTO did not differ significantly ($t = -1.243$, $P = 0.254$) between females of the two species (Fig. 1).

3.2 Counts of Androgen Receptor Immunoreactive Cells

For both species there were no significant effects of body size (as measured by body mass) on AR-positive cell counts. In the blue-male species (*S. undulatus*), the relationship between body mass and individual average (of left and right hemispheres) cell counts for each of the three brain regions were all non-significant combining sexes (POA: $F_{1,8} = 1.826$, $P = 0.212$; VMH: $F_{1,8} = 1.609$, $p = 0.240$; HAB: $F_{1,8} = 0.538$, $p = 0.484$). Similarly, in the white-male species (*S. virgatus*) there were no significant relationships between body mass and individual cell counts (POA: $F_{1,8} = 1.757$, $p = 0.222$; VMH: $F_{1,7} = 0.509$, $p = 0.499$; HAB: $F_{1,8} = 3.609$, $p = 0.094$). Hence, we did not include body size as a covariate in the analyses comparing cell counts.

Many cells in the brains of *Sceloporus* lizards were immunopositive for the AR antibody (Fig. 2) and we found significant differences, for some sex and species comparisons, in the numbers of AR-like-ir cells in the sample of brain sections we assessed for POA and VMH but not for the habenula (Fig. 3).

In the POA (Fig. 3), the effect of sex on AR cell counts was significant in the ANOVA ($F_{1,16} = 7.508$, $p = 0.015$), but neither the effect of species ($F_{1,16} = 2.261$, $p = 0.152$) nor the interaction between sex and species ($F_{1,16} = 2.85$, $p = 0.111$) were significant. Males of the blue-male species had the highest mean number of AR-ir cells for all of four groups, and pair-wise protected comparisons revealed that the mean number of AR-ir cells was higher in males than in females for this blue-male species ($p = 0.006$). By contrast, there was no sex difference for the white-male species ($p = 0.472$). Finally, the males differed significantly between species ($p = 0.03$) but not the females ($p = 0.903$).

In the VMH (Fig. 3), mean AR cell counts again differed significantly for some comparisons. In the ANOVA, the interaction between sex and species was significant ($F_{1,15} = 6.383$, $P = 0.023$). Thus, we did pair-wise multiple comparisons with an overall significance level of 0.05 and found that there was only one significant difference in mean AR-positive cell counts: in the blue-male species, males had a higher mean than females ($p = 0.011$). By contrast, in the white-male species there was no significant sex difference ($p = 0.436$). Comparing between the species revealed no significant difference for females ($p = 0.136$), and a trend for males to differ ($p = 0.058$), with *S. undulatus* males again having higher counts in this brain region.

In the third area, the Hb (Fig. 3), there were potentially some differences in numbers of AR positive cells. The effect of species was significant ($F_{1,16} = 4.507$, $p = 0.0497$), with the white-male species having the highest mean counts. There was no significant effect of sex ($F_{1,16} = 0.145$, $p = 0.709$), and the interaction between sex and species was not significant ($F_{1,16} = 1.47$, $p = 0.242$). Of all four groups, the trend was for females of the white-male species (*S. virgatus*) to have the highest mean AR cell counts.

4. Discussion

4.1 Plasma testosterone concentrations

Testosterone is the major androgen in male lizards [45], and a role for testosterone in male aggression is supported in some species by correlations between seasonal changes in plasma androgens and territorial aggression [38, 43], and by manipulative studies [46, 66, 73]. Class, sex and species differences in aggression in vertebrates can correlate with differences in plasma androgens [21, 33, 63, 74]. Acoustic signaling, combat behavior and morphologies used in fighting in a clade within the frog genus *Rana* vary, and evolutionary loss or reduction of these male traits, which are seasonally activated in these species, correlates with reduced plasma androgen level [18], as do evolutionary reductions in aggression and jaw musculature in plethodontid salamander species [15]. In ornate tree lizards, males with higher frequency and intensity of aggression had elevated levels of circulating testosterone [35]. In these tree lizards, testosterone appears necessary for expression of aggressive behavior but variation in circulating testosterone in adult males does not, by itself, explain individual variation in frequency or intensity of aggression [34].

Here we report differences in plasma TESTO that map on to previously-described differences in aggression. Males in the blue-male species with high male-aggression (*S. undulatus*) had significantly higher plasma TESTO than conspecific females. By contrast, males and females in the white-male species (*S. virgatus*) did not differ in circulating TESTO concentrations in blood samples taken during the breeding season. Androgen can play many roles in female vertebrates [61], and TESTO can be relatively high at certain stages of the ovulatory cycle in lizards [e.g., 69, 76]. Thus these intermediate levels of TESTO in female *S. virgatus* could play several roles. Finally, as predicted by species differences in male-male aggression, we found that males of the two species also differed in average plasma TESTO, with males of the white-male species (*S. virgatus*) having a lower mean level. Integrating centers may be affected by androgens, and steroid hormones also may affect perception of [37] and response to stimuli [2, 29, 39, 64] that elicit aggression. Hence, lower circulating levels could contribute to the observed differences in male-male aggression.

4.2 Brain androgen receptors

For both the POA and the VMH regions, males had higher ARlike-ir cell counts than females in the blue-male species (high male aggression), but there was no significant sex difference in the white-male species, with low male aggression and small sex difference in aggression. Thus species differences in the degree of sex differences in aggression correlate with differences in ARlike-ir cell counts in the subsamples we assessed for both these regions that are important in social behavior, but not in the habenula, a putative control region. Our work is limited because we did not assess cell counts throughout the entire POA or the entire AH. However, because we consistently counted cells in sections taken from the anterior third of each brain region assessed, these data suggest that there may be differences with the more anterior portions of these two important brain regions.

Our work is consistent with other studies of reptiles finding androgen receptor labeling or AR mRNA expression in the POA and the VMH [12, 23, 44, 52, 55, 64]. In vertebrates the VMH is often implicated in mediating female-typical behaviors. However, in some species, males have greater hypothalamic AR mRNA labeling in some subregions of the ventromedial nucleus [e.g., 33, 55, 33]. In some lizards, the dorsolateral subdivision of the VMH may be involved in integrating afferent and efferent information within the neural aggression-control circuit [34]. Kabelik and colleagues [34] suggest this because, in response to both exposure to or performance of aggression, male ornate tree lizards

(*Urosaurus ornatus*) exhibited increased estimates of neural activity in this part of the VMH, as assessed by immunopositive cell counts for phosphorylated cyclic AMP response element binding protein (pCREB).

We detected a modest difference in ARlike immunopositive cells counts in the habenulae, with the white species (*S. virgatus*) having significantly (species effect: $p = 0.0497$) higher counts than the blue-male species. The habenulae receive inputs from, among other areas, the pineal region [27] and the bed nucleus of the stria terminalis [42]. Cells positive for steroid hormone receptors occur in the habenulae of other vertebrates [14, 42, 77]. In our study, there *S. virgatus* differs from many other oviparous *Sceloporus* in the United States in only having one clutch of eggs per year. They have a very predictable and precisely-timed period of breeding activity, that occurs the last 2–3 weeks of May [60, personal observation]. This suggests that the timing of reproduction could be regulating photoperiodically, and habenular size may reflect the importance of photoperiodic cues in the precise timing of reproduction. A role for androgens in the circadian circuit had been found, and AR have been documented in the suprachiasmatic nucleus of rodents, for example [36] and in the habenulae of seasonally-breeding newts [14].

Other brain areas that can mediate aggression should be examined in *Sceloporus*. For example, other limbic brain nuclei have been implicated in the control of aggression in reptiles [26,48,62,65] and other brain areas potentially involved in aggression in vertebrates differ in the presence of steroid hormones or their receptors [e.g., 56, 78]. Two such regions are the nucleus sphericus, and portions of the adjacent amygdala which relays vomeronasal information [e.g., 40]. In prairie voles, the lateral habenula also receives arginine-vasopressin (AVP) projections from the bed nucleus of the stria terminalis, and these projection are sexually-dimorphic [42]. The vasotocinergic system plays diverse roles in vertebrate social behavior [50], including reptiles [e.g., 32] and should also be examined in *Sceloporus*. Differences in brain estrogen receptors may also contribute to species differences in aggression [67, 70], and should be explored in this system. Both AR and ER mRNA-containing cells can occur in olfactory regions of the cortex and in the main and accessory olfactory bulbs [e.g., 14, 57], suggesting steroid hormonal modulation of olfactory and vomeronasal sensory information. Chemical cues and the vomeronasal organ are important in reptilian social behavior [28]. Steroid hormone receptors in the VNO pathways also should be explored in these lizards, as we have shown species differences in visual display behavior of males in response to conspecific chemical signals [31].

4.3 Conclusions

Here we report differences in plasma TESTO that map on to previously-described sex and species differences in aggression. Similarly, assessing portions of two key brain regions, the POA and the VMH, revealed differences in brain sensitivity to androgens, as measured by counts of cells immunopositive for an AR antibody. These differences also paralleled these sex and species differences in aggression.

HIGHLIGHTS

- Two lizard species previously shown to differ in color and visual signaling related to breeding season aggression were studied.
- High male aggression species had high mean plasma testosterone compared to males and females in the low aggression species.
- Androgen receptor cell counts in POA and VMH hypothalamic regions paralleled sex and species differences in aggression.

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Figure 1.

Mean ($\pm$ 1 SE) plasma androgen concentrations in *Sceloporus undulatus* (males with high aggression) and *S. virgatus* (males with low aggression) sampled during their respective breeding seasons when aggression is maximal.

Figure 2.

Androgen receptors as assessed using fluorescent immunohistochemistry in (left panel) the preoptic area (POA), (middle panel) the ventral medial hypothalamus (VMH), and (right panel) a control region, the habenula (Hb), in males and female of two *Sceloporus* lizard species. First row, male *S. undulatus*; second row, female *S. undulatus*; third row, male *S. virgatus*; fourth row, female *S. virgatus*. Scale bar the same for all images.

Figure 3.

Mean (+ 1SE) counts of cells immunopositive for androgen receptors in the preoptic area (POA), ventral medial hypothalamus (VMH), and habenula (Hb) in the brains of male and female *Sceloporus undulatus* (males blue, high aggression; females white, low aggression) and *S. virgatus* (males and females both white, low aggression).