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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: *Sceloporus undulatus* (males blue, high aggression; females white, low aggression) and *Sceloporus 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.

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 [9,10,11,68]. Further, color signals can co-vary with aggressive behavior both within and across species [30]: in *Sceloporus undulatus* adult males display permanent blue abdominal patches during aggression, but females lack the patches and exhibit little aggression. By contrast, in *Sceloporus 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

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lizards, as in other vertebrates, there are sexual and seasonal dimorphisms in the POA [12,49,72]. 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 [35]. Forebrain distribution of cells immunoreactive for the androgen receptor has been described for *S. undulatus* [44], but sex and species differences remain unquantified.

2. Materials 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 [45]. 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 h 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 2400 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 and 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. Analyzes 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 4 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 20 min, 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 [56,78]. Brain sections were fixed with 4% paraformaldehyde for 2 min and washed with 0.1 M PBS three times and incubated in 5% normal donkey serum for 1 h at 4 °C. Then they were incubated in PG21 rabbit antibody (Affinity BioReagents, CO 1:1000) 48 h at 4 °C. Sections were washed with PBS and incubated in secondly antibody, Cy3 donkey anti rabbit (Jackson Immuno Research 1:200) for 2 h 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., [7,18,56] 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 40× 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 [25,35,59]. 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 24 μm-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