Social boldness in male green anole lizards correlates with baseline vasopressin activity

David Kabelik*[1], Allison R. Julien[1], Brandon R. Waddell[1]; Mitchell A. Batschelett[1]; Lauren A. O'Connell[2]

[1]Department of Biology & Program in Neuroscience, Rhodes College, Memphis TN 38112 USA

[2]Department of Biology, Stanford University, Stanford, CA, 94305, USA

Key words: boldness, vasopressin, vasotocin, reptile, lizard, steroid hormones

* Please send correspondence to:

David Kabelik
Department of Biology
Rhodes College
2000 N Parkway
Memphis, TN 38112
USA
kabelikd@rhodes.edu
901-843-3699 (phone)
901-843-3565 (fax)

Abstract

Across species, individuals within a population differ in their level of boldness in social encounters with conspecifics. This boldness phenotype is often stable across both time and social context (e.g., reproductive versus agonistic encounters). Various neural and hormonal mechanisms have been suggested as underlying these stable phenotypic differences, which are often also described as syndromes, personalities, and coping styles. Most studies examining the neuroendocrine mechanisms associated with behavioral boldness examine subjects after they have engaged in a social interaction, whereas baseline neural activity that may predispose behavioral variation is understudied. The present study tests the hypotheses that physical characteristics, steroid hormone levels, and baseline variation in Ile^3-vasopressin (VP, a.k.a., Arg^8-vasotocin) signaling predispose social boldness. Behavioral boldness in agonistic and reproductive contexts was extensively quantified in male green anole lizards (*Anolis carolinensis*), an established research organism for social behavior research that provides a crucial comparison group to investigations of birds and mammals. We found high stability of boldness across time, and between agonistic and reproductive contexts. Next, immunofluorescence was used to colocalize VP neurons with phosphorylated ribosomal protein S6 (pS6), a proxy marker of neural activity. VP-pS6 colocalization within the paraventricular and supraoptic nuclei of the hypothalamus was inversely correlated with aggression boldness, but not reproductive behavior boldness. Our findings suggest that baseline vasopressin release, rather than solely context-dependent release, plays a role in predisposing individuals toward stable levels of aggressive boldness toward conspecifics by inhibiting behavioral output in these contexts.

Introduction

Humans are often described as being introverted, extraverted, or somewhere in between. Such variation in stable social boldness similarly exists within numerous animal species, and has also been described as a behavioral syndrome, personality, or coping style (Koolhaas et al., 2010; Réale et al., 2010; Sih et al., 2004). These terms encapsulate the notion of linked social traits that are expressed across various environments, including both social and nonsocial contexts. Indeed, evidence in some species demonstrates heritability of behavioral boldness (Ballew et al., 2017; Mont et al., 2018; Scherer et al., 2017), suggesting a genetic basis for the stability of individual differences across time. Likewise, the level of an individual's boldness is often consistent across social contexts (Colléter and Brown, 2011; Koolhaas et al., 2010; Qu et al., 2018; Reaney and Backwell, 2007), where individuals that may be shy within a reproductive social encounter may also be shy within an agonistic encounter (Kabelik et al., 2021). Such consistency of behavioral phenotype hints at common neural mechanisms regulating behavioral boldness across a variety of contexts.

The mechanisms that underlie behavioral boldness likely involve the social decision-making network (Newman, 1999; O'Connell and Hofmann, 2012, 2011) and its various neuroendocrine mediators (Baugh et al., 2012; Félix et al., 2020; Ketterson and Nolan Val, 1999) that are conserved across vertebrates. The mediators linked to individual variability in behavioral phenotype include steroid hormones (Koolhaas et al., 2010; Sluyter et al., 1996; Tudorache et al., 2018; Veenema et al., 2004, 2003) and various neuropeptide and neurotransmitter systems (Thörnqvist et al., 2019; Veenema et al., 2004), as well as associated receptors (Alfonso et al., 2019; Kabelik et al., 2021; Kanitz et al., 2019). Although much of the research on the neuroendocrine basis of behavioral boldness has been conducted in mammals and fish, here we examine these traits in a reptilian model system, the green anole lizard (*Anolis carolinensis*). The study of behavioral neuroscience in reptiles lags behind that of other

vertebrate groups and invertebrates (Kabelik and Hofmann, 2018; Taborsky et al., 2015), despite lizards being important for evolutionary comparisons among animal taxa, especially amniotic vertebrates (mammals, birds, and reptiles). Green anoles are a longstanding model for behavioral neuroendocrinology research (Lovern et al., 2004), due to their readily quantifiable social behavioral displays, natural seasonal variability in hormone levels, and natural stress responsivity. A social decision-making network has been described in reptiles (Kabelik et al., 2018), and social boldness has been shown to be stable across contexts and over time in anole lizard studies (Kabelik et al., 2021; Putman et al., 2019).

Various neuroendocrine variables have been related to the expression of social behaviors in lizards, including neuropeptides and sex steroid hormones (Dunham and Wilczynski, 2014; Hartline et al., 2017; Kabelik et al., 2013, 2008b; Kabelik and Crews, 2017; Kabelik and Magruder, 2014; Korzan et al., 2001; Korzan and Summers, 2004; Larson and Summers, 2001; Smith and Kabelik, 2017; Watt et al., 2007; Woolley et al., 2004a, 2004b, 2001). Here, we focus on the potential regulation of behavioral boldness by the vasopressin (VP; Ile^{3}-vasopressin, a.k.a. Arg^{8}-vasotocin) system, as well as circulating steroid hormones. VP has been shown to have various effects on social behavioral expression, and stress reactivity, across species (Albers, 2015; Carter, 2017; Goodson and Kabelik, 2009; Kelly et al., 2011; Walton et al., 2010; Wilczynski et al., 2017). Depending on the involved brain regions of VP release, the effects on social behavior may be completely opposed to each other, such as in the case of displayed aggression in rats (Veenema et al., 2010). Research in anole lizards suggests that VP has an inhibitory effect on components of the social decision-making network (Kabelik et al., 2018). Although many studies focus on activity of VP neurons and receptors during a social encounter (e.g., Kabelik et al., 2013), information about baseline activity of VP neurons in individuals with variable boldness is unknown.

Here, we test the hypotheses that physical traits, circulating steroid hormones, and baseline VP neuron activity are associated with individual variation in behavioral boldness in the male green anole. We first examined the stability of behavioral boldness in both reproductive and agonistic encounters. We then waited for one day before collecting blood samples and brains to eliminate neuroendocrine changes correlated with recent participation in a social behavior interaction. We quantified hormone levels via enzyme-linked immunosorbent assay and used immunofluorescence to label VP neurons and their colocalization with phosphorylated ribosomal protein S6 (pS6), a proxy marker of neural activation (Knight et al., 2012). We predicted that circulating glucocorticoid levels and the baseline activity of VP neurons (%VP-pS6 colocalization) would reflect the neuroendocrine state that predisposes that individual toward boldness or shyness.

Materials and Methods

Subjects

Twenty-two male green anoles (*Anolis carolinensis*) were obtained from a commercial supplier and served as our focal subjects. They were singly housed within terraria (30.5 cm H x 26 cm W x 51 cm L) and kept in breeding season conditions: long-day (14 light:10 dark) full-spectrum lighting, 12 hours of supplemental heat provided 5 cm above one end of a wire-mesh terrarium lid by means of a 60-W incandescent light bulb, and thrice-weekly feeding with crickets. Additional males and females from our housing colony were used as stimulus animals in social interactions. All procedures involving live animals were conducted according to federal regulations and approved by the Institutional Animal Care and Use Committee at Rhodes College.

Social behavior boldness assessment

Behavioral trials were carried out in May and June of 2013. The focal males were assessed for boldness within two social encounter scenarios – a reproductive encounter with two adult conspecific females and an agonistic encounter with a size-matched (within 3 mm snout-vent length) conspecific stimulus male. In each case, the stimulus animals were placed into the focal male's terrarium and behaviors were scored for 10 minutes. Two females were used in the reproductive encounter to maximize the probability of eliciting reproductive behaviors from the focal male. We recorded the frequency (sum of behaviors per 10-min session) and latency to first performance (minute of first occurrence of any listed behavior) of the following behaviors: head bob bout, push-up bout, dewlap extension bout, dewlap extension bout with push up, chase, and copulate. Focal males that failed to display any behaviors were assigned the maximum latency score of 10 min. The maximum intensity of behavioral display was also scored from 0-3 based on the highest achieved category: no display, display only, chase, and copulate. Behaviors in the agonistic encounter were scored as in the reproductive encounter, except that biting of the stimulus male replaced copulation as the highest intensity behavior.

The reproductive encounter was conducted with three separate pairs of females and the agonistic encounter with three separate stimulus males, over the course of a week. Only one trial was conducted per day. Stimulus animals were only used once per day. The entire procedure was then repeated during the subsequent week, toward the same three pairs of females and the same three stimulus males. Conducting the behavioral observations a second time allowed us to determine the stability of each boldness measure across time.

Bold-shy categorization

To generate each boldness score, we conducted principal components analyses (PCA) to reduce the average behavioral latency, frequency, and intensity scores from the given encounter scenarios (reproductive or agonistic), separately for week 1 and for week 2, each into a single value. For example, in male-female trials from week 1, the behavioral latency, frequency, and intensity scores for each focal male were averaged across the three trials in which he took part, to generate an “average reproductive latency”, “average reproductive frequency”, and “average reproductive intensity” score. These average scores were then included in the PCA and generated a single PCA component that we here call BoldnessToFemalesWeek1. In each scenario, the resulting analysis generated a single PCA component with an eigenvalue > 1, and in each case, this component was highly positively correlated with average frequency and intensity scores, and negatively with average latency scores ($r > \pm 0.80$, $p < 0.001$ for each). BoldnessToFemalesWeek1 explained 81% of the behavioral variation in those trials, BoldnessToFemalesWeek2 (from week 2 data) explained 75% of the variation from those data, BoldnessToMalesWeek1 (from week 1 agonistic encounters) explained 83% of the variation in those data, and BoldnessToMalesWeek2 explained 85% of the variation in behavioral frequency, latency, and intensity values from the week 2 agonistic encounter data. As week 1 and week 2 data were highly correlated, we also created a variable that averaged reproductive boldness from weeks 1 and 2 (AverageBoldnessToFemales), as well as agonistic boldness from weeks 1 and 2 (AverageBoldnessToMales).

Tissue harvesting

Prior to handling for blood and brain harvesting, focal subjects were left undisturbed in their home terraria for 1 day following their last behavioral trial. We euthanized focal males by cutting through the spinal column and immediately collected trunk blood for hormone analyses

(average collection time from first handling was 162 ± 3.2 s). The blood was kept at 4°C until centrifugation. The brain was then rapidly dissected and fixed by overnight submersion in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C, followed by cryoprotection with 30% sucrose in 0.1 M phosphate-buffered saline (PBS). The body (minus the head) was then weighed, after which the testes were dissected from the body and also weighed. Brains were sectioned into two series, at a section thickness of 50 µm, on a Microm HM 520 cryostat (Thermo Scientific).

Hormone analyses

Blood samples were centrifuged and plasma (averaging 62 ± 2.7 µl) was frozen at -80°C until hormone analysis. We quantified testosterone (ADI-900-065; sensitivity 5.67 pg/mL), estradiol (ADI-900-008; sensitivity 28.5 pg/mL), progesterone (ADI-900-011; sensitivity 8.57 pg/mL), and cortisol (ADI-900-071; sensitivity 56.72 pg/mL) using enzyme-linked immunosorbent assay (ELISA) kits (Enzo Life Sciences, Farmingdale, NY). The cortisol kit cross-reacts with corticosterone at 28%, representing a general glucocorticoid assay, albeit with lower-than-typical sensitivity. We re-suspended 7 µl of plasma in 203 µL of the appropriate assay buffer and ran each sample in duplicate as per manufacturer's instructions. Samples were run across two plates for each hormone and the inter-assay variation across plates and the intra-assay variance for each plate is as follows: testosterone (inter: 5.6%; intra: 5.6% and 6.1%), estradiol (inter: 4.1%; intra: 3.7% and 8.5%), progesterone (inter: 4.7%; intra: 2.3% and 4.7%), and cortisol (inter: 6.4%; intra: 3.8% for both plates). Five samples were inadvertently excluded from the analysis. Hormone results were generally consistent with previously reported levels in this species (Greenberg and Crews, 1990).

Immunohistochemistry

Immunohistochemical processing was conducted as in previous studies (Hartline et al., 2017; Kabelik et al., 2018, 2014, 2013; Kabelik and Magruder, 2014). Briefly, we processed one series of brain sections with 1:250 dilution of rabbit anti-pS6 antibody (#2211, Cell Signaling Technology), 1:5000 dilution of guinea pig anti-vasopressin antibody (T-5048, Peninsula Laboratories), and 1:2000 dilution of sheep anti-tyrosine hydroxylase antibody (NB300-110, Novus Biologicals; part of a separate study). The sections were subsequently processed with donkey anti-sheep secondary antibody conjugated to Alexa Fluor 488 at 3 µl/ml and (Life Technologies), donkey-anti rabbit secondary antibody conjugated to Alexa Fluor 555 at 5 µl/ml (Life Technologies), and donkey-anti guinea pig DyLight 647 at 16 µl/ml (Jackson ImmunoResearch). Preadsorption with excess VP (4100576, Bachem) and pS6 blocking peptide (1220S, Cell Signaling Technology) antibody eliminated signal (**Supplementary Fig. 1**). We targeted pS6 rather than Fos because preliminary research demonstrated extremely low levels of Fos expression within VP neurons under baseline conditions.

Microscopy and Image Analyses

An LSM 700 Confocal microscope and Zen 2010 software (Carl Zeiss), using a 20X objective, were used to capture z-stacks of photomicrographs at 5 µm intervals, in a grid that was later stitched together. A maximum intensity projection created a two-dimensional image. Individual colors were exported as separate layers using AxioVision 4.8 (Carl Zeiss), and these were stacked as overlaid monochromatic layers in Photoshop (Adobe Systems). Layers in the stack could thus be toggled on and off to determine signal colocalization. Analyses were conducted by individuals blind to treatment groups.

We examined VP cells within the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus (**Fig. 1**). Only cells that could be clearly visualized with VP-signal in the cytoplasm and a darker nucleus were examined. Total VP cell counts could not be accurately obtained due to damage to some tissue sections, and many overlapping cells, especially within the SON. An average of 39.32 ± 4.93 (mean ± S.E.) cells in the PVN and 28 ± 5.85 cells in the SON were analyzed per subject.

Statistical analyses

PCA and Pearson correlation analyses were run using IBM SPSS (version 22). Corrections for multiple comparisons were made using Benjamini-Hochberg calculations (Benjamini and Hochberg, 1995). Scatterplots were made with ggplot2 (version 3.3.3) in RStudio (version 1.4.1106) running R (version 4.1.0). Hormone levels were ln-transformed to meet assumptions of parametric analyses.

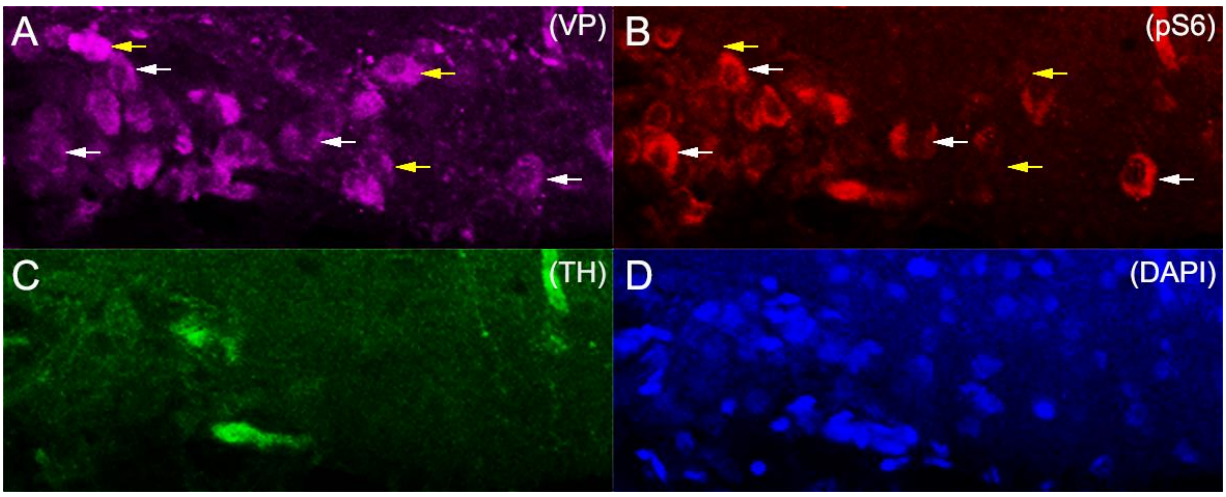


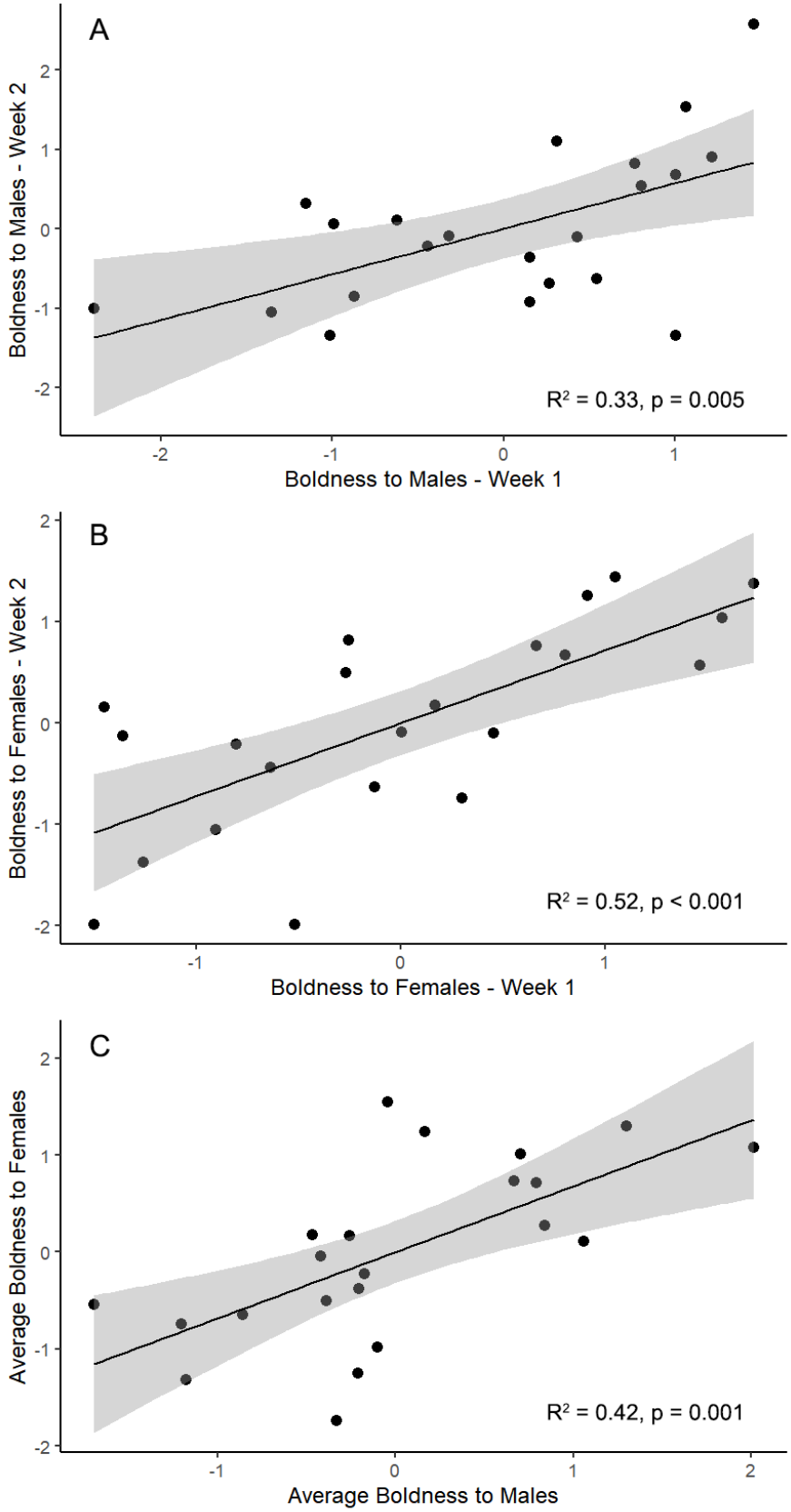
Figure 1. Immunofluorescence within the SON. VP-immunoreactive cells (A) were scored as colocalized (white arrows) or not colocalized (yellow arrows) with pS6-immunoreactive signal

(B). Nonspecific signal due to the presence of blood cells was disregarded because those cells would also cause autofluorescence within the tyrosine hydroxylase (TH) layer (C; captured for a separate study). A DAPI-stained layer was also captured (D).

Results

Behavioral boldness is stable over time and correlated across contexts

Average behavioral boldness was found to be stable across weeks (**Fig. 2**). BoldnessToMalesWeek1 was highly correlated with BoldnessToMalesWeek2 ($r=0.57$, $N=22$, $p=0.005$). BoldnessToFemalesWeek1 was even more highly correlated with BoldnessToFemalesWeek2 ($r=0.72$, $N=22$, $p<0.001$). The averaged boldness data across weeks were then used to compare boldness across social contexts, where AverageBoldnessToFemales correlated strongly with AverageBoldnessToMales ($r=0.65$, $N=22$, $p=0.001$).


A
Boldness to Males - Week 2
Boldness to Males - Week 1
R² = 0.33, p = 0.005
B
Boldness to Females - Week 2
Boldness to Females - Week 1
R² = 0.52, p < 0.001
C
Average Boldness to Females
Average Boldness to Males
R² = 0.42, p = 0.001

244

Figure 2. Stability of behavioral boldness. Boldness scores were correlated across weeks within agonistic contexts involving encounters with three separate conspecific intruder males across two separate weeks (A), as well as within reproductive contexts involving encounters with three separate pairs of conspecific females across separate weeks (B). Average boldness scores across the two weeks in the agonistic context were highly correlated with average boldness score within the reproductive context (C).

Behavioral boldness is generally not correlated with physical traits or steroid hormone levels

Behavioral boldness to males and females was unrelated to physical or hormonal measures except for a positive correlation between testes mass and AverageBoldnessToFemales (**Table 1**). Similarly, neither VP-pS6 colocalization within the PVN, nor in the SON, correlated with any physical characteristics or hormone levels (p>0.05 for all).

Table 1. Results of correlations between measures of boldness and physical and hormonal measures. Displayed are the Pearson correlation coefficient (r), the sample size (N), and the probability of significance (P). No correlations were significant following correction for multiple comparisons.

	AverageBoldnessToMales			**AverageBoldnessToFemales**		
	r	N	P	r	N	P
Physical Variables:						
snout-vent length (mm)	-0.02	22	0.94	-0.13	22	0.55
body-minus-head mass (g)	-0.10	22	0.67	-0.10	22	0.67
testes mass (g)	0.31	22	0.16	0.47	22	0.026
testosterone (ng/ml)	0.21	17	0.42	0.29	17	0.25
estradiol (ng/ml)	0.12	17	0.65	0.31	17	0.22
progesterone (ng/ml)	0.12	17	0.65	0.28	17	0.28
glucocorticoids (ng/ml)	-0.20	17	0.45	0.17	17	0.51

Behavioral boldness to males was associated with vasopressin activity in the PVN

AverageBoldnessToMales was negatively correlated with VP-pS6 colocalization (**Fig. 3**) within the PVN (r=-0.51, N=22, p=0.014) and the SON (r=-0.52, N=22, p=0.014).

AverageBoldnessToFemales was not correlated with VP-pS6 colocalization in either the PVN (r=-0.24, N=22, p=0.29) or the SON (r=-0.09, N=22, p=0.69).

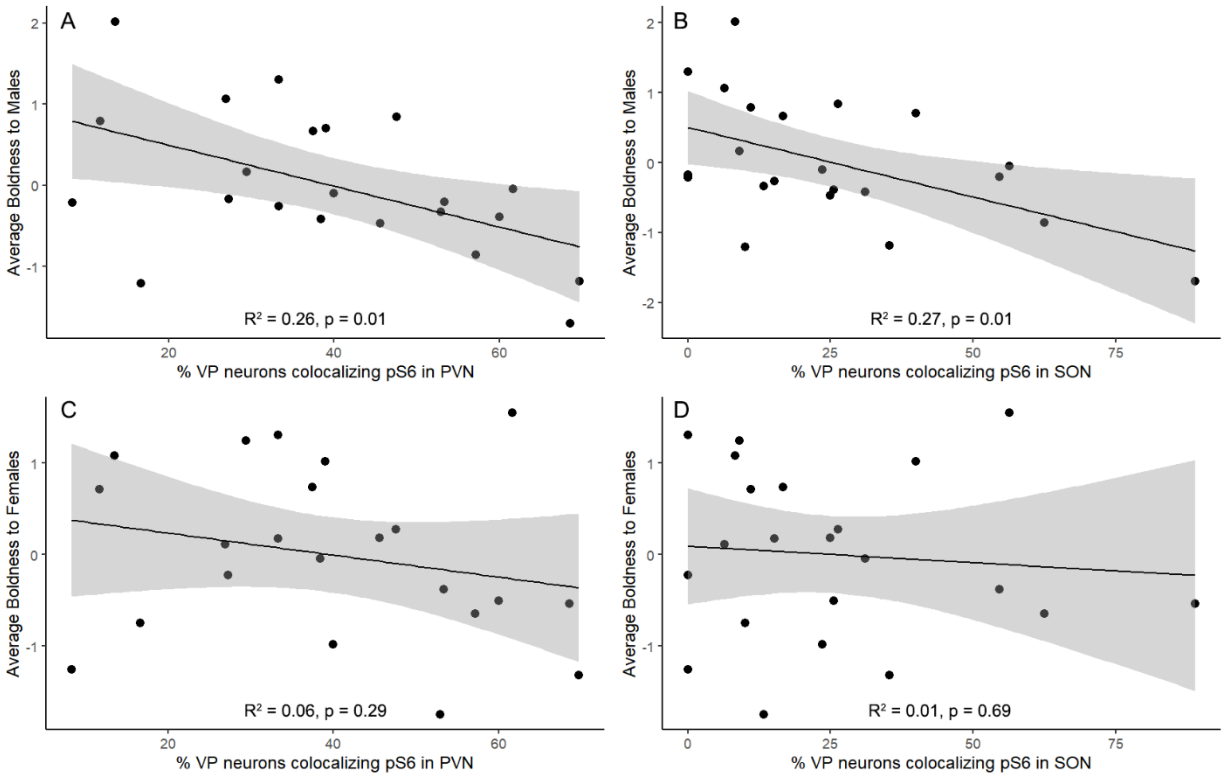


Figure 3. Behavioral boldness relative to VP-pS6 colocalization. Average boldness scores toward males within an agonistic context (A, B) but not females within a reproductive context (C, D) correlated negatively with the percentage of VP neurons in the PVN and SON that were pS6 positive.

Discussion

Our results demonstrate that boldness in male green anoles is stable across social contexts and time and that baseline levels of VP neural activity are associated with behavioral

boldness of male green anoles within an agonistic context, presumably due to differential levels of VP release. VP is a neuromodulator that has previously been shown to have causal effects on aggression (Terranova et al., 2017), and our study suggests that basal VP activity helps to determine individual differences in aggressive behavior output. Our results are in line with previous findings showing a negative correlation between neuronal VP activity and activation of social decision-making network nodes in the closely related brown anole, especially within agonistic contexts (Kabelik et al., 2018). Interestingly, although behavioral boldness is correlated between agonistic and reproductive contexts, VP activity does not correlate well with reproductive boldness suggesting that other mechanisms may underlie the cross-context correlation.

Stability of behavioral boldness

Our results demonstrate stability of behavioral boldness across weeks and social contexts. The correlation of boldness scores across contexts suggests a shared neural network that regulates general social behavioral output. Correlated boldness measures are often referred to as behavioral syndromes (Colléter and Brown, 2011; Koolhaas et al., 2010; Qu et al., 2018; Reaney and Backwell, 2007). The stability of boldness across time, on the other hand, supports the notion that these traits are at least partly hard-wired, as would be expected if boldness has strong heritable components, as has been suggested by previous studies (Ballew et al., 2017; Mont et al., 2018; Scherer et al., 2017). Various selective pressures appear to maintain variability in behavioral boldness within populations (Koolhaas et al., 2010; Smith and Blumstein, 2010).

Social boldness is unrelated to physical characteristics and circulating steroid hormone levels

Although a relationship between body size and boldness has been demonstrated in reptiles, this relationship was observed in juvenile keelback snakes emerging from shelter (e.g., Mayer et al., 2016), and we did not find any such relationships in the present study of adult green anoles. Instead, our results were very much in line with those of Kabelik et al. (2021), where behavioral boldness was not found to correlate with measures of body size. This was true for both boldness within agonistic and reproductive contexts. The one physical variable that did show a correlation trend with boldness was testes mass. However, this result is inconsistent with the findings of Kabelik et al. (2021) which included a larger sample size and did not find any such relationship, and the present finding did not survive correction for multiple comparisons.

In terms of steroid hormone levels, we had initially expected a correlation between behavioral boldness and glucocorticoid hormone levels. The reason for this prediction was that a sub-set of studies addressing behavioral boldness focus primarily on active versus passive coping styles to stressors, and some of these studies have found differences in glucocorticoid levels between individuals differing in coping style (Koolhaas et al., 2010; Sluyter et al., 1996). However, as in Kabelik et al. (2021), we found no relationship between circulating glucocorticoid levels and behavioral boldness in the present green anole study. It is nevertheless important to note that our hormone measures were from baseline diurnal plasma samples, and thus we cannot exclude the possibility that nocturnal or stress-evoked glucocorticoid levels may still relate to behavioral boldness. This is especially true given the fact that VP is a releasing hormone for adrenocorticotropic hormone, including in birds (Cornett et al., 2013), thus suggesting a similarly conserved role in reptiles.

We originally also hypothesized that sex steroid hormones may influence behavioral boldness in male green anoles, because previous lizard studies demonstrated their involvement in the regulation of social behaviors. For instance, in male tree lizards, circulating testosterone correlates with aggression (Kabelik et al., 2006) and testosterone and progesterone treatments

causally promote aggression (Kabelik et al., 2008b; Weiss and Moore, 2004). Additionally, in male brown anoles, the anti-androgen cyproterone acetate reduced display behaviors toward conspecific males and females (Tokarz, 1995). In male side-blotched lizards, the more aggressive morph type has been shown to possess higher levels of testosterone (Sinervo et al., 2000). However, in the present study, we did not find any correlations between baseline sex steroid hormone levels and measures of behavioral boldness. Our findings were again in line with Kabelik et al. (2021). However, that study did detect differences in androgen receptor expression in the ventromedial hypothalamus between bold and shy males, suggesting that activational androgen signaling may nevertheless be involved in regulating social behavior boldness. Furthermore, organizational sex steroid levels likely also play a role in determining adult boldness.

Building upon previous VP research in reptiles

Previous research examining VP cell numbers and optical densities in tree lizards found no correlations between these variables and measures of aggression frequency or intensity (Kabelik et al., 2008c). However, other lizard studies provided findings suggestive of an involvement of VP in the regulation of social behaviors. For instance, neural VP expression was found to be higher in dominant green anoles than in subordinate animals (Hattori and Wilczynski, 2009). Furthermore, rather than solely examining VP expression, a brown anole study examining the colocalization of VP neurons with Fos (another measure of neural activity) showed increased activation of these neurons following sexual and aggressive behavioral encounters (Kabelik et al., 2013). While these studies found links between VP activity and behavior, it was not clear whether VP activity causally impacted behavioral expression, or whether neural input from the perception of and interaction with a conspecific may have led to the observed changes. The goal of the present study was thus to examine VP neuron activity in

the absence of a conspecific, but within male green anoles that had already been extensively screened for behavioral boldness, and whose behavioral boldness stability was established. Although not a test of causality, this approach allows us to ascertain the state of the vasopressinergic activity that precedes a behavioral encounter, and thus likely predisposes individuals toward greater or lesser behavioral expression once a conspecific in perceived.

Experiments directly testing the causality of VP on behavior in lizards have also been conducted, though with inconclusive results (Campos et al., 2020; Dunham and Wilczynski, 2014). This may be partially due to logistic difficulties of targeted central injections, as these manipulations were via intraperitoneal injection. Thus, the resultant effects may be indirect due to peripheral binding of VP to smooth muscle, kidney, or pituitary receptors, rather than direct central manipulations. These green anole studies collectively found that VP manipulation decreased aggressive display to a mirror (though not to a conspecific), and increased tongue flicking and chemical display, but the VP treatment manipulation also elevated circulating glucocorticoid levels. In another study, intraperitoneal administration of VP and the VP receptor antagonist Manning compound both failed to alter displayed aggression to a conspecific in male tree lizards (Kabelik, 2006).

Related to the present study, a recent green anole study examined gene expression in various brain regions of the five most bold and five most shy males from a distribution of 57 animals (Kabelik et al., 2021). Interestingly, rather than regions containing VP neurons, the area containing the most expression differences between bold and shy males was the ventromedial hypothalamus. The genes in this region that were differentially expressed between the most bold and most shy males included androgen receptors. Testosterone, a ligand for these receptors, is known to regulate aggression-associated VP receptors in the ventrolateral hypothalamus (Delville et al., 1996). Androgens may perhaps also regulate VP receptors in the lateral ventromedial hypothalamus, a region both containing high densities of androgen

receptors and showing aggressive display-inducted activation in lizards (Kabelik et al., 2008a; Rosen et al., 2002). Unfortunately, due to a lack of VP gene annotation in the green anole genome, the Kabelik et al. (2021) study could not determine whether nonapeptides including VP were differentially expressed between bold and shy males, though no differences in VP receptor expression in the ventromedial hypothalamus was detected.

Conclusions

Behavioral boldness in male green anoles was found to be stable across weeks, as well as between agonistic and reproductive contexts. Baseline levels of VP activity (VP-pS6 colocalization) within both the PVN and SON were found to correlate inversely with the boldness of aggression toward males, though not with reproductive boldness toward females. Behavioral boldness was unrelated to measures of body size or circulating levels of glucocorticoids or sex steroids. Baseline VP release therefore likely modulates portions of the social decision-making network that regulate male aggression, and levels of VP release may determine individual variation in behavioral boldness during agonistic encounters.

Acknowledgements

LAO acknowledge that Stanford University resides on the ancestral and unceded land of the Muwekma Ohlone Tribe.

Funding

We gratefully acknowledge support from Rhodes College and the James T. and Valeria B. Robertson Chair in Biological Sciences to DK and the National Institutes of Health [DP2HD102042] to LAO. LAO is New York Stem Cell Foundation – Robertson Investigator.

Declaration of competing interest

The authors have no competing interest to declare.

Data Accessibility

Data from the analyses in this manuscript can be found at https://doi.org/10.6084/m9.figshare.16607780.v1.

References

Albers, H.E., 2015. Species, sex and individual differences in the vasotocin/vasopressin system: Relationship to neurochemical signaling in the social behavior neural network. Front. Neuroendocrinol. https://doi.org/10.1016/j.yfrne.2014.07.001

Alfonso, S., Sadoul, B., Gesto, M., Joassard, L., Chatain, B., Geffroy, B., Bégout, M.-L., 2019. Coping styles in European sea bass: The link between boldness, stress response and neurogenesis. Physiol. Behav. 207, 76–85. https://doi.org/https://doi.org/10.1016/j.physbeh.2019.04.020

Ballew, N.G., Mittelbach, G.G., Scribner, K.T., 2017. Fitness consequences of boldness in juvenile and adult largemouth bass. Am. Nat. 189, 396–406. https://doi.org/10.1086/690909

Baugh, A.T., Schaper, S. V, Hau, M., Cockrem, J.F., de Goede, P., Oers, K. van, 2012. Corticosterone responses differ between lines of great tits (*Parus major*) selected for divergent personalities. Gen. Comp. Endocrinol. 175, 488–494. https://doi.org/https://doi.org/10.1016/j.ygcen.2011.12.012

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Campos, S.M., Rojas, V., Wilczynski, W., 2020. Arginine vasotocin impacts chemosensory

behavior during social interactions of Anolis carolinensis lizards. Horm. Behav. 124, 104772. https://doi.org/https://doi.org/10.1016/j.yhbeh.2020.104772

Carter, C.S., 2017. The oxytocin-vasopressin pathway in the context of love and fear. Front. Endocrinol. (Lausanne). https://doi.org/10.3389/fendo.2017.00356

Colléter, M., Brown, C., 2011. Personality traits predict hierarchy rank in male rainbowfish social groups. Anim. Behav. 81, 1231–1237. https://doi.org/https://doi.org/10.1016/j.anbehav.2011.03.011

Cornett, L.E., Kang, S.W., Kuenzel, W.J., 2013. A possible mechanism contributing to the synergistic action of vasotocin (VT) and corticotropin-releasing hormone (CRH) receptors on corticosterone release in birds. Gen. Comp. Endocrinol. 188, 46–53. https://doi.org/https://doi.org/10.1016/j.ygcen.2013.02.032

Delville, Y., Mansour, K.M., Ferris, C.F., 1996. Testosterone facilitates aggression by modulating vasopressin receptors in the hypothalamus. Physiol. Behav. 60, 25–29. https://doi.org/https://doi.org/10.1016/0031-9384(95)02246-5

Dunham, L.A., Wilczynski, W., 2014. Arginine vasotocin, steroid hormones and social behavior in the green anole lizard (*Anolis carolinensis*). J. Exp. Biol. 217, 3670–3676. https://doi.org/10.1242/jeb.107854

Félix, A.S., Cardoso, S.D., Roleira, A., Oliveira, R.F., 2020. Forebrain transcriptional response to transient changes in circulating androgens in a cichlid fish. G3 Genes, Genomes, Genet. 10, 1971–1982. https://doi.org/10.1534/g3.119.400947

Goodson, J.L., Kabelik, D., 2009. Dynamic limbic networks and social diversity in vertebrates: From neural context to neuromodulatory patterning. Front. Neuroendocrinol. https://doi.org/10.1016/j.yfrne.2009.05.007

Greenberg, N., Crews, D., 1990. Endocrine and behavioral responses to aggression and social dominance in the green anole lizard, *Anolis carolinensis*. Gen. Comp. Endocrinol. 77, 246–255. https://doi.org/https://doi.org/10.1016/0016-6480(90)90309-A

Hartline, J.T., Smith, A.N., Kabelik, D., 2017. Serotonergic activation during courtship and aggression in the brown anole, *Anolis sagrei*. PeerJ 2017, e3331. https://doi.org/10.7717/peerj.3331

Hattori, T., Wilczynski, W., 2009. Comparison of arginine vasotocin immunoreactivity differences in dominant and subordinate green anole lizards. Physiol. Behav. 96, 104–107. https://doi.org/10.1016/j.physbeh.2008.09.010

Kabelik, D., 2006. Neural mechanisms underlying the effects of testosterone on aggressive behavior in the tree lizard, *Urosaurus ornatus*. ProQuest Diss. Theses. Arizona State University, Ann Arbor.

Kabelik, D., Alix, V.C., Burford, E.R., Singh, L.J., 2013. Aggression- and sex-induced neural activity across vasotocin populations in the brown anole. Horm. Behav. 63, 437–446. https://doi.org/10.1016/j.yhbeh.2012.11.016

Kabelik, D., Alix, V.C., Singh, L.J., Johnson, A.L., Choudhury, S.C., Elbaum, C.C., Scott, M.R., 2014. Neural activity in catecholaminergic populations following sexual and aggressive interactions in the brown anole, *Anolis sagrei*. Brain Res. 1553, 41–58. https://doi.org/10.1016/j.brainres.2014.01.026

Kabelik, D., Crews, D., 2017. Hormones, Brain, and Behavior in Reptiles, in: Hormones, Brain and Behavior: Third Edition. pp. 171–213. https://doi.org/10.1016/B978-0-12-803592-4.00027-4

Kabelik, D., Crombie, T., Moore, M.C., 2008a. Aggression frequency and intensity, independent of testosterone levels, relate to neural activation within the dorsolateral subdivision of the ventromedial hypothalamus in the tree lizard *Urosaurus ornatus*. Horm. Behav. 54, 18–27. https://doi.org/10.1016/j.yhbeh.2007.09.022

Kabelik, D., Hofmann, H.A., 2018. Comparative neuroendocrinology: A call for more study of reptiles! Horm. Behav. 106, 189–192. https://doi.org/10.1016/j.yhbeh.2018.10.005

Kabelik, D., Julien, A.R., Ramirez, D., O'Connell, L.A., 2021. Social boldness correlates with brain gene expression in male green anoles. Horm. Behav. 133, 105007. https://doi.org/10.1016/j.yhbeh.2021.105007

Kabelik, D., Magruder, D.S., 2014. Involvement of different mesotocin (oxytocin homologue) populations in sexual and aggressive behaviours of the brown anole. Biol. Lett. 10, 20140566. https://doi.org/10.1098/rsbl.2014.0566

Kabelik, D., Weiss, S.L., Moore, M.C., 2008b. Steroid hormones alter neuroanatomy and aggression independently in the tree lizard. Physiol. Behav. 93, 492–501. https://doi.org/10.1016/j.physbeh.2007.10.008

Kabelik, D., Weiss, S.L., Moore, M.C., 2008c. Arginine vasotocin (AVT) immunoreactivity relates to testosterone but not territorial aggression in the tree lizard, Urosaurus ornatus. Brain. Behav. Evol. 72, 283–294. https://doi.org/10.1159/000174248

Kabelik, D., Weiss, S.L., Moore, M.C., 2006. Steroid hormone mediation of limbic brain plasticity and aggression in free-living tree lizards, *Urosaurus ornatus*. Horm. Behav. 49, 587–597. https://doi.org/10.1016/j.yhbeh.2005.12.004

Kabelik, D., Weitekamp, C.A., Choudhury, S.C., Hartline, J.T., Smith, A.N., Hofmann, H.A., 2018. Neural activity in the social decision-making network of the brown anole during reproductive and agonistic encounters. Horm. Behav. 106, 178–188. https://doi.org/10.1016/j.yhbeh.2018.06.013

Kanitz, E., Tuchscherer, M., Otten, W., Tuchscherer, A., Zebunke, M., Puppe, B., 2019. Coping style of pigs is associated with different behavioral, neurobiological and immune responses to stressful challenges. Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2019.00173

Kelly, A.M., Kingsbury, M.A., Hoffbuhr, K., Schrock, S.E., Waxman, B., Kabelik, D., Thompson, R.R., Goodson, J.L., 2011. Vasotocin neurons and septal V1a-like receptors potently modulate songbird flocking and responses to novelty. Horm. Behav. 60, 12–21. https://doi.org/10.1016/j.yhbeh.2011.01.012

Ketterson, E.D., Nolan Val, J., 1999. Adaptation, exaptation, and constraint: A hormonal perspective. Am. Nat. 154, S4–S25. https://doi.org/10.1086/303280

Knight, Z.A., Tan, K., Birsoy, K., Schmidt, S., Garrison, J.L., Wysocki, R.W., Emiliano, A., Ekstrand, M.I., Friedman, J.M., 2012. Molecular profiling of activated neurons by phosphorylated ribosome capture. Cell 151, 1126–1137. https://doi.org/10.1016/j.cell.2012.10.039

Koolhaas, J.M., de Boer, S.F., Coppens, C.M., Buwalda, B., 2010. Neuroendocrinology of coping styles: Towards understanding the biology of individual variation. Front.

Neuroendocrinol. 31, 307–321. https://doi.org/10.1016/j.yfrne.2010.04.001

Korzan, W.J., Summers, C.H., 2004. Serotonergic response to social stress and artificial social sign stimuli during paired interactions between male *Anolis carolinensis*. Neuroscience 123, 835–845. https://doi.org/https://doi.org/10.1016/j.neuroscience.2003.11.005

Korzan, W.J., Summers, T.R., Ronan, P.J., Renner, K.J., Summers, C.H., 2001. The role of monoaminergic nuclei during aggression and sympathetic social signaling. Brain. Behav. Evol. 57, 317–327. https://doi.org/10.1159/000047250

Larson, E.T., Summers, C.H., 2001. Serotonin reverses dominant social status. Behav. Brain Res. 121, 95–102. https://doi.org/https://doi.org/10.1016/S0166-4328(00)00393-4

Lovern, M.B., Holmes, M.M., Wade, J., 2004. The green anole (*Anolis carolinensis*): A reptilian model for laboratory studies of reproductive morphology and behavior. ILAR J. https://doi.org/10.1093/ilar.45.1.54

Mayer, M., Shine, R., Brown, G.P., 2016. Bigger babies are bolder: effects of body size on personality of hatchling snakes. Behaviour 153, 313–323. https://doi.org/https://doi.org/10.1163/1568539X-00003343

Mont, C., Hernandez-Pliego, P., Cañete, T., Oliveras, I., Río-Álamos, C., Blázquez, G., López-Aumatell, R., Martínez-Membrives, E., Tobeña, A., Flint, J., Fernández-Teruel, A., Mott, R., 2018. Coping-style behavior identified by a survey of parent-of-origin effects in the rat. G3 Genes, Genomes, Genet. 8, 3283–3291. https://doi.org/10.1534/g3.118.200489

Newman, S.W., 1999. The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann. N. Y. Acad. Sci. 877, 242–257. https://doi.org/10.1111/j.1749-6632.1999.tb09271.x

O'Connell, L.A., Hofmann, H.A., 2012. Evolution of a vertebrate social decision-making network. Science (80-.). 336, 1154–1157. https://doi.org/10.1126/science.1218889

O'Connell, L.A., Hofmann, H.A., 2011. The vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. J. Comp. Neurol. 519, 3599–3639. https://doi.org/10.1002/cne.22735

Putman, B.J., Azure, K.R., Swierk, L., 2019. Dewlap size in male water anoles associates with consistent inter-individual variation in boldness. Curr. Zool. 65, 189–195. https://doi.org/10.1093/cz/zoy041

Qu, J., Fletcher, Q.E., Réale, D., Li, W., Zhang, Y., 2018. Independence between coping style and stress reactivity in plateau pika. Physiol. Behav. 197, 1–8. https://doi.org/https://doi.org/10.1016/j.physbeh.2018.09.007

Réale, D., Dingemanse, N.J., Kazem, A.J.N., Wright, J., 2010. Evolutionary and ecological approaches to the study of personality. Philos. Trans. R. Soc. B Biol. Sci. 365, 3937–3946. https://doi.org/10.1098/rstb.2010.0222

Reaney, L.T., Backwell, P.R.Y., 2007. Risk-taking behavior predicts aggression and mating success in a fiddler crab. Behav. Ecol. 18, 521–525. https://doi.org/10.1093/beheco/arm014

Rosen, G., O'Bryant, E., Matthews, J., Zacharewski, T., Wade, J., 2002. Distribution of androgen receptor mRNA expression and immunoreactivity in the brain of the green anole lizard. J. Neuroendocrinol. 14, 19–28. https://doi.org/10.1046/j.0007-1331.2001.00735.x

Scherer, U., Kuhnhardt, M., Schuett, W., 2017. Different or alike? Female rainbow kribs choose males of similar consistency and dissimilar level of boldness. Anim. Behav. 128, 117–124. https://doi.org/https://doi.org/10.1016/j.anbehav.2017.04.007

Sih, A., Bell, A., Johnson, J.C., 2004. Behavioral syndromes: an ecological and evolutionary overview. Trends Ecol. Evol. 19, 372–378. https://doi.org/10.1016/j.tree.2004.04.009

Sinervo, B., Miles, D.B., Frankino, W.A., Klukowski, M., DeNardo, D.F., 2000. Testosterone, endurance, and Darwinian fitness: Natural and sexual selection on the physiological bases of alternative male behaviors in side-blotched lizards. Horm. Behav. 38, 222–233. https://doi.org/10.1006/hbeh.2000.1622

Sluyter, F., Korte, S.M., Bohus, B., Van Oortmerssen, G.A., 1996. Behavioral stress response of genetically selected aggressive and nonaggressive wild house mice in the shock-probe/defensive burying test. Pharmacol. Biochem. Behav. 54, 113–116. https://doi.org/10.1016/0091-3057(95)02164-7

Smith, A.N., Kabelik, D., 2017. The effects of dopamine receptor 1 and 2 agonists and antagonists on sexual and aggressive behaviors in male green anoles. PLoS One 12. https://doi.org/10.1371/journal.pone.0172041

Smith, B.R., Blumstein, D.T., 2010. Behavioral types as predictors of survival in Trinidadian guppies (*Poecilia reticulata*). Behav. Ecol. 21, 919–926. https://doi.org/10.1093/beheco/arq084

Taborsky, M., Hofmann, H.A., Beery, A.K., Blumstein, D.T., Hayes, L.D., Lacey, E.A., Martins, E.P., Phelps, S.M., Solomon, N.G., Rubenstein, D.R., 2015. Taxon matters: promoting integrative studies of social behavior: NESCent Working Group on Integrative Models of Vertebrate Sociality: Evolution, Mechanisms, and Emergent Properties. Trends Neurosci. 38, 189–191. https://doi.org/10.1016/J.TINS.2015.01.004

Terranova, J.I., Ferris, C.F., Albers, H.E., 2017. Sex differences in the regulation of offensive aggression and dominance by Arginine-vasopressin. Front. Endocrinol. (Lausanne). https://doi.org/10.3389/fendo.2017.00308

Thörnqvist, P.-O., McCarrick, S., Ericsson, M., Roman, E., Winberg, S., 2019. Bold zebrafish (Danio rerio) express higher levels of delta opioid and dopamine D2 receptors in the brain compared to shy fish. Behav. Brain Res. 359, 927–934. https://doi.org/https://doi.org/10.1016/j.bbr.2018.06.017

Tokarz, R.R., 1995. Importance of androgens in male territorial acquisition in the lizard *Anolis sagrei*: an experimental test. Anim. Behav. 49, 661–669. https://doi.org/10.1016/0003-3472(95)80199-5

Tudorache, C., Slabbekoorn, H., Robbers, Y., Hin, E., Meijer, J.H., Spaink, H.P., Schaaf, M.J.M., 2018. Biological clock function is linked to proactive and reactive personality types. BMC Biol. 16, 148. https://doi.org/10.1186/s12915-018-0618-0

Veenema, A.H., Beiderbeck, D.I., Lukas, M., Neumann, I.D., 2010. Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. Horm. Behav. 58, 273–281. https://doi.org/https://doi.org/10.1016/j.yhbeh.2010.03.006

Veenema, A.H., Koolhaas, J.M., De Kloet, E.R., 2004. Basal and stress-induced differences in HPA axis, 5-HT responsiveness, and hippocampal cell proliferation in two mouse lines.

Ann. N. Y. Acad. Sci. 1018, 255–265. https://doi.org/10.1196/annals.1296.030

Veenema, A.H., Meijer, O.C., De Kloet, E.R., Koolhaas, J.M., Bohus, B.G., 2003. Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. Horm. Behav. 43, 197–204. https://doi.org/10.1016/S0018-506X(02)00013-2

Walton, J.C., Waxman, B., Hoffbuhr, K., Kennedy, M., Beth, E., Scangos, J., Thompson, R.R., 2010. Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. Neuropharmacology 58, 126–134. https://doi.org/10.1016/j.neuropharm.2009.07.018

Watt, M.J., Forster, G.L., Korzan, W.J., Renner, K.J., Summers, C.H., 2007. Rapid neuroendocrine responses evoked at the onset of social challenge. Physiol. Behav. 90, 567–575. https://doi.org/https://doi.org/10.1016/j.physbeh.2006.11.006

Weiss, S.L., Moore, M.C., 2004. Activation of aggressive behavior by progesterone and testosterone in male tree lizards, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 136, 282–288. https://doi.org/https://doi.org/10.1016/j.ygcen.2004.01.001

Wilczynski, W., Quispe, M., Muñoz, M.I., Penna, M., 2017. Arginine vasotocin, the Social Neuropeptide of Amphibians and Reptiles. Front. Endocrinol. (Lausanne). 8, 186. https://doi.org/10.3389/fendo.2017.00186

Woolley, S.C., Sakata, J.T., Crews, D., 2004a. Tyrosine hydroxylase expression is affected by sexual vigor and social environment in male *Cnemidophorus inornatus*. J. Comp. Neurol. 476, 429–439. https://doi.org/10.1002/cne.20236

Woolley, S.C., Sakata, J.T., Crews, D., 2004b. Evolutionary insights into the regulation of courtship behavior in male amphibians and reptiles. Physiol. Behav. 83, 347–360. https://doi.org/10.1016/j.physbeh.2004.08.021

Woolley, S.C., Sakata, J.T., Gupta, A., Crews, D., 2001. Evolutionary changes in dopaminergic modulation of courtship behavior in *Cnemidophorus* whiptail lizards. Horm. Behav. 40, 483–489. https://doi.org/10.1006/hbeh.2001.1713