1 **Aggressive but not reproductive boldness in male green anole lizards**

2 **correlates with baseline vasopressin activity**

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9 Key words: vasotocin, reptile, lizard, steroid hormones

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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 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 boldness during social encounters. 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. Vasopressin-pS6 colocalization within the paraventricular and supraoptic nuclei of the hypothalamus was inversely correlated with boldness of aggressive behaviors, but not of reproductive behaviors. Our findings suggest that baseline vasopressin release, rather than solely context-dependent release, plays a role in predisposing individuals toward stable levels of displayed aggression toward conspecifics by inhibiting behavioral output in these contexts.

43   **Introduction**

44   Humans are often described as being introverted, extraverted, or somewhere in

45   between. Such variation in a stable boldness measure similarly exists within numerous animal

46   species, and has also been described as a behavioral syndrome, personality, or coping style

47   (Koolhaas et al., 2010; Réale et al., 2010; Sih et al., 2004). These terms encapsulate the notion

48   of linked social traits that are expressed across various environments, including both social and

49   nonsocial contexts. Indeed, evidence in some species demonstrates heritability of displayed

50   boldness (Ballew et al., 2017; Mont et al., 2018; Scherer et al., 2017), suggesting a genetic

51   basis for the stability of individual differences across time. Likewise, the level of an individual's

52   boldness is often consistent across social contexts (Colléter and Brown, 2011; Koolhaas et al.,

53   2010; Qu et al., 2018; Reaney and Backwell, 2007), where individuals that may be shy within a

54   reproductive social encounter may also be shy within an agonistic encounter (Kabelik et al.,

55   2021). Such consistency of behavioral phenotype hints at common neural mechanisms

56   regulating boldness across a variety of contexts.

57   The mechanisms that underlie boldness during social encounters likely involve the social

58   decision-making network (Newman, 1999; O'Connell and Hofmann, 2012, 2011) and its various

59   neuroendocrine mediators (Baugh et al., 2012; Félix et al., 2020; Ketterson and Nolan Val,

60   1999) that are conserved across vertebrates. The mediators linked to individual variability in

61   behavioral phenotype include steroid hormones (Koolhaas et al., 2010; Sluyter et al., 1996;

62   Tudorache et al., 2018; Veenema et al., 2004, 2003), various neuropeptide and

63   neurotransmitter systems (Thörnqvist et al., 2019; Veenema et al., 2004), and their associated

64   receptors (Alfonso et al., 2019; Kabelik et al., 2021; Kanitz et al., 2019). Although much of the

65   research on the neuroendocrine basis of boldness in social contexts has been conducted in

66   mammals and fish, here we examine these traits in a reptilian model system, the green anole

67   lizard (*Anolis carolinensis*). The study of behavioral neuroscience in reptiles lags behind that of

3

68   other vertebrate groups and invertebrates (Kabelik and Hofmann, 2018; Taborsky et al., 2015),

69   despite lizards being important for evolutionary comparisons among animal taxa, especially

70   amniotic vertebrates (mammals, birds, and reptiles). Green anoles are a longstanding model for

71   behavioral neuroendocrinology research (Lovern et al., 2004) due to their readily quantifiable

72   social behavioral displays, natural seasonal variability in hormone levels, and natural stress

73   responsivity. A social decision-making network has been described in reptiles (Kabelik et al.,

74   2018), and boldness has been shown to be stable across contexts and over time in anole lizard

75   studies (Kabelik et al., 2021; Putman et al., 2019).

76   Various neuroendocrine variables have been related to the expression of social

77   behaviors in lizards, including neuropeptides and sex steroid hormones (Dunham and

78   Wilczynski, 2014; Hartline et al., 2017; Kabelik et al., 2013, 2008b; Kabelik and Crews, 2017;

79   Kabelik and Magruder, 2014; Korzan et al., 2001; Korzan and Summers, 2004; Larson and

80   Summers, 2001; Smith and Kabelik, 2017; Watt et al., 2007; Woolley et al., 2004a, 2004b,

81   2001). Here, we focus on the potential regulation of boldness by the vasopressin (VP; Ile^3-

82   vasopressin, a.k.a. Arg^8-vasotocin) system, as well as circulating steroid hormones.

83   Vasopressin has been shown to have various effects on social behavioral expression, and

84   stress reactivity, across species (Albers, 2015; Carter, 2017; Goodson and Kabelik, 2009; Kelly

85   et al., 2011; Kelly and Goodson, 2014a; Walton et al., 2010; Wilczynski et al., 2017). Depending

86   where in the brain VP is released, the effects on social behavior may be completely opposed to

87   each other, such as in the case of displayed aggression in rats (Veenema et al., 2010).

88   Research in anole lizards suggests that VP has an inhibitory effect on components of the social

89   decision-making network (Kabelik et al., 2018). Although many studies focus on activity of VP

90   neurons and receptors during a social encounter (e.g., Kabelik et al., 2013), information about

91   baseline activity of VP neurons in individuals varying in boldness is unknown.

4

92   Here, we test three hypotheses about the regulation of boldness with social contexts of

93   male green anoles. First, we test the hypothesis that the physical size of males relates to their

94   boldness during social encounters. Body size has been shown to be predictive of boldness in

95   some but not all species (Adriaenssens and Johnsson, 2011; Brown and Braithwaite, 2004;

96   Mayer et al., 2016; Smith and Blumstein, 2010), and thus we predicted either a positive

97   association or no association between these variables. Second, we test the hypothesis that

98   circulating levels of steroid hormones regulate boldness within social behavior interactions. As

99   testosterone and progesterone have been linked to aggression (Kabelik et al., 2008b; Weiss

100 and Moore, 2004) and glucocorticoids to boldness in other species (Koolhaas et al., 2010;

101 Sluyter et al., 1996), we predicted that steroid hormone levels would predict behavioral boldness

102 in the present study. Third, we tested the hypothesis that central release of VP modulates

103 boldness within social interactions. As VP is associated with both affiliative and agonistic

104 behavior (Kelly and Goodson, 2014b), we predicted a relationship between these variables, but

105 not predict a specific direction of this correlation.

106 We first examined the stability of boldness in both reproductive and agonistic

107 encounters across a period of two weeks. We then waited for one day before collecting blood

108 samples and brains to eliminate neuroendocrine changes correlated with recent participation in

109 a social behavior interaction. We quantified hormone levels via enzyme-linked immunosorbent

110 assay and used immunofluorescence to label VP neurons and their colocalization with

111 phosphorylated ribosomal protein S6 (pS6), a proxy marker of neural activation in response to

112 stimulation, as well as baseline neural activity (Cao et al., 2011; Klingebiel et al., 2017; Knight et

113 al., 2012). We predicted that circulating steroid hormone levels and the baseline activity of VP

114 neurons (%VP-pS6 colocalization) would reflect the neuroendocrine state that predisposes that

115 individual toward boldness or shyness.

116

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 for three weeks prior to experimentation 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. Animals were fed three times per week with crickets and cages were misted twice-daily with deionized water. 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.

Behavior assays

Behavioral trials were carried out in May and June of 2013. 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

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141   maximum latency score of 10 min. The maximum intensity of behavioral display was also

142   scored from 0-4 based on the highest achieved category: no display, headbob only, pushup

143   and/or dewlap display, chase, and copulate. Behaviors in the agonistic encounter were scored

144   as in the reproductive encounter, except that biting of the stimulus male replaced copulation as

145   the highest intensity behavior.

146   The reproductive encounter was conducted with three separate pairs of females and the

147   agonistic encounter with three separate stimulus males over the course of a week. Only one trial

148   was conducted per day. Stimulus animals were only used once per day. The entire procedure

149   was then repeated during the subsequent week, toward the same three pairs of females and the

150   same three stimulus males. Conducting the behavioral observations a second time allowed us

151   to determine the stability of each boldness measure across time.

152

153   *Bold-shy categorization*

154   To generate each boldness score, we conducted principal components analyses (PCA)

155   to reduce the average behavioral latency, frequency, and intensity scores from the given

156   encounter scenarios (reproductive or agonistic), separately for week 1 and for week 2, each into

157   a single value. For example, in male-female trials from week 1, the behavioral latency,

158   frequency, and intensity scores for each focal male were averaged across the three trials to

159   generate an "average reproductive latency", "average reproductive frequency", and "average

160   reproductive intensity" score. These average scores were then included in the PCA and

161   generated a single PCA component that we here call BoldnessToFemalesWeek1. In each

162   scenario, the resulting analysis generated a single PCA component with an eigenvalue > 1, and

163   in each case, this component was highly positively correlated with average frequency and

164   intensity scores, and negatively with average latency scores (r > ± 0.80, p < 0.001 for each).

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165   BoldnessToFemalesWeek1 explained 81% of the behavioral variation in those trials,

166   BoldnessToFemalesWeek2 (from week 2 data) explained 75% of the variation from those data,

167   BoldnessToMalesWeek1 (from week 1 agonistic encounters) explained 83% of the variation in

168   those data, and BoldnessToMalesWeek2 explained 85% of the variation in behavioral

169   frequency, latency, and intensity values from the week 2 agonistic encounter data. As week 1

170   and week 2 data were highly correlated, we also created a variable that averaged reproductive

171   boldness from weeks 1 and 2 (AverageBoldnessToFemales), as well as agonistic boldness from

172   weeks 1 and 2 (AverageBoldnessToMales).

173

174   *Tissue harvesting*

175   Prior to handling for blood and brain harvesting, focal subjects were left undisturbed in

176   their home terraria for 1 day following their last behavioral trial. We euthanized focal males by

177   cutting through the spinal column and immediately collected trunk blood for hormone analyses

178   (average collection time from first handling was 162 ± 3.2 s). The blood was kept at 4°C until

179   centrifugation. The brain was then rapidly dissected and fixed by overnight submersion in 4%

180   paraformaldehyde in 0.1 M phosphate buffer at 4°C, followed by cryoprotection with 30%

181   sucrose in 0.1 M phosphate-buffered saline (PBS). The body (minus the head) was then

182   weighed, after which the testes were dissected from the body and weighed. Brains were

183   sectioned into two series, at a section thickness of 50 µm, on a Microm HM 520 cryostat

184   (Thermo Scientific).

185

186   *Hormone analyses*

187   Blood samples were centrifuged and plasma (averaging 62 ± 2.7 µl) was frozen at -80°C

188   until hormone analysis. We quantified testosterone (ADI-900-065; sensitivity 5.67 pg/mL),

189   estradiol (ADI-900-008; sensitivity 28.5 pg/mL), progesterone (ADI-900-011; sensitivity 8.57

190   pg/mL), and cortisol (ADI-900-071; sensitivity 56.72 pg/mL) using enzyme-linked

191   immunosorbent assay (ELISA) kits (Enzo Life Sciences, Farmingdale, NY). The cortisol kit

192   cross-reacts with corticosterone at 28%, representing a general glucocorticoid assay, albeit with

193   lower-than-typical sensitivity. We re-suspended 7 µl of plasma in 203 µL of the appropriate

194   assay buffer and ran each sample in duplicate as per manufacturer's instructions. Samples

195   were run across two plates for each hormone and the inter-assay variation across plates and

196   the intra-assay variance for each plate is as follows: testosterone (inter: 5.6%; intra: 5.6% and

197   6.1%), estradiol (inter: 4.1%; intra: 3.7% and 8.5%), progesterone (inter: 4.7%; intra: 2.3% and

198   4.7%), and cortisol (inter: 6.4%; intra: 3.8% for both plates). Five samples were inadvertently

199   excluded from the analysis. Hormone results were generally consistent with previously reported

200   levels in this species (Greenberg and Crews, 1990; Young et al., 1991).

201

202   *Immunohistochemistry*

203   Immunohistochemical processing was conducted as in previous studies (Hartline et al.,

204   2017; Kabelik et al., 2018, 2014, 2013; Kabelik and Magruder, 2014). Briefly, we processed one

205   series of brain sections with 1:250 dilution of rabbit anti-pS6 antibody (#2211, Cell Signaling

206   Technology), 1:5000 dilution of guinea pig anti-vasopressin antibody (T-5048, Peninsula

207   Laboratories), and 1:2000 dilution of sheep anti-tyrosine hydroxylase antibody (NB300-110,

208   Novus Biologicals; part of a separate study). The sections were subsequently processed with

209   donkey anti-sheep secondary antibody conjugated to Alexa Fluor 488 at 3 µl/ml and (Life

210   Technologies), donkey-anti rabbit secondary antibody conjugated to Alexa Fluor 555 at 5 µl/ml

211   (Life Technologies), and donkey-anti guinea pig DyLight 647 at 16 µl/ml (Jackson

212   ImmunoResearch). Preadsorption with excess VP (4100576, Bachem) and pS6 blocking

213   peptide (1220S, Cell Signaling Technology) antibody eliminated signal (**Supplementary Fig. 1**).

9

214   We targeted pS6 rather than Fos because preliminary research demonstrated extremely low

215   levels of Fos expression within VP neurons under baseline conditions.

216

217   *Microscopy and Image Analyses*

218   An LSM 700 Confocal microscope and Zen 2010 software (Carl Zeiss), using a 20X

219   objective, were used to capture z-stacks of photomicrographs at 5 μm intervals, in a grid that

220   was later stitched together. A maximum intensity projection created a two-dimensional image.

221   Individual colors were exported as separate layers using AxioVision 4.8 (Carl Zeiss), and these

222   were stacked as overlaid monochromatic layers in Photoshop (Adobe Systems). Layers in the

223   stack could thus be toggled on and off to determine signal colocalization. Analyses were

224   conducted by individuals unaware of treatment groups.

225   We examined VP cells within the paraventricular nucleus (PVN) and the supraoptic

226   nucleus (SON) of the hypothalamus (**Fig. 1**). Only cells that could be clearly visualized with VP-

227   signal in the cytoplasm and a darker nucleus were examined. VP cell counts could not be

228   accurately obtained due to damage to some tissue sections and many overlapping cells,

229   especially within the SON. We estimated VP cell density by examining the average number of

230   cells per section across the three most densely populated sections. An average of 39.32 ± 4.93

231   (mean ± S.E.) cells in the PVN and 28 ± 5.85 cells in the SON were analyzed per subject.

232

233   *Statistical analyses*

234   Statistical analyses (Pearson's correlations; repeated-measures analysis of variance,

235   ANOVA; Friedman test; PCA) were run using IBM SPSS (version 22). Corrections for multiple

236   comparisons were made using Benjamini-Hochberg calculations (Benjamini and Hochberg,

237   1995). Scatterplots and boxplots were made with ggplot2 (version 3.3.3) in RStudio (version

238   1.4.1106) running R (version 4.1.0). Hormone levels were ln-transformed to meet assumptions

239   of parametric analyses.

240

241   **Results**

242   *Boldness is stable over time and correlated across contexts*

243   Relative boldness was found to be stable across weeks (**Fig. 2**).

244   BoldnessToMalesWeek1 was highly correlated with BoldnessToMalesWeek2 (r=0.57, N=22,

245   p=0.005). This was despite a general drop in aggression frequency and intensity, and rise in

246   aggression latency across the six testing sessions, possibly due to habituation (p<0.01 for all

247   three variables, see **Supplementary Figs. 2-4**). Similarly, BoldnessToFemalesWeek1 was

248   highly correlated with BoldnessToFemalesWeek2 (r=0.72, N=22, p<0.001). Reproductive

249   behavior frequency, intensity, and latency did not differ across testing sessions (p>0.05 for all,

250   see **Supplementary Figs. 5-7**). Because the week 1 and week 2 boldness PCA scores were

251   highly correlated, the average scores from both weeks were then used to compare boldness

252   across social contexts, where AverageBoldnessToFemales correlated strongly with

253   AverageBoldnessToMales (r=0.65, N=22, p=0.001).

254

255   *Boldness is generally not correlated with physical traits or steroid hormone levels*

256   Boldness to males and females was unrelated to physical or hormonal measures except

257   for a positive correlation between testes mass and AverageBoldnessToFemales (**Table 1**).

258   Similarly, neither VP-pS6 colocalization within the PVN, nor in the SON, correlated with any

259   physical characteristics or hormone levels (p>0.05 for all).

260

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

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).

Boldness to males and females was not associated with measures of total VP cell number

The average PVN VP cell density across the three most densely populated sections was unrelated to displayed boldness to males (r=-0.05, N=22, p=0.84) and females (r=0.22, N=22, p=0.33). This was likewise true for SON VP cell density and displayed boldness to males (r=-0.19, N=20, p=0.42) and females (r=-0.02, N=20, p=0.95). Similar analyses for average VP counts per section, or VP counts on the single most densely populated section likewise showed no relationship to displayed boldness (p>0.27 for all).

Discussion

12

281   In this study, we tested the hypotheses that physical size, circulating steroid hormone

282   levels, and central release of VP regulate displayed boldness in reproductive and agonistic

283   social encounters. Our results support the third hypothesis, where baseline levels of VP activity

284   are associated with boldness of male green anoles within an agonistic context, presumably due

285   to differential levels of VP release. However, our results do not support our first two hypotheses

286   relating to physical size and steroid hormones levels being associated with boldness. We also

287   demonstrate that boldness in male green anoles is stable across social contexts and time.

288   Vasopressin is a neuromodulator that has previously been shown to have causal effects

289   on aggression (Kelly and Goodson, 2014b; Terranova et al., 2017), and our study supports the

290   notion that basal VP activity helps to determine individual differences in aggressive behavior

291   output. Our results are in line with previous findings showing a negative correlation between

292   neuronal VP activity and activation of social decision-making network nodes in the closely

293   related brown anole, especially within agonistic contexts (Kabelik et al., 2018). Interestingly,

294   although boldness measures are correlated between agonistic and reproductive contexts, VP

295   activity does not correlate well with reproductive boldness suggesting that other mechanisms

296   may underlie the cross-context correlation.

297

298   *Stability of boldness*

299   Our results demonstrate stability of displayed boldness across weeks and social

300   contexts. The correlation of boldness scores across contexts suggests a shared neural network

301   that regulates general social behavioral output. Correlated boldness measures are often

302   referred to as behavioral syndromes (Colléter and Brown, 2011; Koolhaas et al., 2010; Qu et al.,

303   2018; Reaney and Backwell, 2007). The stability of boldness across time, on the other hand,

304   supports the notion that these traits are at least partly hard-wired, as would be expected if

305   boldness has strong heritable components, as has been suggested by previous studies (Ballew

306   et al., 2017; Mont et al., 2018; Scherer et al., 2017). Various selective pressures appear to

307   maintain variability in exhibited boldness within populations (Koolhaas et al., 2010; Smith and

308   Blumstein, 2010).

309

310   *Boldness is unrelated to physical characteristics*

311   Although a relationship between body size and boldness has been demonstrated in

312   reptiles, this relationship was observed in juvenile keelback snakes emerging from shelter (e.g.,

313   Mayer et al., 2016), and we did not find any such relationships in the present study of adult

314   green anoles. Instead, our results were very much in line with those of Kabelik et al. (2021),

315   where boldness of social displays was not found to correlate with measures of body size. This

316   was true for both boldness within agonistic and reproductive contexts. The one physical variable

317   that did show a correlation trend with boldness was testes mass. However, this result is

318   inconsistent with the findings of Kabelik et al. (2021) that included a larger sample size and did

319   not find any such relationship. Moreover, the present finding did not survive correction for

320   multiple comparisons.

321

322   *Boldness is unrelated to circulating steroid hormone levels*

323   We predicted that glucocorticoids would correlate with boldness in green anole lizards

324   based on studies that found differences in glucocorticoid levels between individuals differing in

325   active versus passive coping styles (Koolhaas et al., 2010; Sluyter et al., 1996). However, as in

326   Kabelik et al. (2021), we found no relationship between circulating glucocorticoid levels and

327   displayed boldness during social interactions in the present green anole study. It is nevertheless

328   important to note that our hormone measures were from baseline diurnal plasma samples, and

14

329   thus we cannot exclude the possibility that nocturnal or stress-evoked glucocorticoid levels may

330   still relate to exhibited boldness. This is especially true given the fact that VP is a releasing

331   hormone for adrenocorticotropic hormone, including in birds (Cornett et al., 2013), thus

332   suggesting a similarly conserved role in reptiles.

333   We originally also hypothesized that sex steroid hormones may influence boldness in

334   male green anoles because previous lizard studies demonstrated their involvement in the

335   regulation of social behaviors. For instance, in male tree lizards, circulating testosterone

336   correlates with aggression (Kabelik et al., 2006) and testosterone and progesterone treatments

337   causally promote aggression (Kabelik et al., 2008b; Weiss and Moore, 2004). Additionally, in

338   male brown anoles, the anti-androgen cyproterone acetate reduced display behaviors toward

339   conspecific males and females (Tokarz, 1995). In male side-blotched lizards, the more

340   aggressive morph type has also been shown to possess higher levels of testosterone (Sinervo

341   et al., 2000). However, in the present study, we did not find any correlations between baseline

342   sex steroid hormone levels and measures of displayed boldness within social contexts, which is

343   in line with Kabelik et al. (2021). However, that study did detect differences in androgen receptor

344   expression in the ventromedial hypothalamus between bold and shy males, suggesting that

345   activational androgen signaling may nevertheless be involved in regulating the boldness of

346   social behaviors. Furthermore, organizational sex steroid levels likely also play a role in

347   determining levels of adult boldness.

348

349   *Building upon previous VP research in reptiles*

350   Previous research examining VP cell numbers and optical densities in tree lizards found

351   no correlations with aggression frequency or intensity (Kabelik et al., 2008c). However, other

352   lizard studies provided findings suggestive of an involvement of VP in the regulation of social

15

353   behaviors. For instance, neural VP expression was found to be higher in dominant green anoles

354   than in subordinate animals (Hattori and Wilczynski, 2009). Furthermore, rather than solely

355   examining VP expression, a brown anole study examining the colocalization of VP neurons with

356   Fos (another measure of neural activity) showed increased activation of these neurons following

357   sexual and aggressive behavioral encounters (Kabelik et al., 2013). While these studies found

358   links between VP activity and behavior, it was not clear whether VP activity causally impacted

359   behavioral expression, or whether neural input from the perception of and interaction with a

360   conspecific may have led to the observed changes. The goal of the present study was thus to

361   examine VP neuron activity in the absence of a conspecific, but within male green anoles whose

362   stable boldness was established. Although not a test of causality, this approach allows us to

363   ascertain the state of the vasopressinergic activity that likely precedes a behavioral encounter to

364   predispose individuals toward greater or lesser behavioral expression once a conspecific in

365   perceived. It should be noted, however, that the repeated testing to establish boldness could

366   itself have long-lasting effects on VP chemoarchitecture and activity, much like the androgenic

367   changes observed in the winner effect seen in some species (Fuxjager and Marler, 2010).

368   Experiments directly testing the causality of VP on behavior in lizards have also been

369   conducted, though with inconclusive results (Campos et al., 2020; Dunham and Wilczynski,

370   2014). This may be partially due to logistic difficulties of targeted central injections, as these

371   manipulations were via intraperitoneal injection. Thus, the resultant effects may be indirect due

372   to peripheral binding of VP to smooth muscle, kidney, or pituitary receptors, rather than direct

373   central manipulations. These green anole studies collectively found that VP manipulation

374   decreased aggressive display to a mirror (though not to a conspecific), increased tongue flicking

375   and chemical display, but also circulating glucocorticoid levels (the effects of which are then

376   difficult to disentangle from those of VP itself). In another study, intraperitoneal administration of

377   VP and the VP receptor antagonist Manning compound both failed to alter displayed aggression

378   to a conspecific in male tree lizards (Kabelik, 2006).

379   Related to the present study, a recent green anole study examined gene expression in

380   various brain regions of the five most bold and five most shy males from a distribution of fifty-

381   seven animals (Kabelik et al., 2021). Interestingly, rather than regions containing VP neurons,

382   the area containing the most expression differences between bold and shy males was the

383   ventromedial hypothalamus. The androgen receptor was among the genes differentially

384   expressed in this region, with increased expression in bold males. Testosterone, a ligand for

385   these receptors, is known to regulate aggression-associated VP receptors in the ventrolateral

386   hypothalamus (Delville et al., 1996). Androgens may also regulate VP receptors in the lateral

387   ventromedial hypothalamus, a region both containing high densities of androgen receptors and

388   showing aggressive display-inducted activation in lizards (Kabelik et al., 2008a; Rosen et al.,

389   2002). Unfortunately, due to a lack of VP gene annotation in the green anole genome, the

390   Kabelik et al. (2021) study could not determine whether nonapeptides including VP were

391   differentially expressed between bold and shy males, though no differences in VP receptor

392   expression in the ventromedial hypothalamus was detected.

393

394   *Ties to VP research in other vertebrate taxa*

395   Apart from VP activity, the number of VP neurons present in a brain region also

396   influences the amount of VP that can be released from that cell population. The number of VP

397   neurons present in the bed nucleus of the stria terminalis of certain songbird species (as well as

398   VP receptor densities in the lateral septum, a target site of these neurons) correlates positively

399   with the degree of sociality (ranging from territorial to colonial) of the species (Goodson et al.,

400   2006; Goodson and Wang, 2006). However, VP cells in the bed nucleus of the stria terminalis

401 were barely detectable in a related brown anole study (Kabelik et al. 2013), and we were not

402 able to discern any VP cells in that brain region in this green anole study. Vasopressin neurons

403 of the PVN also play a related role in the regulation of social behaviors (Kelly and Goodson,

404 2014c) and knockdown of these neurons reduces gregariousness and alters displayed

405 aggression in zebra finches (Kelly and Goodson, 2014a). Within the latter study, knockdown of

406 VP in the PVN of male zebra finches caused increased aggression to opposite-sex individuals,

407 while the same manipulation resulted in decreased aggression in females. While our study

408 found no effects of neuron number within the PVN (or SON) on boldness, our results are

409 nevertheless in line with those of Kelly and Goodson (2014a). Here, VP activity in the green

410 anole PVN was correlated with decreased boldness in aggressive encounters (albeit toward a

411 same-sex individual), which is consistent with those neurons reducing aggression in male zebra

412 finches. However, the role of PVN VP neurons is complex, difficult to understand, and likely both

413 sex- and species-specific. For instance, findings in male song sparrows find increased PVN VP

414 activation following participation in a simulated agonistic encounter (Goodson and Evans, 2004),

415 and aggression intensity is positively associated with PVN VP activity in male brown anoles

416 (Kabelik et al., 2013). However, in goldfish, VP inhibits social approach when released within a

417 hindbrain circuit (Thompson and Walton, 2004; Walton et al., 2010), which may be separate

418 from forebrain circuitry regulating agonistic and anxiety related behaviors. This notion of

419 separate cell groups exerting separate functions on social behavior is further supported in fish

420 by work on African cichlids, where VP expression was found to be higher in gigantocellular cells

421 of territorial than nonterritorial males, though a reverse finding was present in parvocellular

422 neurons (Greenwood et al., 2008). Both of these cell groups are found within the preoptic area,

423 an ancestral common region of VP production which gave rise to separate disparate populations

424 in anamniotes (Goodson and Kabelik, 2009). In amniotes, both magnocellular and parvocellular

425 VP neurons are present in the PVN (Kabelik et al., 2008c; Kawakami et al., 2021; Panzica et al.,

426 1999), and this heterogeneity of cell types with separate functions may be one reason for the

18

427   different functions and behavioral relationships attributed to VP neurons of the PVN across

428   studies.

429

Conclusions

431   Boldness of social interactions in male green anoles was found to be stable across

432   weeks, as well as between agonistic and reproductive contexts. Baseline levels of VP activity

433   (VP-pS6 colocalization) within both the PVN and SON were found to correlate inversely with the

434   boldness of aggression toward males, though not with reproductive boldness toward females.

435   Boldness was unrelated to measures of body size, circulating levels of glucocorticoids or sex

436   steroids, or measures of VP cell number in the PVN and SON. The finding that VP activity

437   correlates inversely with boldness suggests that VP modulates portions of the social decision-

438   making network that regulate male aggression, and levels of VP release help determine

439   individual variation in boldness during agonistic encounters. This hypothesis must be taken with

440   caution, however, as a possible alternate hypothesis also exists, in that the extensive testing

441   involved in determining measures of boldness could have produced long-lasting changes to VP

442   neuronal activity. Further research will be required to help differentiate between these

443   possibilities.

444

19

453

Declaration of competing interest

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455   The authors have no competing interest to declare.

456

Data Accessibility

457

458   Data from the analyses in this manuscript can be found at

459   https://doi.org/10.6084/m9.figshare.16607780.

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729

730   **Figure captions**

731   **Figure 1. Immunofluorescence within the SON.** VP-immunoreactive cells (A) were scored as

732   colocalized (white arrows) or not colocalized (yellow arrows) with pS6-immunoreactive signal

733   (B). Nonspecific signal due to the presence of blood cells was disregarded because those cells

734   would also cause autofluorescence within the tyrosine hydroxylase (TH) layer (C; captured for a

735   separate study). A DAPI-stained layer was also captured (D).

736

737   **Figure 2. Stability of boldness.** Boldness scores were correlated across weeks within

738   agonistic contexts involving encounters with three separate conspecific intruder males across

739   two separate weeks (A), as well as within reproductive contexts involving encounters with three

740   separate pairs of conspecific females across separate weeks (B). Average boldness scores

741   across the two weeks in the agonistic context were highly correlated with average boldness

742   score within the reproductive context (C).

743

744   **Figure 3. Boldness of social behavior interactions relative to VP-pS6 colocalization.**

745   Average boldness scores toward males within an agonistic context (A, B) but not females within

746   a reproductive context (C, D) correlated negatively with the percentage of VP neurons in the

747   PVN and SON that were pS6 positive.

748

749

27

A

$R^2 = 0.33$, p = 0.005

B

$R^2 = 0.52$, p < 0.001

C

$R^2 = 0.42$, p = 0.001