# Social boldness correlates with brain gene expression in male green anoles

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## Abstract

Within populations, some individuals tend to exhibit a bold or shy social behavior phenotype relative to the mean. The neural underpinnings of these differing phenotypes – also described as syndromes, personalities, and coping styles – is an area of ongoing investigation. Although a social decision-making network has been described across vertebrate taxa, most studies examining activity within this network do so in relation to exhibited differences in behavioral expression. Our study instead focuses on constitutive gene expression in bold and shy individuals by isolating baseline gene expression profiles that influence social boldness predisposition, rather than those reflecting the results of social interaction and behavioral execution. We performed this study on male green anole lizards (*Anolis carolinensis*), an established model organism for behavioral research, which provides a crucial comparison group to investigations of birds and mammals. After identifying subjects as bold or shy through repeated reproductive and agonistic behavior testing, we used RNA sequencing to compare gene expression profiles between these groups within various forebrain, midbrain, and hindbrain regions. The ventromedial hypothalamus had the largest group differences in gene expression, with bold males having increased expression of calcium channels and neuroendocrine receptor genes compared to shy males. Conversely, shy males express more integrin alpha-10 in the majority of examined regions. There were no significant group differences in physiology or hormone levels. Our results highlight the ventromedial hypothalamus as an important center of behavioral differences across individuals and provide novel candidates for investigation into the regulation of individual variation in social behavior phenotype.

## Introduction

Individuals vary widely in their social boldness. Some individuals perform many high intensity behaviors within moments of participating in a novel social encounter, while others hesitantly engage in few, low-intensity interactions. Often, social boldness is consistent across different social environments (Coppens et al., 2010; Stamps and Groothuis, 2010). Although a continuum of such behavioral propensity usually exists within a population, we can categorize individuals at each end of such a continuum as either behaviorally 'bold' or 'shy' based on the latency, frequency, and intensity of exhibited social behaviors across contexts. Such behavioral phenotypes, also referred to as behavioral syndromes, personalities, or coping styles (Koolhaas et al., 1999; Réale et al., 2010; Sih et al., 2004), often manifest as correlated suites of behavioral outputs, presumably due to regulation by shared neural underpinnings. The neural substrates that lead an individual toward exhibiting a bold or shy phenotype likely rely on brain regions involved in social decision-making (Newman, 1999; O'Connell and Hofmann, 2011, 2012), and neuroendocrine mediators of these circuits (Baugh et al., 2012; Félix et al., 2020; Ketterson and Nolan Val, 1999). Although numerous neural systems have been associated with social behavioral output, the bases of stable bold-shy behavioral phenotypes remain poorly understood.

Among vertebrates, the lack of understanding of neuroendocrine regulators of behavioral phenotypes is especially true among non-avian reptiles, as they are the least studied vertebrate taxonomic group (Kabelik and Hofmann, 2018), despite serving as an important evolutionary comparison group, especially for amniotic vertebrates. A social decision-making network has been described in reptiles (Kabelik et al., 2018), and various neuroendocrine variables have been related to the expression of social behaviors in lizards (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; Sarah C. Woolley et al., 2004; Woolley et al., 2001; Sarah C Woolley et al., 2004). However, many potential regulators of social boldness remain unexamined. In this study, we compare neural gene expression from various brain regions of male green anoles (*Anolis carolinensis*) that exhibit stable bold and shy phenotypes in order to identify potential regulatory variables. Green anoles are a longstanding model for social behavior investigation (Lovern et al., 2004), and they have recently become a model for comparative genomic investigation (Alföldi et al., 2011), making them an ideal subject species for the present study. We focus here on male green anoles because they exhibit high levels of both reproductive and aggressive behaviors, and our aim was to differentiate individuals based on boldness within both contexts.

While many studies of social boldness examine gene expression resulting from the performance of specific social behaviors (e.g., Mukai et al., 2009; Wong et al., 2012; Zayed & Robinson, 2012), or by adoption of a dominant or submissive status within a social hierarchy (e.g., Eastman et al., 2020; Renn et al., 2008), here we examine differences in baseline neural gene expression among subjects that have been extensively screened within different social contexts and assigned to a bold or shy phenotype category. This design eliminates gene expression differences associated with expressed behavioral output and instead places focus on the neural state differences that predispose individuals toward bold or shy behavioral outputs prior to engaging in a behavioral encounter with a conspecific. Additionally, the examined males are housed individually and thus hold identical home ‘territories’, eliminating social status-related gene transcription. We selected five bold and five shy individuals for our analysis and compared gene expression profiles between these experimental groups across four forebrain, one midbrain, and one hindbrain region. These individuals did not differ in body size or baseline steroid hormone levels. However, our gene expression analyses show differential regulation of integrin alpha-10 across brain regions of bold versus shy subjects, and a prominent role for

calcium channels and various neuroendocrine factors within the ventromedial hypothalamus, including androgen and secretin receptors.

## Materials and Methods

### *Subjects*

Fifty-seven focal male green anoles (*Anolis carolinensis*) were obtained from a commercial supplier. These males were housed singly 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 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*

Focal males were each assessed three times with different conspecifics for social boldness within each of three social encounter scenarios – reproductive encounter, agonistic encounter as a resident, and agonistic encounter as an intruder. Thus, each focal male's behavior was scored in nine separate 10-min behavioral encounters, and a maximum of one social encounter per focal male was run per day. The *reproductive behavior* scenario involved two conspecific adult females simultaneously placed into the focal male's terrarium. Two females were used 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. The *agonistic encounter as a resident* scenario involved a size-matched (within 3 mm snout-vent length) adult conspecific male intruder being placed within the focal male's terrarium. Behaviors were scored as in the reproductive encounter, except that biting of the stimulus male replaced copulation as the highest intensity behavior. The *agonistic encounter as an intruder* scenario involved the focal male being taken out of his terrarium and placed into the terrarium (home territory) of a size-matched adult conspecific male. Behavioral scoring was the same as in the previous agonistic scenario. Stimulus animals were also only used once per day, and no behavioral trials involved the repeated pairing of the same subjects.

*Bold-shy categorization*

We conducted principal components analysis (PCA) using SPSS Statistics 22 (IBM) to reduce the average behavioral latency, frequency, and intensity scores from each of the three social behavior interaction scenarios into a single value. In each scenario, the resulting analysis generated a single PCA axis with an eigenvalue > 1, and in each case, this axis was highly positively correlated with average frequency and intensity scores, and negatively with average latency scores ($r>\pm0.73$, $p<0.001$ for each). This PCA axis 1 explained 65% of the behavioral variation in the reproductive boldness trial, 79% of the variation in the agonistic trial as resident, and 80% of the variation in the agonistic trial as intruder. We used these PCA axes to correlate boldness across behavioral scenarios. We also took an average of these three PCA axes to use in selecting bold and shy individuals for the RNAseq portion of this study. Because the average

PCA axis 1 score differed across the three behavioral testing blocks ($F(2,54)=4.23$, $p=0.02$), we ranked focal males based on this average PCA principal component axis 1 score within each behavioral block. We then chose the highest and lowest scoring focal male within each block, as well as the next highest and next lowest scoring focal male in two of the three blocks. This resulted in selection of the five most socially 'bold' and five most 'shy' males out of the 57 focal males screened for behavioral consistency.

*Tissue harvesting and brain tissue punching*

Prior to handling for blood and brain harvesting, focal subjects were left undisturbed in their home terraria for 24 h. 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, placed within a microcentrifuge tube filled with Tissue Tek (Sakura) cutting medium, and frozen under dry ice (average time from first handling to freezing of brain was 544 ± 8.4 s). The body (minus the head) was then weighed, after which the testes were dissected from the body and also weighed. Brains were sectioned at 100 µm on a Microm HM 520 cryostat (Thermo Scientific). The sections were laid onto glass microscope slides resting on a metal block within the cryostat at -19°C. Tissue punches of selected areas were obtained using a Stoelting brain punch set with the aid of a dissecting microscope (Olympus SZX7) mounted above the cryostat. Brain punches were placed into Trizol (Invitrogen) and frozen at -80°C. Either 1 mm or 1.5 mm tissue punches were used to obtain tissue from selected brain regions. These were as follows: POA-LS, a region including the preoptic area, anterior hypothalamus, paraventricular nucleus of the hypothalamus, and septal nuclei; HIP, a region of the medial cortex, which is at least partly homologous to the mammalian hippocampus (Desfilis et al., 2018; Striedter, 2016; Tosches et al., 2018); DVR, including the subcortical pallium (dorsal

ventricular ridge, including amygdaloid nuclei) as well as striatum; VMH, the ventromedial hypothalamus; MID, the midbrain tegmentum; HIND, the pons and rostral medulla, though not cerebellum. Brain regions were determined by reference to multiple atlases and publications (Bruce and Braford, 2009; Butler and Hodos, 2005; Greenberg, 1982; Hoops et al., 2018; Jarvis, 2008; Kabelik et al., 2014; Lopez et al., 1992; Naik et al., 1981; O'Connell and Hofmann, 2011; Rosen et al., 2002; ten Donkelaar, 1998).

*Hormone analyses*

Blood samples were centrifuged and plasma (averaging 77 ± 3.3 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. 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). 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. Hormone results were generally consistent with previously reported levels in this species (Greenberg and Crews, 1990).

*RNA sequencing and analyses*

Brain RNA was extracted using Trizol according to manufacturer's instructions (Thermo Fisher Scientific). Poly-adenylated RNA was isolated from each sample using the NEXTflex PolyA Beads purification kit (Perkin Elmer). Strand-specific libraries with unique barcodes were prepared using the NEXTFLEX Rapid Directional RNA-Seq kit 2.0 according to manufacturer's instructions (Perkin Elmer). Libraries were pooled in equal molar amounts and sequenced on an Illumina HiSeq 2500 to obtain roughly 40 million reads per sample.

We first applied quality and adaptor trimming to the raw reads using Trim Galor! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; parameters: trim_galore --paired --phred33 --length 36 -q 5 --stringency 5 --illumina -e 0.1). Reads were then aligned using kallisto (Bray et al., 2016) with default parameters to the *Anolis carolinensis* cDNA reference transcriptome (Anolis_carolinensis.AnoCar2.0.cdna.all.fa.gz) downloaded from Ensembl (May 2020). Read counts were combined into a single matrix. Differences in gene expression within each brain region were calculated using DESeq2 (Love et al., 2014) within the *in silico* Trinity pipeline ($p<0.05$, 4-fold change). We corrected p-values for multiple hypothesis testing and considered transcripts with false discovery rate (FDR) correct p-values $<0.05$ significantly differentially expressed. We performed a gene ontology enrichment analysis for differentially expressed genes in the ventromedial hypothalamus using the PANTHER (version 14; http://pantherdb.org/; Mi et al., 2019). Data visualizations were made in RStudio (version 1.3.1056) running R (version 3.5.2). Boxplots and bar charts were made with ggplot2 (version 3.3.0) and the volcano plot was generated using EnhancedVolcano (version 1.0.1).

*Statistical analyses*

Some behavioral scores and all hormone levels were ln-transformed to meet assumptions of parametric analyses. Data reduction was conducted using PCA, and the comparison of the average resultant score across behavioral testing blocks was conducted

using one-way analysis of variance. Correlations among behavioral scores were conducted using Pearson's r. Behavioral and physical differences between bold and shy focal males were examined via independent-samples t-tests, except for behavioral intensity measure, which was compared using a Mann-Whitney U test. Scatterplots of PCA scores were made with ggplot2 (version 3.3.0) in RStudio (version 1.3.1056) running R (version 3.5.2).

*Data availability*

Data from the behavior and hormone analyses, as well as RNA sequencing analysis, including count matrices, GO enrichment analyses, and differential expression statistics, are available in the Supplementary Excel File. Raw sequencing reads are available on the Sequence Read Archive (submission pending acceptance).

## Results

*Correlated behavioral traits are stable within individuals*

Individual differences in social boldness are relatively stable across different types of social encounters. In **Figure 1**, we present correlations between focal males in the reproductive, agonistic as a resident, and agonistic as an intruder scenario, reflecting latency, frequency, and intensity measures reduced into a single principal component axis for each scenario. We found positive correlations between boldness scores across all behavioral scenarios: reproductive boldness and boldness as the resident in an agonistic trial ($r=0.55$, $N=57$, $p<0.001$), between boldness as the resident in an agonistic trial and boldness as the intruder in an agonistic trial ($r=0.50$, $N=57$, $p<0.001$), and between reproductive boldness and boldness as the intruder in an agonistic trial ($r=0.29$, $N=57$, $p=0.026$).

*Behavioral, but not physiological, traits differ between bold and shy individuals*

Relative to shy males, the bold males showed a higher average frequency of reproductive behaviors, agonistic behaviors as a resident, and agonistic behaviors as an intruder. Similarly, the bold males exhibited lower average latencies to first reproductive behavior, to first agonistic behavior as a resident, and to first agonistic behavior as an intruder. Bold males also exhibited higher average behavioral intensities to females, as resident males in agonistic trials, and as intruder males in agonistic trials. However, behavioral boldness was not correlated with physical or hormonal characteristics. Bold and shy focal males did not differ in snout-vent length, or body-minus-head mass (body was weighed after brain removal, so as not to delay freezing of brain tissue). Likewise, these groups did not differ in testes mass, or in circulating testosterone, estradiol, progesterone, or glucocorticoid levels.

**Table 1**. Mean and standard error (S.E.) values for behavioral and physical variables of bold and shy male green anoles. No physical but all behavioral variables differ between bold and shy groups. Parametric comparisons state the t statistic (t) and degrees of freedom (df); nonparametric comparisons state the *U* statistic (*U*) and the sample size (N); both state the probability of significance (P) at α=0.05.

| | Shy mean | Shy S.E. | Bold mean | Bold S.E. | t/*U* | Df/N | P |
|---|---|---|---|---|---|---|---|
| ***Behavioral Variables:*** | | | | | | | |
| reproductive behavior frequency (#/10 min) | 11.1 | 2.62 | 27.9 | 1.72 | -5.36 | 8 | **0.001** |
| resident agonistic frequency (#/10 min) | 4.3 | 2.86 | 38.4 | 6.08 | -5.07 | 8 | **0.001** |
| intruder agonistic frequency (#/10 min) | 7.7 | 2.71 | 32.3 | 3.58 | -5.50 | 8 | **0.001** |
| reproductive behavior latency (min) | 3.7 | 0.80 | 1.1 | 0.06 | 3.44 | 8 | **0.009** |
| resident agonistic latency (min) | 6.9 | 1.07 | 1.0 | 0.00 | 10.36 | 8 | **<0.001** |
| intruder agonistic latency (min) | 7.6 | 0.81 | 1.6 | 0.24 | 7.13 | 8 | **<0.001** |
| reproductive behavior intensity (0-4 scale) | 0.9 | 0.07 | 1.5 | 0.17 | 1.50 | 10 | **0.016** |
| resident agonistic intensity (0-4 scale) | 0.7 | 0.35 | 2.3 | 0.29 | 1.50 | 10 | **0.016** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| intruder agonistic intensity (0-4 scale) | 0.5 | 0.22 | 2.0 | 0.13 | 0.00 | 10 | **0.008** |
| | | | | | | | |
| ***Physical Variables:*** | | | | | | | |
| snout-vent length (mm) | 6.0 | 0.07 | 6.0 | 0.10 | 0.00 | 8 | 1.00 |
| body mass (g) | 3.3 | 0.07 | 3.4 | 0.08 | -0.75 | 8 | 0.48 |
| testes mass (g) | 0.1 | 0.00 | 0.1 | 0.01 | -0.26 | 8 | 0.80 |
| testosterone (ng/ml) | 2.2 | 1.08 | 1.5 | 0.14 | 0.30 | 8 | 0.77 |
| estradiol (ng/ml) | 9.3 | 5.68 | 5.2 | 1.78 | 0.68 | 8 | 0.74 |
| progesterone (ng/ml) | 0.6 | 0.06 | 0.8 | 0.21 | -0.97 | 8 | 0.14 |
| glucocorticoids (ng/ml) | 7.0 | 3.14 | 4.8 | 0.59 | 0.25 | 8 | 0.81 |

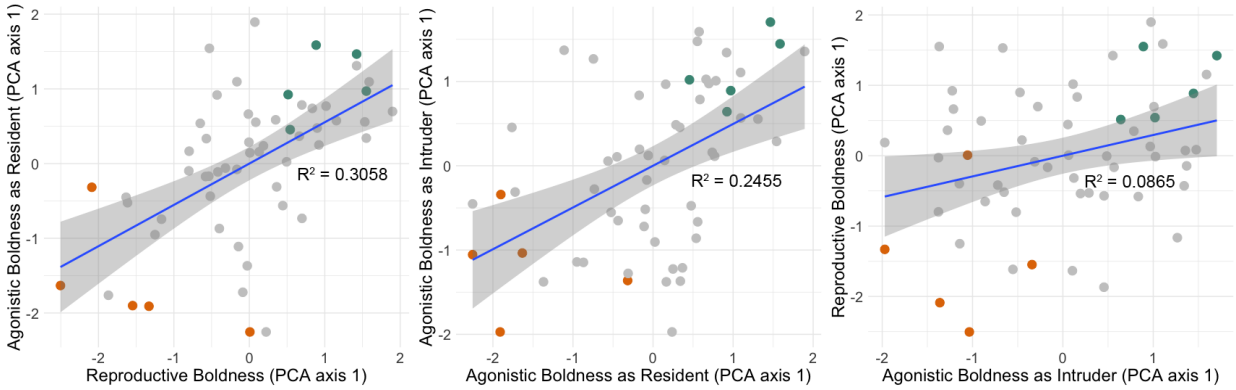


**Figure 1. Focal male green anole lizards exhibit stable social boldness phenotypes.** (Left) Average boldness in three reproductive encounters correlates strongly with average boldness as the resident male in three separate agonistic encounters. (Middle) Average boldness as the resident male correlates strongly with average boldness as an intruder within three separate agonistic encounters. (Right) Average boldness as the intruder male in three separate agonistic encounters correlates weakly with average reproductive boldness to three pairs of females. Social boldness is represented by PCA axis 1 values, which are positively correlated with average behavioral frequency and intensity, and negatively correlated with average latency to display, within each behavioral encounter scenario. Trials were carried out in three blocks. One to two focal males with the highest combined PCA values per block ('bold', shown in blue-green), and one to two focal males with the lowest combined PCA values per block ('shy', shown in orange) were selected for the bold-shy neural RNA sequencing comparison.

*Boldness is associated with gene upregulation in the ventromedial hypothalamus*

We measured baseline gene expression in bold and shy individuals across six brain regions that contribute to social decision-making and are functionally conserved across vertebrates (Kabelik et al., 2018; Newman, 1999; O'Connell and Hofmann, 2011, 2012;

Thompson et al., 2008; Walton et al., 2010) (**Figure 2A**; Supplemental Excel File). The number of differentially expressed genes across brain regions were relatively few (average of 8), with the exception of the ventromedial hypothalamus, where 608 genes were differentially expressed. Across brain regions, only integrin alpha-10 (*itga10*) was consistently downregulated in bold individuals compared to shy individuals (**Figure 2B**), with the exception of the dorsal ventricular ridge where expression of this gene was not detected.

Since the ventromedial hypothalamus had a drastically different patterns in baseline gene expression between bold and shy individuals, we explored these patterns in more detail. There were 601 genes upregulated and 7 downregulated in bold individuals compared to shy individuals (**Figure 2C**). We examined gene otology annotations for differentially expressed genes and found enriched molecular function of calcium channel activity ($p=5.04x10^{-4}$). Indeed, at least seven voltage-gated calcium channel genes were upregulated in bold individuals (**Figure 2D**). While many differentially expressed genes are unannotated and labeled as “novel transcripts”, we noted several that have established roles in regulating behavior or have a log fold change of greater than 5. This includes the androgen receptor (*ar*, p=0.014) and the two subunits of the NMDA receptor, *grin1a* (p<0.001) and *grin 2b* (p<0.001). Expression of integrin alpha-10 was downregulated in bold individuals (p=0.017), similar to other brain regions. Finally, some genes had a large fold change increased in bold individuals, including a potassium channel (*kcnh1-like*, p>0.001), the secretin receptor (*sctr*, p=0.02), and an unknown g protein-coupled receptor (p=0.003).

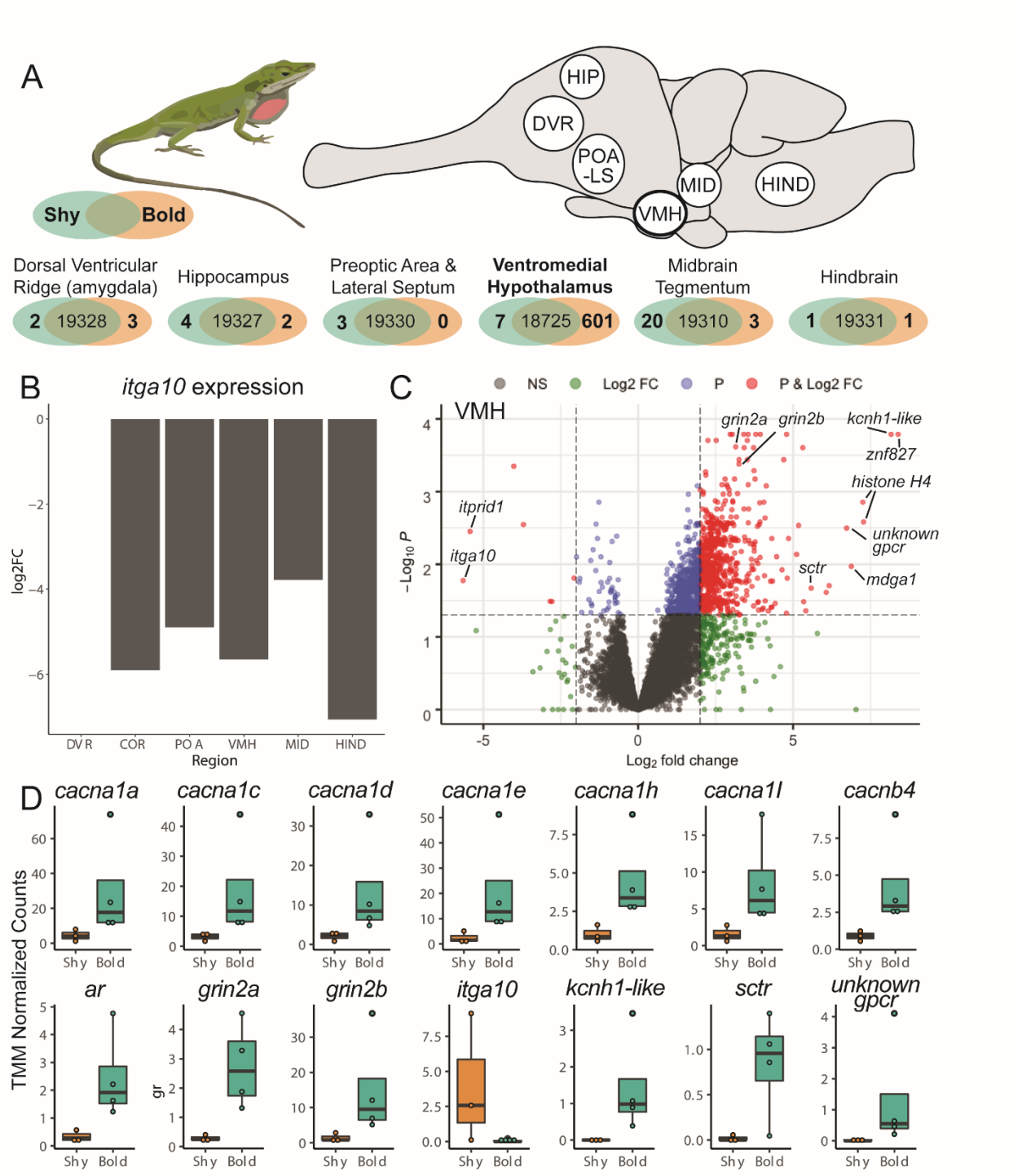


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**Figure 2. Baseline brain gene expression in bold and shy anoles highlights the ventromedial hypothalamus. (A)** RNA sequencing was used to quantify gene expression in six different brain regions in bold (orange) and shy (blue-green) individuals**.** The number of differentially expressed genes in each brain region is shown in the Venn diagrams. **(B)** Integrin alpha-10 (*itga10*) was downregulated in bold anoles across almost all brain regions. The log fold change (log2FC) is shown in bar plots. **(C)** A volcano plot highlights the genes that are differentially expressed in the ventromedial hypothalamus, where the cutoff for significance (red dots beyond the dashed lines) is $p<0.05$ with false discovery rate correction and a log fold change of two or greater. **(D)** Select differentially expressed genes are shown, including voltage-gated calcium channels (top) and other genes previous linked to behavior and/or with large fold changes in expression (bottom). Boxplots show rectangles as the lower and upper quartiles (with the median as the line) and whiskers that indicate the maximum and minimum values; individual data points are shown as dots.

## Discussion

Most behaviorally linked gene expression studies examine changes resulting from participation in behavioral trials or the establishment of dominant or subordinate status within a social hierarchy. However, such comparisons make it difficult to ascertain what variables are predisposing animals to exhibit a bold or shy phenotype in the first place, as such studies will also detect gene transcription differences that result from the perception of conspecifics and performance of varied levels of behaviors toward other individuals. Hence, to remove perception-related and performance-based gene expression, and thus focus on neural differences that predispose individuals toward bold or shy behavioral profiles, we examined male green anoles under baseline conditions. These anoles had been previously screened under three different social conditions, and repeatedly within each condition. We found highly stable boldness phenotypes, which transcended social context. That is, in relation to shy males, bold males tended to behave more quickly and to exhibit greater numbers and higher intensities of courtship behaviors within reproductive trials, and of aggressive behaviors within agonistic trials. This correlation of social boldness across contexts is in line with expectations for consistent behavioral phenotypes (Coppens et al., 2010; Stamps and Groothuis, 2010). Thus, our gene expression analysis investigates neural correlates of a general social boldness.

*Social boldness phenotype is unrelated to circulating steroid hormone levels*

While there were many behavioral differences between bold and shy individuals in this study, we did not find any group differences in circulating hormone levels or body size. We expected testosterone, estradiol, progesterone, or glucocorticoid levels to correlate with individual differences in behavior. Previous findings suggesting steroidal regulation of behavioral expression include research on male tree lizards, which found correlations between circulating testosterone levels and aggressive behavior display frequency and intensity (Kabelik et al., 2006). Manipulative studies in the same species demonstrated that both testosterone and progesterone promote faster, more frequent, and more intense displays of aggression (Kabelik et al., 2008b; Weiss and Moore, 2004). Similarly, in male side-blotched lizards, circulating testosterone levels are higher in the more aggressive morph (Sinervo et al., 2000). However, while we cannot rule out organizational effects of steroid hormones on social behavior phenotype in male green anoles, we find no evidence for activational effects of any of these steroid hormones on boldness phenotype.

Studies examining other vertebrate taxa have found mixed associations between circulating steroid hormone levels and behavioral boldness. For example, testosterone treatment in African striped mice (*Rhabdomys pumilio*) increases boldness behavior (Raynaud and Schradin, 2014), and acute 17a-ethinylestradiol (an estrogen mimic) decreases boldness behavior in Siamese fighting fish (*Betta splendens*). However, aromatase inhibitors decrease boldness in female Siamese fighting fish, highlighting a role for local hormone synthesis within the brain that may regulate behavioral phenotypes. As aromatase was not differentially expressed between bold and shy males within this study, functional manipulations would be required to determine a causal role for steroid hormone synthesis in the brain and any relationships with individual variation in behavior.

Studies attempting to link hormones to behavioral phenotypes often measure hormone levels after a behavioral stimulus is presented, but our study focused instead on baseline levels of hormones. Similar to our finding in green anoles, bold and shy male zebrafish do not differ in baseline cortisol levels (Oswald et al., 2012), suggesting that baseline hormone levels may be unlinked to behavioral phenotypes. Instead, hormones may influence behavioral syndromes through differential abundance of their receptors within the brain.

*Differentially expressed genes and a regulatory role for the ventromedial hypothalamus*

When comparing forebrain, midbrain, and hindbrain regions of bold versus shy male green anole lizards, we found the greatest number of differentially expressed genes within the ventromedial hypothalamus, a node within the social decision-making neural network (O'Connell and Hofmann, 2011). We were initially surprised to find so few genes differentially expressed within other brain regions, but the patterns here represent a constitutive state, rather than a response to a behavioral stimulus that would be expected to induce a greater change in gene expression across brain regions. The ventromedial hypothalamus has been implicated in the regulation of both reproductive and agonistic behaviors, making it a logical location for regulation of general social behavior boldness. For instance, both copulatory and agonistic conditions have been shown to upregulate markers of neural activity within the Syrian hamster ventromedial hypothalamus, although copulation tends to induce more c-Fos expression (an immediate early gene product and proxy marker of neural activity) in the medial portions of the nucleus, while agonistic situations tend to increase c-Fos in the lateral ventromedial hypothalamus (Kollack-Walker and Newman, 1995). Additionally, stimulation of the lateral portions of the rat ventromedial hypothalamus has been shown to elicit aggressive responses (Kruk, 1991). The ventromedial hypothalamus therefore seems a likely social behavior integration center, owing to its regulatory role in various types of social behavior expression.

A prominent category of differentially expressed genes within the ventromedial hypothalamus was voltage-gated calcium channels. Voltage-gated calcium channels regulate both intracellular calcium levels as well as general neuronal excitability, and have been linked to a number of neuropsychiatric symptoms in humans, including bipolar disorder, depression, and attention deficit hyperactivity disorder (Kabir et al., 2017). In addition to voltage-gated calcium channels, we also found that expression of ligand-gated calcium channel NMDA subunits is upregulated in bold individuals. NMDA receptor regulation has been linked to behavioral boldness in birds (Audet et al., 2018), suggesting the tuning of calcium channel expression may be a conserved feature of behavioral variability. Indeed, increased intracellular calcium levels induce signaling cascades that can lead to changes in transcription, such as the phosphorylation of cyclic adenosine monophosphate response element binding protein (CREB), which has been linked to synaptic, neuronal, and behavioral plasticity (Hofmann, 2003). In tree lizards, the dorsolateral portions of the ventromedial hypothalamus show increased neural activity as measured by an increase in pCREB following an agonistic encounter (Kabelik et al., 2008a). Thus, a number of neural activity-regulating channels differ in baseline gene expression between bold and shy males, highlighting neuronal excitability in the ventromedial hypothalamus as a contributor to stable individual variation in behavior.

In the ventromedial hypothalamus, we also observed increased gene expression of a few neuromodulators with strong ties to behavior. For example, although we did not find significant differences in testosterone levels between bold and shy individuals, we did find an increase in androgen receptor expression in bold individuals. This finding is exciting given that androgen receptor presence within the ventromedial hypothalamus occurs within its dorsolateral aspect (Rosen et al., 2002), the same region of the ventromedial hypothalamus that has been previously linked to the expression of aggressive behavior in male tree lizards (Kabelik et al., 2008a). Apart from androgen receptor gene expression, we also found an increase in

399 expression of the secretin receptor in bold individuals. Secretin receptor expression is
400 widespread in the brain and although central secretin signaling is primarily known for its role in
401 regulating neurodevelopment and memory function via effects on synaptic plasticity, secretin
402 has also been shown to regulate anxiety and associated behaviors (Nishijima et al., 2006; Wang
403 et al., 2019), as well as activity of reproductive circuitry (Csillag et al., 2019). Secretin has been
404 shown to regulate GABAergic transmission to gonadotropin-releasing hormone-producing cells
405 (Csillag et al., 2019), as well as affecting the firing rate of over 50% of examined paraventricular
406 hypothalamus neurons in rat *in vivo* studies (Chen et al., 2013). These studies suggest a role for
407 secretin as a widespread modulator of neural function, and as such, secretin may also regulate
408 social behavior via modulation of neuronal activity within the ventromedial hypothalamus.

409 Across most brain regions, integrin alpha-10 was the one gene consistently
410 downregulated in bold individuals. Integrins are typically associated with neuronal development,
411 as they can detect and transmit mechanical force on extracellular matrices into an intracellular
412 signal (Takada et al., 2007). The role of integrin alpha-10 is not well understood, especially in
413 the context of behavior. Integrin alpha-10 in humans is associated with the 1q21.1 chromosomal
414 region, which when deleted leads to thrombocytopenia absent radius (TAR) (Brunetti-Pierri et
415 al., 2008). Genome wide association studies in humans has also linked integrin alpha-10 to
416 bipolar disorder (Pedroso et al., 2012). Thus, a role for integrin alpha-10 in natural variation in
417 behavioral strategies beyond that of human disease is a promising avenue of future research.

418

419 **Conclusion**

420 Our study focuses on constitutive differences across bold or shy individuals by isolating
421 baseline hormone levels and brain gene expression profiles that influence social boldness
422 predisposition, rather than those reflecting the results of social interaction and behavioral

423 execution. We found that correlated behavioral traits were not driven by differences in steroid
424 hormone levels or body size, as these were consistent across treatment groups. Instead, brain
425 gene expression differences strongly relate to social boldness and likely reflect variables
426 involved in the neural circuitry that regulates social boldness. Specifically, we found that
427 baseline differences between bold and shy males were associated with gene expression in the
428 ventromedial hypothalamus, where expression of voltage-gated calcium channels, the androgen
429 receptor, and the secretin receptor were increased in bold individuals. We suggest that studies
430 should include examination of the ventromedial hypothalamus as a potential regulator of social
431 behavior boldness in reptiles as well as across other vertebrate taxa.

432

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436

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441

442 **Declaration of competing interest**

443 The authors have no competing interest to declare.

444

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