5-11-2015

Arginine Vasotocin and Social Behavior: Endocrine Effects and Reciprocal Interactions in Anolis carolinensis

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Arginine vasotocin (AVT; or its mammalian homologue arginine vasopressin) is a potent regulator of social behavior in many species. Examining its behavioral and endocrinological effects in different species can provide insight into its general mechanisms in regulating social behavior and physiology. These experiments were aimed at gaining insights into the role of AVT and its interactions with other steroid hormones in the regulation of social behavior including aggression and courtship as well as the effect of aggressive behavior itself on AVT expressing cells in the brains, using the green anole lizard as the research subject. Little is known about the role of AVT in reptilian behavior. First, the effect of exogenous AVT on social behavior (mirror and paired aggression, courtship) and circulating steroids was assessed. I found that AVT increased corticosterone (CORT) in all animals and tended to reduce aggressive behavior as has been reported for other
territorial species. AVT did not perceptibly alter male courtship but did increase the display behavior of untreated females paired with treated males. Next, endocrine and behavioral effects of different AVT doses were considered as well as the impact of AVT on steroid hormones in single housed animal. I found that high doses of AVT increased CORT more in aggressing than courting animals although no effects on behavior were observed. I also found that AVT stimulates CORT release in animals without a behavior challenge. Then, I examined the influence of AVT on aggressive behavior in animals with a CORT-synthesis inhibitor to establish a non-stress hormone dependent role for AVT. AVT tended to reduce aggressive behavior even in the presence of a CORT-synthesis inhibitor. Next, AVT immunoreactive cell counts were compared in animals with varying numbers of exposures to an aggression inducing versus neutral stimulus. I found that AVT immunoreactive cell number in the preoptic area (POA) increased in animals with five aggressive exposures. Findings from this dissertation suggest a possible role for AVT in the regulation of aggressive behavior and highlight the importance of considering the glucocorticoid response when considering AVT effects.

INDEX WORDS: Aggression, Anolis carolinensis, Arginine vasotocin, Corticosterone, Courtship, Neuroendocrinology, Social behavior, Testosterone
ARGININE VASOTOCIN AND SOCIAL BEHAVIOR: ENDOCRINE EFFECTS AND RECIPROCAL INTERACTIONS IN *ANOLIS CAROLINENSIS*

by

LESLIE A. DUNHAM

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2015
DEDICATION

To Deb who showed me how...

To David who always listens...

To Bryce who inspires me to be awesome...

To my brother who fearlessly lives for his dreams...

And to my mom and dad – whose love and confidence

and incredible patience have made every moment of it possible.
ACKNOWLEDGEMENTS

The number of people who deserve acknowledgement for their impact on my education and science and life over the past years is truly staggering and humbling. First and foremost, this dissertation would not have been possible without the mentorship of Walt Wilczynski whose openness to all ideas and encouragement of exploration in the lab has been a liberating experience. Walt's perspective on the necessity for a healthy work/life balance is refreshing and inspiring and I believe it is what has made him such a success in his career. While I am quite certain he did not know what he was getting into when he took me on, he has always accepted me, quirks and all, and for that, I could never begin to thank him enough. I would also like to thank my committee members: Laura Carruth, Kim Huhman, and Geert de Vries, for putting things into perspective and helping me to see the big picture. Thank you for your time and patience.

I would be incredibly remiss in forgetting to thank Bill Hopkins who deserves the credit for helping me to find GSU and Walt. His lab was my first experience with research and the summers I spent working with him at Yerkes remain some of the greatest and most fulfilling of my life. I would never have pursued a PhD without his encouragement.

Thank you also to the NI support staff and faculty as well as the DAR team who have made the last years such a joy. Thank you especially to Liz Weaver for your work with the B&B Fellowship, it has been an honor to be a part of it, and to Mary Karom for all of the talks and technical advice and for having the scintillation counter repaired for me on multiple occasions.
I must acknowledge the members of the Wilczynski lab, past and present with whom I have had the pleasure of working and learning from over the years, including Lynn Almli, Michael Black, Carmen Canete, Anna Creighton, Chisom Ezeoke, Megan Gall, Scarlet Salem, and Deep Shukla. I also need to thank the awesome group of undergrads I have been fortunate enough to mentor including Krystal Archer, Javonte Carter, Hillary Doyle, Anandhi Martin, Skylar McHenry, Reagan Norris, Michael Sabula, and Sheena Palmer. They all suffered through hours and hours of lizard behavior scoring and the data presented here would not have been possible without them. David and Deb, I did not forget you! You both have been more than lab mates to me, you have been my lifeline and my sounding board and truly incredible friends. To David, thank you for always listening and for dropping everything to help me with the crisis of the day. To Deb... I cannot even really begin - I could write this dissertation’s worth and more about what your friendship and mentorship has meant to me. I am so proud of the success you have achieved in your own career and my life would truly not be the same had our paths not crossed.

Thank you to all of the other wonderful friends I have made at GSU who have provided the much-needed reprieve when “science is broken” - especially Kate, Elizabeth, Lori, Mac, Alex, and Christy. A special thanks must go to Bryce Chung, my go to coffee date, northbound chauffer, and general all around incredible guy. You inspire me every single day. To my non-GSU family including the Berry girls and book club ladies, Team Nasty, and my Jazzercise girls - you have kept me going the whole way through, thank you!

Finally, to my mom, dad, and brother - knowing your support was just a phone call away made this possible. I finally made it; I hope I have made you proud.
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LIST OF ABBREVIATIONS

III  Third ventricle
AA  Amygdala area
ACTH  Adrenocorticotropic Hormone
ANOVA  Analysis of variance
AVP  Arginine vasopressin
AVT  Arginine vasotocin
BNST  Bed nucleus of the stria terminalis
CORT  Corticosterone
CRF  Corticotropin Releasing Factor
CPM  Counts per minute
DCTX  Dorsal cortex
DM  Dorsomedial nucleus
ELISA  Enzyme-linked immunosorbent assay
hAVT  High-AVT dose
HPA  Hypothalamic-pituitary-adrenal
IHC  Immunohistochemistry
IP  Intraperitoneal
IR  Immunoreactive
lAVT  Low-AVT dose
LCTX  Lateral Cortex
LFB  Lateral forebrain bundle
LHA  Lateral hypothalamus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>LV</td>
<td>Lateral ventricle</td>
</tr>
<tr>
<td>mAVT</td>
<td>Mid-AVT dose</td>
</tr>
<tr>
<td>MCTX</td>
<td>Medial cortex</td>
</tr>
<tr>
<td>MeA</td>
<td>Medial amygdala</td>
</tr>
<tr>
<td>MET</td>
<td>Metyrapone</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>OC</td>
<td>Optic chiasm</td>
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<tr>
<td>POA</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>PPMC</td>
<td>Pearson-product moment correlation</td>
</tr>
<tr>
<td>PPN</td>
<td>Periventricular preoptic nucleus</td>
</tr>
<tr>
<td>PVN/AH</td>
<td>Paraventricular nucleus/Anterior hypothalamus</td>
</tr>
<tr>
<td>SC</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>V1a</td>
<td>Vasotocin 1a receptor</td>
</tr>
<tr>
<td>V1b</td>
<td>Vasotocin 1b receptor</td>
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<tr>
<td>VEH</td>
<td>Vehicle</td>
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1 INTRODUCTION

An individual’s ability to respond adaptively to ever changing environmental and social conditions is critical for survival as these responses can secure access to prime territories where food and mates are abundant and thus are important for survival and fitness. When a perceived threat or mating opportunity is encountered, the animal’s capacity to evaluate the context of the interaction and to determine the appropriate behavioral response is critical and likely results from the interactions of many internal and external signals (Crews, 1987; Wingfield et al., 1998). An important challenge in behavioral neuroscience is to understand the contributions of anatomy, physiology, and experience on the expression of social behavior. The interplay of these factors likely contributes to the wealth of behavioral diversity that is observed both between and within species and across the life span. The goal of this dissertation is to consider the relationship among arginine vasotocin (AVT; a regulator of social behavior in many species), social behavior, and steroid hormones in green anole lizards. In addition, the role of AVT in regulating social behavior, and the role of social behavior experience in changing the AVT system will also be considered.

1.1 Neuroendocrinology of Social Behavior

1.1.1 Arginine vasotocin

Arginine vasotocin and its mammalian homologue arginine vasopressin (AVP) are neurohypophysial hormones produced primarily by cells located in the supraoptic and paraventricular nuclei (SON and PVN, respectively) of the hypothalamus. Peripherally,
AVT and AVP are released from the posterior pituitary and act on both the kidneys and blood vessels to regulate water balance and blood pressure (McCann et al., 2002). Central release of AVT and AVP from parvocellular neurons located within several regions of the brain including the PVN, bed nucleus of the stria terminalis (BNST), medial amygdala (MeA), and suprachiasmatic nucleus (SCN) potently affects social behavior (including aspects of courtship and aggression) in virtually every species examined thus far (for reviews, see: Albers, 2012; Balment et al., 2006; Boyd, 2012; Caldwell et al., 2008; Goodson and Bass, 2001; Rose and Moore, 2002). While there is a wealth of data describing the stimulatory effects of AVP on mammalian affiliative and paternal behaviors, I will focus primarily on the role of AVT in modulating courtship and aggressive display behaviors in non-mammalian vertebrates for the purposes of this dissertation.

1.1.1.1 Aggression

The effects of AVT on aggressive behavior can be highly variable and context dependent. Research in a variety of species has demonstrated the importance of the social spacing of a species (e.g. territorial vs. gregarious [Goodson, 1998a; Goodson and Adkins-Regan, 1999; Goodson and Evans, 2004; Lema and Nevitt, 2004]), the behavioral phenotype of the individual (e.g. territorial vs. non-territorial morphs [Semsar et al., 2001]; dominant vs. subordinate [Goodson et al., 2009a]), and the specific context of the interaction (e.g. sexually naïve vs. experienced [Winslow et al., 1993]; territory defense vs. mate competition [Goodson et al., 2009a]) in influencing how AVT modulates aggressive behavior. For example, AVT increases aggression in socially gregarious zebra finches (Goodson and Adkins-Regan, 1999) while the opposite effect is observed when AVT is given to typically territorial species - the field sparrow and violet eared waxbill (Goodson,
Even within a species, AVT can have different effects on aggressive behavior depending on the behavior phenotype or morph of each individual. For instance, AVT increases aggression in non-territorial morphs of bluehead wrasse but significantly reduces it in their territorial counterparts (Semsar et al., 2001). These data indicate that the relationship between social behavior and AVT can be highly complex; to date, the role of AVT in regulating the expression of behavior in reptiles is not well known (Woolley et al., 2004).

1.1.1.2 Courtship

AVT stimulates reproduction-associated and courtship display behaviors in a variety of non-mammalian vertebrates. Early studies in the roughskin newt demonstrated that injection with AVT increases male clasping behavior while infusions of AVP antagonists and anti-AVT serum reduce courtship (Moore and Miller, 1983; Moore and Zoeller, 1979). Courtship display behaviors such as advertisement calling in frogs (Boyd, 1994; Burmeister et al., 2001; Kime et al., 2007; Marler et al., 1995; Propper and Dixon, 1997; Ten Eyck, 2005; Tito et al., 1999) and attending behavior in fish (Salek et al., 2002; Santangelo and Bass, 2010; Semsar et al., 2001) are also affected. In zebra finches, exogenous AVT fails to significantly increase directed song (Goodson and Adkins-Regan, 1999; Goodson et al., 2004), however the number of AVT-immunoreactive (AVT-ir) cells in the BNST correlates positively with the number of courtship displays performed (Goodson et al., 2009b). In addition, positive affiliative interactions have been shown to increase Fos activity in BNST AVT/AVP populations in chickens and mice (Ho et al., 2010; Xie et al., 2011). Similar evidence has emerged in the brown anole lizard in which the number of “social engagement behaviors” toward a female was positively correlated with co-localization of AVT peptide
and Fos expression in several behaviorally significant brain regions (POA, BNST, and PVN) suggesting an important relationship between courtship and activation of the AVT system (Kabelik et al., 2013).

1.1.2 Testosterone

Testosterone is a steroid hormone derived from cholesterol and produced in the gonads of both males and females. Production is stimulated based on signals by the hypothalamus which trigger the release of the luteinizing hormone from the pituitary gland as part of the hypothalamic-pituitary-gonadal axis and negative feedback from the gonadal steroids themselves helps to maintain appropriate physiological levels (for review, see: Nelson et al., 1990). Sex, season, and body condition are important for determining levels in many species. Additionally, testosterone can be converted into estrogen by the enzyme aromatase which is found both centrally (for review, see Lephart, 1996) and peripherally, and this conversion can have important implications for social behavior (see Trainor et al., 2006; Wingfield et al., 2001). In general, T is considered an important hormone in the regulation and expression of social behavior.

1.1.2.1 Aggression

Testosterone effects on aggression have been examined in many species, including green anole lizards (Adkins and Schlesinger, 1979; Giammanco et al., 2005; Soma, 2006; Wingfield et al., 1987). Offensive or territorial aggression is reduced in castrated animals (Adkins and Schlesinger, 1979; Albert et al., 1986) and aggression can be rescued by treatment with exogenous T. This is not always the case as was observed in castrated prairie voles where aggression levels were comparable to intact animals (Demas et al.,
In green anoles, castration decreases territorial aggression and it can be rescued with testosterone replacement (Adkins and Schlesinger, 1979; Crews et al., 1978). However, the reduction of aggression with castration appears to have a contextual component as this effect is most robustly observed not when animals are in their home cage or territory, but when they are placed into an unfamiliar cage (Crews, 1974).

1.1.2.2 Courtship

In some species, the expression of normal courtship behavior appears to be testosterone dependent (Beach and Inman, 1965; Burmeister and Wilczynski, 2001; Schmidt, 1966; Wada, 1981). However, this is not necessarily true in species which display a dissociated reproduction pattern (for review, see Crews, 1984) where castration at any time may have little effect on the expression of courtship behavior (Crews et al., 1984). The site of action for testosterone’s effect on courtship behavior appears to be localized strongly to the anterior hypothalamus. Administration of T into the anterior hypothalamus of castrated birds elicits courtship behavior (Adkins-Regan, 1981; Barfield, 1971; for reviews, see: Ball et al., 2004; Balthazart and Ball, 2007; Fusani, 2008).

1.1.2.3 Effects on AVT/AVP

There are many examples of androgen effects on AVT/AVP in the literature. First, major sex differences have been observed in a variety of species (for reviews: Albers, 2014; De Vries, 2008; De Vries and Panzica, 2006) where males have both more cells and more dense AVT/AVP-immunoreactive (-ir) fibers. Further, it has been demonstrated that castration significantly reduces AVT/AVP-ir in specific regions such as the bed nucleus of the stria terminalis and the lateral septum (De Vries et al., 1985; De Vries and Panzica,
2006) and receptors may also be affected (Grozhik et al., 2014; Young et al., 2000). Natural declines in testosterone as observed in seasonally reproductive species are also associated with declines in AVT/AVP-ir in some steroid sensitive regions (Buijs et al., 1986; Rasri et al., 2008) and these changes seem to be testosterone dependent.

1.1.3 Corticosterone

Corticosterone (cortisol in primates) is the primary glucocorticoid produced by reptiles, birds, amphibians, and rodents in response to stress. It is secreted primarily from the adrenal glands as a result of the activation the hypothalamic-pituitary-adrenal (HPA) axis that consists of a cascade of chemical signals beginning with the release of corticotropin releasing hormone (CRH) from the hypothalamus immediately after encountering a stressor. CRF then induces the release of adrenocorticotropic hormone (ACTH) from the pituitary gland that stimulates CORT synthesis and release (see section 1.2 for more details on the HPA axis). Corticosterone is derived from cholesterol and is converted from 11-deoxycorticosterone by the steroidogenic enzyme 11-β hydroxylase (Bury and Sturm, 2007). Evidence suggests that this enzymatic machinery is present in brain tissues such as the hippocampus, opening the possibility of central synthesis and local release (Higo et al., 2011; Mellon and Deschepper, 1993). The primary goal of glucocorticoid release is to allow an organism to adapt to a stressor, e.g. environmental or social, and promote a return to homeostasis. Due to its promotion of homeostasis and energy conservation, corticosterone can have a potent impact on social behavior (both aggressive and courtship).
1.1.3.1 Aggression

In some species, increased corticosterone has been associated with reduced aggressive behavior, presumably an evolutionary tactic meant to conserve energy resources and avoid injury. However, the evidence supporting this is variable (for review, see: Summers et al., 2005b). For example, studies in several non-mammalian vertebrates have found that treatment with corticosterone implants resulted in a decrease in the frequency and/or intensity of aggressive displays (Fish: Øverli et al., 2002; Lizards: DeNardo and Licht, 1993; Tokarz, 1987), however in animals exposed to an aggression inducing stimulus without hormonal manipulation, no correlations were observed between circulating corticosterone levels and the number of aggressive display behaviors in green anole lizards (Yang and Wilczynski, 2003). In rats and mice, treatment with CORT just 2 -20 minutes prior to an aggressive encounter has been shown to reduce attack latency and increase aggressive behavior (Brain et al., 1971; Haller et al., 1997; Mikics et al., 2004; Poole and Brain, 1973).

1.1.3.2 Courtship

Elevated corticosterone can also lead to disruptions in normal reproductive functioning. In the roughskin newt, treatment with either corticosterone or CRH reduced clasping behavior in a dose dependent manner and pretreatment with metyrapone, a CORT synthesis inhibiter, rescued courtship behavior (Moore and Miller, 1984). In two species of toads, CORT treatment inhibited calling and elicited a change to “satellite” or non-call based mating tactics (Leary et al., 2006) although CORT did not change calling behavior in the green tree frog (Burmeister et al., 2001).
1.1.3.3 Interactions with AVT/AVP

It is well established that AVT can promote the release of corticosterone from the adrenals by further stimulating the HPA axis resulting in increased circulating ACTH (Gibbs, 1986; see 1.2 below for more details). There is, however, evidence suggesting that the overall behavioral actions of AVT/AVP and CORT may be in opposition. For example, AVT stimulates courtship behavior while CORT tends to suppress it, and priming the system with one can block the effects of the other (Burmeister et al., 2001; Coddington and Moore, 2003). There are physiologically important interactions between AVT/AVP and steroid hormones and these interactions are likely to be critical influences on the expression of social behavior.

1.2 Hypothalamic-Pituitary-Adrenal Axis

When a perceived stressor is encountered, e.g. an intruding male, the HPA axis is activated. The primary goal of this system is to stimulate a coping response to the perturbation and subsequently to promote a return to a homeostatic condition after a threat has passed (McEwen, 2007). Corticotropin releasing hormone (CRH) released from the hypothalamus into the median eminence portal vein is a primary hormone in HPA axis responsivity. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland that is responsible for initiating the synthesis and release of glucocorticoids (e.g. corticosterone) from the adrenal cortex in response to the stressor. In addition to CRH, arginine vasopressin, or its non-mammalian analogue arginine vasotocin, also has the ability to influence this HPA activation and response in potentially complex ways. Release of AVP from parvocellular neurons in the paraventricular nucleus (PVN) and magnocellular neurons in the supraoptic nucleus (SON) leads to increases in
ACTH secretion (Antoni, 1993) and it is estimated that at least half of the CRF-positive neurons in the PVN co-express AVP in rats and humans (Mouri et al., 1993; Whitnall, 1987). In humans, AVP treatment has been shown to increase ACTH release (Salata et al., 1988) and rats treated with an AVP antagonist show a blunted ACTH response after 20 minutes of ether stress (Rivier and Vale, 1983). Overall, the body of evidence suggests that CRH and AVP/AVT work synergistically and that both are necessary to achieve maximum activation of the HPA circuit (AVP: DeBold et al., 1984; Dinan and Scott, 2005; Favrod-Coune et al., 1993; Gillies et al., 1982; AVT: Baker et al., 1996) and that AVP/AVT may be especially important in regulating the adaptation of the HPA axis to chronic stress (Aguilera et al., 2008).

1.3 Social Behavior Neural Network

The social behavior neural network comprises a number of brain regions that are functionally connected and implicated in the control of a variety of behaviors including courtship and affiliation, chemical and vocal communication, and aggression (Albers, 2012; Albers, 2014; Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2012). Mounting evidence from studies representing a breadth of species demonstrate overlapping activity among populations of neurons in the amygdala, lateral septum, preoptic area, and various hypothalamic nuclei, leading to the idea that the pattern of activity across multiple nuclei in this network contributes to variability in social behavior and behavioral responses (Newman, 1999).

In order to be considered for inclusion as a “node” in the social behavior neural network, a population of neurons must 1) be sensitive to gonadal hormones (specifically express steroid hormone receptors although this criteria for inclusion may not be
necessary; Albers, 2014), 2) be implicated in the control of multiple social behaviors, and 3) be reciprocally connected with other regions of the social behavior network (Albers, 2012; Albers, 2014; Newman, 1999). Neuropeptides present within the various nodes of the social behavior neural network are likely important for coordinating the activity across nodes. AVT/AVP is an excellent example of a neuropeptide present throughout this network that, given its impact on a variety of social behaviors, likely contributes to the functionality and connectivity of the social behavior neural network.

### 1.3.1 Arginine vasotocin and vasopressin in the social behavior neural network

The distribution of AVT/AVP cells has been described in many species (For reviews, see: Caldwell et al., 2008; Goodson and Bass, 2001) and both the cell bodies and receptors have been observed throughout the social behavior neural network in both mammals and non-mammalian vertebrates. Differences in both cell and receptor density and distribution by species (Acharjee et al., 2004; Bester-Meredith et al., 1999; Insel et al., 1994; Leung et al., 2011; Phelps and Young, 2003; Young et al., 1997), sex (De Vries et al., 1984a; De Vries and Panzica, 2006), gonadal hormone state (Boyd, 1997; Buijs et al., 1986; De Vries et al., 1985; De Vries et al., 1984b; De Vries et al., 1986; Kabelik et al., 2008; Miller et al., 1992; Wang and De Vries, 1993), and experience (Delville et al., 1998; Hattori and Wilczynski, 2009; Larson et al., 2006; Qiao et al., 2014) have been described, highlighting the importance of the AVT/AVP system in contributing to the activity of the social behavior neural network and further, social behavior diversity and variability.

The make-up of the AVT system likely contributes to the observed variability in behavioral effects. Differences in the number and type of AVT and AVP cells, the density of their receptors, as well as expression of AVT and AVP peptide mRNA between species
and/or individual behavior phenotypes have been observed. In several species, chronically subjugated or subordinate animals have fewer AVT/AVP containing cells (Delville et al., 1998; Hattori and Wilczynski, 2009; Qiao et al., 2014) and different types of ir-positive cells (gigantocellular versus parvocellular) within the preoptic area have been associated with increased and decreased aggression respectively (Dewan et al., 2008; Dewan and Tricas, 2011).

In green anoles, chronic maintenance of a subordinate social status (10 day pairing) resulted in a decrease in the number of AVT-ir cells in the POA as compared to both dominant and single housed males (no-status; Hattori and Wilczynski, 2009). Similar results have been observed in mandarin voles where the density of AVP-ir cells was significantly lower in the paraventricular nucleus, supraoptic nucleus, lateral hypothalamus, and anterior hypothalamus (Qiao et al., 2014) suggesting that cell number may be sensitive to social experience.

Comparisons between closely related species with different social spacing have revealed that regional distribution of the V1a receptor may contribute to behavior diversity (Bester-Meredith et al., 1999; Young et al., 1997), and social experience can alter V1a receptor density (Albers et al., 2006). In addition to the contributions of V1a to behavior, mice lacking the V1b show reduced aggression (Caldwell et al., 2010; Wersinger et al., 2002). Although the distribution of AVT-ir cells has been described for reptiles (Bons, 1983; Propper et al., 1992; Stoll and Voorn, 1985; Thepen et al., 1987), little is known about the receptor distribution or contributions of these receptors to behavior.
1.4 Model System

Green anole lizards have long been recognized as a powerful model system in which to ask questions about aggression and the mechanisms underlying territorial and other social interactions (Greenberg, 1977; Greenberg et al., 1984; Greenberg and Crews, 1990a; Greenberg and Noble, 1944; Lovern et al., 2004; Lovern and Jenssen, 2003; Summers et al., 2005a). The stereotyped behaviors associated with aggression and courtship have been well documented and defined (Crews, 1975; DeCourcy and Jenssen, 1994; Greenberg, 1977; Husak et al., 2007; Jenssen et al., 2012; Orrell and Jenssen, 2003; Wilczynski et al., 2015). In addition, the ability to stimulate aggressive displays by a variety of means (video display, mirror presentation, live encounter; Baxter Jr et al., 2001; Farrell and Wilczynski, 2006; Macedonia et al., 1994; Yang et al., 2001; Yang and Wilczynski, 2003) allows for nuanced questions of the impact of not only social status on the animal's physiology (through paired aggression with a live conspecific) but also the impact of behavior itself where no status is achieved (mirror aggression).

In addition, the green anole's reliance on primarily visual social signals represents a unique mode in the AVT/AVP literature. The majority of studies examining the effect of AVT/AVP on social behavior focus on species using primarily olfactory (newts and rodents) or auditory (birds and frogs) communication. To understand how AVT might affect behavior in a species with little evidence of olfactory or auditory signaling will ultimately increase our understanding of how these peptides have evolved across many species and social communication modalities.
1.5 Chapter Overviews

The goal of this dissertation is to examine the relationship among AVT, social behavior, and hormones, including the role of AVT in regulating social behavior, and the role of social behavior experience in changing the AVT system. To date, little is known about the effect of AVT on social behavior in any reptile. Further, the contribution of aggressive behavior independent of social status to the AVT system in the social behavior neural network is not clear. Through the experiments described below, I will explore these questions.

1.5.1 Chapter 2

AVT/AVP is a potent regulator of social behavior in many species. It is known to stimulate calling behaviors in frogs as well as attending and courtship behavior in newts and fish (for reviews, see: Balment et al., 2006; Boyd, 2012; Goodson and Bass, 2001; Rose and Moore, 2002). Evidence also suggests that AVT may influence aggressive behavior in a complex way, relating to the social structure of the species the behavioral phenotype of an individual, and the context of the social situation (Kabelik et al., 2009). In general, the evidence suggests that AVT tends to stimulate aggression in typically non-territorial individual/species while suppressing it in their territorial counterparts. The role of AVT in reptile behavior has not been examined to date. This experiment aims to assess the influence of AVT on mirror and paired aggression as well as courtship behavior in green anole lizards. Effects of steroid hormones on AVT are also considered as a possible mechanism through which behavioral effects may occur.
1.5.2 Chapter 3

In green anole lizards, individuals treated with 3 µg/g body weight perform significantly fewer aggressive display bouts when exposed to a mirror while courtship behavior is not affected at this dose (Dunham and Wilczynski, 2014). In addition, AVT treatment resulted in a significant increase in circulating corticosterone regardless of the social behavior stimulus (mirror, male conspecific, or female conspecific). This experiment aims to assess the behavioral and endocrine dose response to AVT as well as the effect of AVT on steroid hormone concentrations in single housed animals not experiencing a behavior challenge.

1.5.3 Chapter 4

Treatment with AVT significantly reduces aggressive behavior displays (Dunham and Wilczynski, 2014). It also significantly increases CORT levels. Evidence suggests that elevated CORT may be correlated with an overall reduction in aggressive responding. For example, animals that become subordinate maintain a higher level of CORT for a longer period of time than do dominant animals (Summers et al., 2003) and animals treated chronically with CORT show fewer aggressive displays (Tokarz, 1987). This experiment aims to determine if the influence of AVT on behavior is entirely CORT dependent by utilizing the CORT synthesis inhibitor, metyrapone.

1.5.4 Chapter 5

Previous research in our lab has demonstrated that subordinate animals have fewer ir-positive AVT cells than dominant animals, singly housed males, or males housed with a
female after 10 days exposure to a conspecific male resulting in a stable social hierarchy (Hattori and Wilczynski, 2009). The contribution of behavior alone, independent of a social status, is not well understood. This experiment aims to determine if aggressive behavior experience contributes to AVT cell number.

1.5.5 Chapter 6

The conclusions from each above chapter are summarized and a general overall discussion is presented.

2 ARGinine vasOTOCIN, STEROID HormONES, AND SOCIAL BEHAVIOR IN THE GREEN ANOLE LIZARD (*Anolis carolinensis*)

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Previously published in *The Journal of Experimental Biology* 217: 3670-3676

2.1 Abstract

Arginine vasotocin (AVT) is a potent regulator of social behavior in many species but little is known about its role in reptilian behavior. Here we examine the effect of exogenous AVT on aggressive responding and courtship behavior in the green anole lizard (*Anolis carolinensis*). Aggressive behavior was stimulated in two ways: 1) mirror presentation (no relative status formed) and 2) size-matched pairs (where a social status is
achieved). To elicit courtship behavior, a novel female was introduced into the home cage of a male. Regardless of the behavior condition, male anoles were injected IP with either reptile ringer’s solution (VEH) or AVT prior to testing. Animals treated with AVT performed fewer aggressive display bouts during mirror presentation but AVT treatment did not affect the overall number of aggressive display bouts within size-matched pairs. Male courtship behavior was not affected by AVT however, untreated females displayed more frequently when paired with an AVT-treated male than a VEH-injected control suggesting that AVT-treated males were more attractive to females. Regardless of behavior condition, AVT injections led to increases in circulating corticosterone. Overall, we found that AVT tended to reduce aggressive behavior as has been reported for other territorial species. AVT did not perceptibly alter male courtship but did increase the display behavior of untreated females paired with treated males. Our study supports a role for AVT in the regulation of reptile social behavior.

2.2 Introduction

Decades of research have established that arginine vasopressin (AVP; found in mammals) and arginine vasotocin (AVT; non-mammalian homologue identified in birds, reptiles, amphibians, and fish) are capable of inducing potent and rapid behavioral changes in a large variety of species (for reviews, see: Albers, 2012; Balment et al., 2006; Caldwell et al., 2008; Goodson and Bass, 2001; Rose and Moore, 2002). The effects of these hormones can contribute significantly to variability in behavior between species as well as among individuals. Among all of the reported influences of AVT/AVP on behavior, their role in
regulating or modulating social behaviors – including aspects of aggression and courtship – has been extensively demonstrated.

The effects of AVT on aggressive behavior can be highly variable and context-dependent. Research in a variety of species has demonstrated the importance of several variables, including the social spacing of the species (e.g. territorial vs. gregarious [Goodson, 1998a, b; Goodson and Adkins-Regan, 1999; Goodson and Evans, 2004; Lema and Nevitt, 2004]), the behavioral phenotype of the individual (e.g. territorial vs. non-territorial morphs [Semsar et al., 2001]; dominant vs. subordinate [Goodson et al., 2009a]), and the specific context of the interaction (e.g. sexually naïve vs. experienced [Winslow et al., 1993]; territory defense vs. mate competition [Goodson et al., 2009a]) in influencing how AVT modulates aggressive behavior, specifically increasing versus decreasing the number and intensity of aggressive behavioral displays. For example, differences in the aggression-modulating effects of AVT have been observed in studies examining closely related species with different social spacing preferences. In socially gregarious zebra finches (Taeniopygia guttata), AVT increases aggression (Goodson and Adkins-Regan, 1999) while the opposite effect was observed when AVT was given to typically territorial species - the field sparrow (Spizella pusilla) and violet eared waxbill (Uraeginthus granatius) (Goodson, 1998b; Goodson, 1998a). Even within a species, AVT can have different effects on aggressive behavior depending on the behavior phenotype or morph of each individual. For instance, AVT increases aggression in non-territorial morphs of bluehead wrasse (Thalassoma bifasciatum) but significantly reduces it in their territorial counterparts (Semsar et al., 2001). In addition, colocalization of the immediate early gene Fos with AVT/AVP cells in several hypothalamic regions of both mammalian and aves
brains correlate negatively with measures of aggression (Goodson and Evans, 2004; Goodson and Kabelik, 2009; Goodson et al., 2012; Ho et al., 2010) suggesting an important role for hypothalamic AVT/AVP cell populations in suppressing aggressive behavior. Overall, these data suggest that there is a tendency for AVP/AVT to suppress aggressive behavior in typically territorial individuals while stimulating it in non-territorial or socially gregarious animals. The mechanism through which these variable effects emerge is not well understood. To date, little is known about the effect of AVT on territorial aggression in any reptile.

AVT stimulates reproduction associated and courtship display behaviors in a variety of non-mammalian vertebrates. Early studies in the roughskin newt (*Taricha granulosa*) demonstrated that injection with AVT increases male clasping behavior while infusions of AVP antagonists and anti-AVT serum reduce courtship (Moore and Miller, 1983; Moore and Zoeller, 1979). Courtship display behaviors such as attending behavior in fish (Salek et al., 2002; Santangelo and Bass, 2010; Semsar et al., 2001) and aspects of calling in several species of frogs including frequency and acoustic characteristics (Burmeister et al., 2001; Kime et al., 2007; Marler et al., 1995) are also affected. In zebra finches, exogenous AVT failed to significantly increase directed song (Goodson and Adkins-Regan, 1999; Goodson et al., 2004), however a positive correlation between AVT-immunoreactive (AVT-ir) cell number in the bed nucleus of the stria terminalis and the number of courtship displays performed was observed (Goodson et al., 2009b). Similar evidence has emerged in the brown anole lizard in which the number of “social engagement behaviors” toward a female was positively correlated with colocalization of AVT peptide and expression of the immediate early gene Fos in several behaviorally significant brain regions (preoptic area,
bed nucleus of the stria terminalis, and the periventricular nucleus) suggesting an important relationship between courtship and activation of the AVT system (Kabelik et al., 2013).

Interactions between AVT/AVP and other behaviorally relevant hormone systems are likely to play a role in the expression of behavior. Steroids such as testosterone (T) and corticosterone (CORT) can potently influence the expression and intensity of courtship and territorial aggression-related behaviors, both individually and through reciprocal interactions (DeNardo and Licht, 1993; Leary et al., 2006; Moore and Miller, 1984; Rose and Moore, 2002; Trainor and Nelson, 2012; Wade, 2005; Wingfield et al., 1998). In the anole brain, AVT-immunoreactivity has been described in several regions known to influence social behavior including the supraoptic nucleus, preoptic area, and anterior hypothalamus, some of which are also steroid sensitive (Hattori and Wilczynski, 2009; Propper et al., 1992). In addition, there is evidence for interactions between AVT/AVP and steroid hormones, providing a potential mechanism through which behavioral variability and context-dependent responses could be achieved. For example, AVT/AVP cells are androgen sensitive and castration reduces immunoreactive cell number (Rodents: Boyd, 1997; Kabelik et al., 2008; Miller et al., 1992; Wang and De Vries, 1993; Reptiles: Kabelik, Weiss, & Moore, 2008).

It is well established that AVT can promote the release of corticosterone from the adrenals by further stimulating the hypothalamic-pituitary-adrenal (HPA) axis resulting in increased circulating adrenocorticotropic hormone (Gibbs, 1986). There is, however, evidence suggesting that the overall behavioral actions of AVT/AVP and CORT may be in opposition. For example, AVT stimulates courtship behavior while CORT suppresses it, and...
priming the system with one can block the effects of the other (Burmeister et al., 2001; Coddington and Moore, 2003). There are physiologically important interactions between AVT/AVP and steroid hormones and these interactions are likely to be critical influences on the expression of social behavior.

Considered together, the current body of research on AVT/AVP and social behavior (courtship and aggression) highlights an incredible range of effects that may contribute to the overwhelming diversity and variability of behaviors observed both between- and within-species. Understanding the relationship between these peptides and behavior in multiple classes of animals is critical to understanding the evolution and complexity of AVT/AVP function. To date, however, little is known about how arginine vasotocin influences social behaviors in any reptiles. The present experiments were designed to examine the influence of AVT on social behavior displays and steroid hormone concentrations in the green anole lizard (*Anolis carolinensis*). This species uses physical displays to send visual signals that can deter other males encroaching on a given territory and to court nearby females for mating opportunities. The green anole’s reliance on the use of primarily visual signals also represents a unique mode of social communication in which to consider a role for AVT/AVP as most studies have utilized species with predominantly chemical and auditory communication systems (Albers, 2012; Caldwell et al., 2008; Goodson and Bass, 2001).

Given the emerging trends observed in mice, rats, and a variety of avian species where high AVT/AVP is associated with subordinate-like behavior and low aggression in territorial species, we hypothesized that administration of exogenous AVT would result in decreased aggressive responding in a territorial reptile. To address this question, we
examined aggression under two testing conditions: mirror presentation (Experiment 1) and conspecific pairing (Experiment 2) in order to determine the effect of AVT on aggressive behavior alone as well as its overall impact on the outcome of an aggressive interaction, i.e. social status. Given the positive relationship between AVT and mating behaviors in many species of amphibians, we also hypothesized that AVT would increase male courtship display behavior (Experiment 3).

### 2.3 Methods

#### 2.3.1 Animals

Seventy-six male and twenty-four female anole lizards were obtained from Charles Sullivan, Inc. (Nashville, TN) and were housed individually in half of a 10 gallon glass aquarium (24x12x18) on a 14L:10D light cycle with an ambient daytime temperature of 26-28°C. Each enclosure contained artificial leaves, a water dish, and an elevated perch. Animals were fed calcium gut-loaded crickets (Ghann’s Cricket Farm; Augusta, GA) three times weekly and water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University.

#### 2.3.2 General methods

For each of the following experiments, animals were allowed to acclimate to the laboratory conditions prior to any behavior test. Previous studies have suggested that the memory for social status relative to a given individual is maintained for 10 days after which time status reversals may occur (Korzan and Summers, 2007). Our males were housed individually for at least 10 days prior to testing in order to account for the possible memory
of social status. While we do not know the history of the animals prior to their arrival in the laboratory, this acclimation period should be sufficient to ensure minimal influence of previous social experience.

Each animal was randomly assigned to one of two treatment conditions: AVT (15 µg AVT/50 µl reptile ringers) or VEH (50 µl reptile ringers). This dose of AVT (Phoenix Pharmaceuticals; Burlingame, CA), approximately 3 µg/g body mass, was adapted from the published effective intraperitoneal (IP) administered dose in roughskin newts (Coddington and Moore, 2003) and green treefrogs (Burmeister et al., 2001). Fifteen minutes after injection, the behavior tests were conducted and filmed for 30 minutes. All behavioral tests were conducted between 10:30am and 1:30pm on the day of testing. Males were then sacrificed via an overdose with IP injected Nembutal. Trunk blood was collected within 3 minutes of capture and stored at -20°C until processing via enzyme-linked immunosorbent assay (ELISA).

2.3.3 Behavior definition and scoring

Aggressive and courtship display behaviors have been thoroughly characterized for this species (Crews, 1975; DeCourcy and Jenssen, 1994; Garcia et al., 2014; Greenberg and Crews, 1990b; Korzan et al., 2000; Lovern and Jenssen, 2003; Yang et al., 2001). Males engaging in aggressive displays perform pushups and head bobs, often in conjunction with extension of the red dewlap throat fan. Pushups occur when the body is moved up and down by flexing of the animal’s forelimbs. Head bobs, in contrast, involved movement of the head only. These behaviors are typically accompanied by lateral compression of the body and nuchal crest extension; causing the challenging male to appear larger overall. Aggressive bouts can further escalate to include jaw sparring and biting, as well as the
formation of a dark “eye spot” posterior to the animal’s orbit. At the conclusion of an aggressive interaction, the ‘loser’ will often develop a brown body color indicative of a subordinate status while the ‘winner’ or dominant animal will remain green. Courtship displays feature several of the same behaviors described above including pushups, head bobs, and dewlap extensions. In addition, males attempt to approach females in order to maximize copulation opportunities. During courtship interactions, females also display to males with bouts of head bobs.

The taped 30-minute trials were scored using Stopwatch + software (www.cbn-atl.org) by individuals blind to the injection condition. The latency to initiate behavior as well as the number of behavioral bouts was recorded. For both aggression and courtship, a single bout is defined as a rapid burst of display-associated behaviors (often including 5-10 instances of head-bobbing and extension of the red dewlap throat fan) followed by a brief pause. Quantification of behavioral bouts was chosen over individual behavior number as discrete display-associated behaviors (dewlap extension and head bob) are rarely performed in isolation but rather occur in highly stereotyped patterns (DeCourcy and Jenssen, 1994; Jenssen et al., 2012; Lovern and Jenssen, 2003; Orrell and Jenssen, 2003), suggesting that bout number provides a more salient measure of the overall level of social signaling. For courtship tests, the behavior of both the injected male and the untreated female were scored to determine if female behavior was impacted by the male’s injection condition. For all experiments, treated males (both VEH and AVT) that failed to perform at least one bout of behavior were considered non-responsive and excluded from the final analysis.
2.3.4  **Hormone analysis**

The plasma samples from all individuals were spiked with approximately 2000 cpm of \(^{3}H\)-CORT to allow for measurement of extraction efficiency. Samples were diluted 1:25, extracted with 3 ml of ether, and dried at 37°C under a nitrogen stream. The extracted samples were reconstituted with assay buffer (1:25-1:100 for testosterone; 1:300-1:750 for corticosterone) and analyzed via ELISA (Caymen Chemical; Ann Arbor, MI) for both T and CORT according to the manufacturer’s instructions. These assays have been previously validated for *Anolis carolinensis* by our lab and others using pooled plasma serial dilutions (Garcia et al., 2012; Yang and Wilczynski, 2003). All plates were run concurrently and each sample was assayed in triplicate. Sample recovery rates were used to correct for extraction efficiency. Overall mean recovery was 88.4% and no corrections were made for samples with greater than 100% recovery. Overall intra-assay coefficients of variation were 6.4% for testosterone and 11% for corticosterone. The assays have a sensitivity of 32 pg/ml for T and 150 pg/ml for CORT according to the manufacturer’s analysis. Testosterone values obtained were lower than previously reported for the green anole, which generally fall within a range of approximately 2-20 ng/ml in breeding condition animals (Husak et al., 2009; Husak et al., 2007). The levels reported here were, however, consistent across all of our experimental groups.

2.3.5  **Experiment 1: Mirror aggression**

Twenty-four individually housed male anole lizards were used for the mirror aggression tests. Previous studies have demonstrated that anoles will reliably and robustly aggress toward their reflected image (Baxter, et al., 2001; Farrell and Wilczynski, 2006). This method of eliciting behavior allows us to examine how AVT affects aggressive displays
when the animal's own behavior output matches the visual input it observes and a relative social status is not achieved.

Following IP injection of the assigned drug, a two-way mirror (Reflection Products, Inc.; Roswell, GA) was placed in front of the home cage of a single-housed male. Behavior displays were captured by a video camera placed directly behind the mirror (Sony Handycam). Of the 24 animals tested, 16 responded to the mirror and were included in the final analyses.

2.3.6 Experiment 2: Paired aggression

In order to determine if AVT had an effect on male aggression in a social contest with another male, and influenced the overall outcome of an aggressive encounter, we conducted paired aggression tests with 14 pairs of male anoles in which one animal received an AVT injection and a size-matched partner (within 0.1 g) received VEH. Fifteen minutes post-injection, both males (one AVT and one VEH-injected) were introduced to a neutral cage and the interaction was recorded for 30 minutes. The neutral cage arena was selected so that neither individual would experience a resident-intruder advantage. Of the 14 pairs tested, only 10 interacted during the aggression trial. These 20 animals were included in the final analyses.

2.3.7 Experiment 3: Courtship

Twenty-four individually housed male anole lizards were injected with AVT or VEH. Fifteen minutes post IP injection, a stimulus female was introduced into the male’s home cage. In the green anole, the primary method for determining female reproductive status is through a post-mortem dissection (Crews, 1973); therefore we cannot guarantee the
receptive state of our stimulus females. The females used however, were recently obtained from the field during the breeding season and were randomly selected for placement with treated males in order to avoid a behavior bias due to female receptivity and motivation. The male and female were allowed to interact for 30 minutes, during which time their behavior was recorded. Of the 24 males tested, courtship behavior was observed in 17 animals and these individuals were included in the analyses. Female behavior was scored only in those trials with responding males.

2.4 Results

2.4.1 Experiment 1: Mirror aggression

A Mann-Whitney test revealed that injection of a 15µg/50µl dose of arginine vasotocin resulted in a significant decrease in the overall number of aggressive display bouts (two-tailed; VEH: n = 6, M = 30.67 bouts, SEM = 7.55; AVT: n = 10, M = 4.60 bouts, SEM = 1.23; U = 4.50, p < 0.01) as compared with ringer injected controls (Figure 2.1). There was no effect of AVT on the latency to initiate the first aggressive bout (two-tailed; VEH: n = 6, M = 363.57 s, SEM = 130.22; AVT: n = 10, M = 429.21 s, SEM = 97.24; t14 = -0.41, p = 0.69). Vasotocin injection did not alter circulating levels of total testosterone (two-tailed; VEH: n = 5, M = 670.44 pg/ml, SEM = 68.37; AVT: n = 10, M = 645.19 pg/ml, SEM = 67.16; t13 = 0.24, p = 0.82; Figure 2.2) but led to a significant increase in total circulating CORT (two-tailed; VEH: n = 6, M = 55.84 ng/ml, SEM = 14.58; AVT: n = 10, M = 282.75 ng/ml, SEM = 35.36; U = 55.00, p < 0.01; Figure 2.3) as compared to control animals.
Overall, CORT was negatively correlated with the number of behavior bouts (r = -0.60, p = 0.02; Figure 2.4).

**2.4.2 Experiment 2: Paired aggression**

When paired aggression was considered, a paired t-test revealed that within a pair, the number of displays did not differ significantly between AVT-injected and controls (two-tailed; VEH: n = 10, M = 57.8 bouts, SEM = 10.62; AVT: n = 10, M = 36.9 bouts, SEM = 8.42; t9 = 1.56, p = 0.15; Figure 2.5). Animals injected with AVT were not significantly more likely to achieve a specific social status although 70% of AVT animals did eventually become subordinate to their VEH-injected partner (two-tailed; χ² = 1.8, p = 0.18). Within pairs, there was no difference in the latency to first aggressive display bout (two-tailed; VEH: n = 10, M = 57.36 s, SEM = 24.23; AVT: n = 10, M = 67.50 s, SEM = 19.06; t9 = -0.35, p = 0.73). AVT treatment did not affect plasma testosterone (two-tailed; VEH: n = 10, M = 660.14 pg/ml, SEM = 139.18; AVT: n = 10, M = 794.41 pg/ml, SEM = 108.00; t18 = -0.762, p = 0.46; Figure 2.6) but individuals injected with AVT had significantly higher circulating CORT (two-tailed; VEH: n = 10, M = 24.11 ng/ml, SEM = 3.88; AVT: n = 10, M = 339.35 ng/ml, SEM = 31.75; U = 100.00, p < 0.01; Figure 2.7). Overall, CORT was negatively correlated with the number of aggressive display bouts (r = -0.47, p = 0.04; Figure 2.8).

**2.4.3 Experiment 3: Courtship**

Courtship display behavior was not significantly affected by injection with 15 µg/50 µl AVT as compared to control animals (two-tailed; VEH: n = 10, M = 14.1 bouts, SEM = 3.95; AVT: n = 7, M = 17.71 bouts, SEM = 5.93; U = 30.50, p = 0.70; Figure 2.9). There was also no effect of AVT on the latency to initiate the first courtship bout (two-tailed; VEH: n =
10, M = 131.56 s, SEM = 37.18; AVT: n = 7, M = 142.53 s, SEM = 65.84; t_{15} = -0.16, p = 0.88).

In courting males, vasotocin injection did not affect testosterone (two-tailed; VEH: n = 6, M = 1234.63 pg/ml, SEM = 626.56; AVT: n = 7, M = 1119.09 pg/ml, SEM = 255.52; t_{11} = 0.18, p = 0.86; Figure 2.10) but did lead to a significant increase in circulating corticosterone (two-tailed; VEH: n = 10, M = 152.09 ng/ml, SEM = 61.62; AVT: n = 7, M = 612.52 ng/ml, SEM = 63.37; U = 5.00, p < 0.01; Figure 2.11). Overall, CORT was not correlated with the number of courtship displays observed (r = 0.16, p = 0.54; Figure 2.12).

We also analyzed the behavior of the untreated females used as stimulus animals for the VEH- and AVT-treated males. Females paired with AVT-treated males performed significantly more courtship displays than those paired with VEH-treated males (two-tailed; VEH paired: n = 10, M = 5 bouts, SEM = 2.80; AVT paired: n = 7, M = 14.29 bouts, SEM = 1.74; U = 9.00, p = 0.01; Figure 2.13).

2.5 Discussion

This study provides evidence for a role of AVT in reptile behavior. A variety of context specific effects have been described in other species and this appears to be the case in the green anole as well. Treatment of territorial species or morphs with AVT leads to a reduction in aggression in a variety of species (Goodson, 1998a, b; Goodson and Adkins-Regan, 1999; Goodson and Evans, 2004; Lema and Nevitt, 2004; Semsar et al., 2001). In green anole males, which form territories and dominant-subordinate relationships, arginine vasotocin administration resulted in reduced aggressive responding when animals were presented with a mirror. This was not the case in a male-male social interaction,
however, and it did not significantly affect the overall outcome in a more natural paired aggression test.

Vasotocin injection resulted in a marked decrease in aggressive behavior during mirror presentation suggesting that high levels of vasotocin are inhibitory to aggression, at least when only visual display cues are available. When size-matched conspecifics were allowed to freely interact, where not only visual displays but also chemical cues and physical stimuli may contribute to the experience of the interaction, AVT administration failed to significantly affect overall aggressive behavior. It is important to consider that in the paired test, both males were displaying to one another, and we assume that some of the behavior each displayed resulted from this ongoing exchange. It could be that the natural behavioral cues of another male and cadence of the interaction may be more important than the effects of AVT on aggression. It is also the case that interaction with a conspecific might involve both offensive and defensive aggression, which is a more complicated situation than the more pure offensive aggression expressed to a mirror. In this sample, AVT-treated males became subordinate in 70% of interactions with VEH-treated individuals. While this does not reach statistical significance (two-tailed $\chi^2$), it suggests to us a trend for individuals with high circulating AVT to be less aggressive overall.

A previous study examining the relationship between social status and AVT-ir cell populations in *Anolis carolinensis* demonstrated that subordinate males had significantly fewer ir-positive AVT cells in the preoptic area as compared to dominant males, singly housed males, and males pair housed with a female, following 10 days of pairing/observation (Hattori and Wilczynski, 2009). While these results may seem to be in conflict with our findings, one possible hypothesis is that reduced ir-cell numbers could
reflect an increase in release of peptides as opposed to a decrease in synthesis (Marler et al., 1999), suggesting that changes in the release of AVT and thus the circulating levels, may contribute to differences in aggression that could ultimately result in a dominance hierarchy. Based on our findings, high levels of circulating AVT are likely suppressive to aggression.

In our study, we found no effect of AVT treatment on courtship behavior in the male green anole. To date, no other studies have examined the role of this hormone in reptile behavior. However, we did find that while the number of male courtship bouts appears unaffected by AVT, untreated females perform more courtship displays to AVT-treated males than to VEH-treated males. It may be that AVT affected the form of the displays, rather than the number of display bouts, in such a way that they were more attractive to females. Furthermore, in these tests, males and females were freely interacting and thus could potentially gather information about their partner not only from visual courtship displays but also through chemical and physical cues. While it has been argued that the chemosensory system is relatively rudimentary in the green anole lizard and likely not a primary mode of communication in this particular species (Greenberg, 1993), its possible role in this female preference for AVT males cannot be ruled out by the present experiment. Many lizards use chemical signals in male-female and male-male interactions (reviewed in Martin and Lopez, 2011), and there are several examples of olfactory cues being used in female choice (Kopena et al., 2011; Labra, 2011; Lopez and Martin, 2005), although at present there is no evidence for this in green anoles. When chemical communication is used, AVT is an important modulator. In red-bellied newts (*Cynops pyrrhogaster*), for example, AVT treatment induces the release of a female-attracting
pheromone (Toyoda et al., 2003). Alternatively, AVT may have changed aspects of the display behavior that were too subtle for our measures to detect. AVT treatment has been shown to alter call characteristics leading to changes in female phonotaxis and mate preference in the túngara frog (*Engystomops pustulosus*; Kime et al., 2007). Future studies are needed to determine what aspects of AVT-treated males are attractive to females. Regardless of what these aspects may be, the fact that more females produced courtship behavior to AVT-treated males suggests that AVT does influence reproductive behavior in reptiles in a way that makes males more attractive to females.

AVT and AVP act as stimulators of the hypothalamic-pituitary-adrenal axis (HPA axis), increasing the release of adrenocorticotropic hormone and ultimately stimulating corticosterone/cortisol release from the adrenal glands. Given this relationship, it is surprising that few studies utilizing exogenous AVT/AVP administration have reported the effects of those treatments on the CORT system, especially considering its potent behavioral effects. Here, we demonstrate that AVT treatment results in a significant increase in circulating CORT. This increase in CORT following AVT treatment is observed regardless of the type of behavior stimulated (aggression or courtship) and the manner in which it is stimulated (mirror or live conspecific pairing). While the plasma used to measure CORT in this study was taken at the conclusion of the behavior test and thus may reflect not only the influence of AVT treatment but also the effect of a behavior challenge, it is important to consider that the behavioral effects observed with AVT treatment may be attributed, at least in part, to high levels of circulating CORT. CORT level was negatively correlated with the number of aggressive display bouts for both mirror presentation and live pairing. We caution that at this point we do not know if the relation between CORT
elevation and decreases in aggressive display bouts is the result of independent and unrelated effects of AVT on both, or if there is a causal relationship. Future studies are needed to determine if AVT treatment affects behavior independently via effects on the brain or if the observed effects work primarily through its activation of the HPA axis and stimulation of CORT release. There is some indication, in mammals, that a small but measurable portion of peripherally administered radiolabeled-AVP can cross the blood brain barrier (Zlokovic et al., 1990). While there have been several studies on the structure and permeability of the blood-brain barrier in the green anole (Kenny and Shivers, 1974; Shivers, 1979; Shivers and Harris, 1984), the specific permeability to AVT has not been examined. In other species where behavioral effects of AVT/AVP have been observed with peripheral injection, similar effects are observed when smaller amounts are delivered directly to the brain. While this is not direct evidence that peripheral AVT/AVP is getting into the brain, it suggests that it likely is crossing over in some amount.

In sum, we demonstrate a role for arginine vasotocin in the control of reptile social behavior that is consistent with its effects in other species. Our data tend to support the hypothesis that AVT inhibits aggression in territorial species. They also indicate that AVT increases the attractiveness of males to females, although we cannot identify the reason for this from our study. In addition, our findings highlight the importance of considering the secondary, peripheral endocrine effects of AVT administration, including the activation of the HPA axis, when interpreting behavioral data. AVT treatment causes a significant elevation of CORT under all conditions. Further investigations are needed to determine if this elevation contributes to changes in aggression seen after AVT treatment.
2.6 Acknowledgements

We thank Deborah Lutterschmidt, Javonte Carter, and Skylar McHenry for their technical assistance and David Sinkiewicz and Bryce Chung for their helpful discussions during the preparation of this manuscript.

2.7 Funding

Supported by the National Science Foundation [IBN 0751573 to W.W.] and the Georgia State University Brains & Behavior Area of Focus.

![Figure 2.1 Effect of AVT on aggressive behavior during mirror presentation](image)

**Figure 2.1 Effect of AVT on aggressive behavior during mirror presentation**

During mirror-stimulated aggression, males treated with AVT perform significantly fewer aggressive display bouts in thirty minutes than VEH-injected males. White indicates VEH and grey denotes AVT-injected animals. Data are presented as mean ± SEM and group
sample sizes are indicated in parentheses. Asterisks indicate statistical significance (p < 0.05).

Figure 2.2 Effect of AVT on plasma testosterone with mirror presentation
AVT did not affect circulating T (pg/ml). White indicates VEH and grey denotes AVT-injected animals. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses.
Figure 2.3 Effect of AVT on plasma corticosterone with mirror presentation
AVT treatment results in significantly more circulating plasma CORT (ng/ml) following mirror aggression. White indicates VEH and grey denotes AVT-injected animals. Data are presented as mean ± SEM and sample sizes are indicated in parentheses. Asterisks indicate statistical significance (p < 0.05).
Corticosterone was negatively correlated with the overall number of aggressive display bouts toward the mirror.

Figure 2.4 Correlation between aggression and corticosterone in mirror presentation
Corticosterone was negatively correlated with the overall number of aggressive display bouts toward the mirror.
Figure 2.5 Effect of AVT on aggressive behavior within pairs

Within pairs, AVT-injected animals did not behave significantly different from their VEH-injected partners. White and grey bars represent VEH- and AVT-injected animals, respectively. Data are presented as mean ± SEM and sample sizes are indicated in parentheses.
Figure 2.6 Effect of AVT on plasma testosterone in paired aggression
There was no effect of AVT treatment on circulating T (pg/ml). White and grey bars represent VEH- and AVT-injected animals, respectively. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses.
Figure 2.7 Effect of AVT on plasma corticosterone in paired aggression
Individuals injected with AVT showed a significant increase in plasma CORT (ng/ml) following a paired aggressive interaction with a VEH-injected partner. White and grey bars represent VEH- and AVT-injected animals, respectively. Data are presented as mean ± SEM and sample sizes are indicated in parentheses. Asterisks indicate statistical significance (p < 0.05).
Figure 2.8 Correlation between aggression and corticosterone in paired aggression
Overall, corticosterone levels were negatively correlated with the number of observed display bouts. Asterisk represents statistical significance ($p < .05$).
Figure 2.9 Effect of AVT on courtship behavior
AVT injection did not affect the number of courtship displays to a female conspecific. White and grey bars represent VEH and AVT-injected animals, respectively. Data are presented as mean ± SEM and sample sizes are indicated in parentheses.
Figure 2.10 Effect of AVT on plasma testosterone in courting males
There was no effect of AVT treatment on plasma T (pg/ml). White and grey bars represent VEH and AVT-injected animals, respectively. Data are presented as mean ± SEM and sample sizes are indicated in parentheses.
Figure 2.11 Effect of AVT on plasma corticosterone in courting males
Individuals injected with AVT had significantly higher circulating CORT (ng/ml) than VEH-injected controls. White and grey bars represent VEH and AVT-injected animals, respectively. Data are presented as mean ± SEM and sample sizes are indicated in parentheses. Asterisks represent statistically significant differences (p < 0.05).
Figure 2.12 Correlation between courtship behavior and corticosterone
There was no correlation between corticosterone and the number of observed courtship displays.

$r = 0.16; \rho = 0.54$
Figure 2.13 Female courtship behavior to AVT- and VEH-treated males
Untreated females paired with an AVT-treated male perform significantly more bouts of courtship displays than untreated females paired with VEH-injected control males. White and grey bars represent females paired with VEH- or AVT-injected males, respectively. Data are presented as mean ± SEM and sample sizes are indicated in parentheses. Asterisks represent statistically significant differences (p < 0.05).
3 BEHAVIORAL AND ENDOCRINE DOSE RESPONSE TO ARGinine VASOTOCIN IN
GREEN ANOLE LIZARDS (Anolis carolinensis)

3.1 Introduction

Arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) have long been implicated in the regulation of social behavior (for reviews, see: Albers, 2012; Balment et al., 2006; Caldwell et al., 2008; Goodson and Bass, 2001; Rose and Moore, 2002). In the mammalian literature, a number of studies suggest a stimulatory role for AVP in appetitive courtship and affiliative behaviors (Cho et al., 1999; Lim and Young, 2004; Winslow et al., 1993; For reviews: Caldwell et al., 2008; Insel, 1997, 2010; Keverne and Curley, 2004). Similar effects have been reported in non-mammalian systems such as the newt, Taricha granulosa where infusions of AVT resulted in increased clasping behavior (Moore and Miller, 1983; Moore and Zoeller, 1979), increased attending behavior in fish (Salek et al., 2002; Santangelo and Bass, 2010; Semsar et al., 2001), and increased calling in frogs (Burmeister et al., 2001; Kime et al., 2007; Marler et al., 1995). The literature for AVP/AVT effects on aggression is far more variable but mounting evidence in non-mammalian species suggests that AVT reduces aggression in territorial species (Goodson, 1998a, b) while increasing it in non-territorial species and morphs (Goodson and Adkins-Regan, 1999; Semsar et al., 2001). In green anole lizards, I have previously demonstrated that AVT has no effect on courtship behavior but results in a significant reduction in aggression to a mirror (Dunham and Wilczynski, 2014). This reduction was not observed when animals were paired with a conspecific. In addition, I found that AVT treatment led to a significant increase in circulating corticosterone, a result consistent with studies
suggesting a stimulatory role for AVT on the hypothalamic-pituitary-adrenal (HPA) axis (described below).

When a perceived stressor is encountered, e.g. an intruding male, the HPA axis is activated. The primary goal of this system is to stimulate a coping response to the perturbation and subsequently to promote a return to a homeostatic condition after a threat has passed (McEwen, 2007). Corticotropin releasing hormone (CRH) released from the hypothalamus into the median eminence portal vein is a primary hormone in HPA axis responsivity. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland that is responsible for initiating the synthesis and release of glucocorticoids (e.g. corticosterone) from the adrenal cortex in response to the stressor. In addition to CRH, arginine vasopressin, or its non-mammalian analogue arginine vasotocin, also has the ability to influence this HPA activation and response in potentially complex ways. Release of AVP from parvocellular neurons in the paraventricular nucleus (PVN) and magnocellular neurons in the supraoptic nucleus (SON) leads to increases in ACTH secretion (Antoni, 1993) and it is estimated that at least half of the CRF-positive neurons in the PVN co-express AVP in rats and humans (Mouri et al., 1993; Whitnall, 1987). In humans, AVP treatment has been shown to increase ACTH release (Salata et al., 1988) and rats treated with an AVP antagonist show a blunted ACTH response after 20 minutes of ether stress (Rivier and Vale, 1983). Overall, the body of evidence suggests that CRH and AVP/AVT work synergistically and that both are necessary to achieve maximum activation of the HPA circuit (AVP: DeBold et al., 1984; Dinan and Scott, 2005; Favrod-Coune et al., 1993; Gillies et al., 1982; AVT: Baker et al., 1996).
The goal of this experiment was to determine 1) if there are dose dependent behavioral and endocrine effects of AVT and 2) the endocrine effects of AVT administration when no social behavior is stimulated. Previous work in our lab has demonstrated that a 3µg/g body weight injection of AVT causes significant increases in circulating corticosterone and a significant decrease in the number of aggressive bouts to a mirror. Effects on courtship behavior were not observed at that dose (Dunham and Wilczynski, 2014). Given the wealth of data from other species outlining an important role for AVT/AVP in courtship behavior, I wanted to determine if dose of AVT was a factor. In addition, few studies have examined the effect of AVT administration on measures of HPA axis activation, such as circulating corticosterone, in any lizard species. Therefore, I wanted to determine if a social behavior challenge was necessary for the elevated corticosterone I have observed following injection with AVT.

3.2 Methods

3.2.1 Animals

Thirty four male anole lizards were obtained from Charles Sullivan, Inc. (Nashville, TN) and were housed individually in half of a 10 gallon glass aquarium (24x12x18) on a 14L:10D light cycle with an ambient daytime temperature of 26-28°C. Each enclosure contained artificial leaves, a water dish, and an elevated perch. Animals were fed calcium gut-loaded crickets (Ghann’s Cricket Farm; Augusta, GA) three times weekly and water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University.
3.2.2  **Experiment 1: AVT dose on mirror aggression and courtship**

3.2.2.1  **Injections**

Fourteen males were assigned to either the mirror aggression or courtship stimulus groups. For both groups, each individual received four injections, one per week for four weeks in a counterbalanced design. Injections of VEH (50μl reptile ringers) or one of three AVT doses: low AVT (lAVT; 5 μg AVT/50 μl reptile ringers or ~ 1μg/g body weight), mid AVT (mAVT; 15 μg AVT/50 μl reptile ringers or ~ 3μg/g body weight), and high AVT (hAVT; 30 μg AVT/50 μl reptile ringers or ~ 10μg/g body weight) were administered IP. The mAVT dose matches the dose used previously to produce behavioral effects on mirror aggression in green anole lizards (Dunham and Wilczynski, 2014).

3.2.2.2  **Mirror aggression**

Seven individually housed male anole lizards were used for the mirror aggression tests. Previous studies have demonstrated that anoles will reliably and robustly aggress toward their reflected image (Baxter, et al., 2001; Farrell and Wilczynski, 2006). This method of eliciting behavior allows us to examine how AVT affects aggressive displays when the animal’s own behavior output matches the visual input it observes and a relative social status is not achieved.

Each animal received one injection (VEH, lAVT, mAVT, hAVT) per week for four consecutive weeks prior to each behavior trial. Following IP injection of the assigned drug and dose, a two-way mirror (Reflection Products, Inc.; Roswell, GA) was placed in front of the home cage of a single-housed male. Behavior displays were captured by a video camera placed directly behind the mirror (Sony Handycam).
3.2.2.3  Courtship

Seven males were used to examine the effect of different AVT doses on courtship behavior. Each animal was given one injection (VEH, lAVT, mAVT, hAVT) per week for four consecutive weeks, prior to each behavior trial. Fifteen minutes post IP injection, a stimulus female was introduced into the male’s home cage. A novel female was introduced in each of the four trials. In green anole lizards, the primary method for determining female reproductive status is through a post-mortem dissection (Crews, 1973); therefore I cannot guarantee the receptive state of my stimulus females. The females used however, were recently obtained from the field during the breeding season and were randomly selected for placement with treated males in order to avoid a behavior bias due to female receptivity and motivation. The male and female were allowed to interact for 30 minutes, during which time their behavior was recorded.

3.2.2.4  Behavior definition and scoring

Aggressive and courtship display behaviors have been thoroughly characterized for this species (Crews, 1975; DeCourcy and Jenssen, 1994; Garcia et al., 2014; Greenberg and Crews, 1990b; Korzan et al., 2000; Lovern and Jenssen, 2003; Yang et al., 2001). Males engaging in aggressive displays perform pushups and head bobs, often in conjunction with extension of the red dewlap throat fan. Pushups occur when the body is moved up and down by flexing of the animal’s forelimbs. Head bobs, in contrast, involved movement of the head only. These behaviors are typically accompanied by lateral compression of the body and nuchal crest extension; causing the challenging male to appear larger overall. Aggressive bouts can further escalate to include jaw sparring and biting, as well as the formation of a dark “eye spot” posterior to the animal’s orbit. At the conclusion of an
aggressive interaction, the ‘loser’ will often develop a brown body color indicative of a subordinate status while the ‘winner’ or dominant animal will remain green. Courtship displays feature several of the same behaviors described above including pushups, head bobs, and dewlap extensions. In addition, males attempt to approach females in order to maximize copulation opportunities. During courtship interactions, females also display to males with bouts of head bobs.

The taped 30-minute trials were scored using Stopwatch + software (www.cbn-atl.org) by individuals blind to the injection condition. The latency to initiate behavior as well as the number of behavioral bouts was recorded. For both aggression and courtship, a single bout is defined as a rapid burst of display-associated behaviors (often including 5-10 instances of head-bobbing and extension of the red dewlap throat fan) followed by a brief pause. Quantification of behavioral bouts was chosen over individual behavior number as discrete display-associated behaviors (dewlap extension and head bob) are rarely performed in isolation but rather occur in highly stereotyped patterns (DeCourcy and Jenssen, 1994; Jenssen et al., 2012; Lovern and Jenssen, 2003; Orrell and Jenssen, 2003), suggesting that bout number provides a more salient measure of the overall level of social signaling. For courtship tests, the behavior of both the injected male and the untreated female were scored to determine if female behavior was impacted by the male’s injection condition. One male never produced a courtship bout and was removed from the experimental analysis.

3.2.2.5 Hormone Assays

The plasma samples from all individuals were spiked with approximately 2000 cpm of [3H]-CORT to allow for measurement of extraction efficiency. Samples were diluted 1:25,
extracted with 3 ml of ether, and dried at 37°C under a nitrogen stream. The extracted samples were reconstituted with assay buffer (1:25 to 1:50 for testosterone; 1:300 to 1:750 for corticosterone) and analyzed via ELISA (Caymen Chemical; Ann Arbor, MI) for both T and CORT according to the manufacturer’s instructions. These assays have been previously validated for Anolis carolinensis by our lab and others using pooled plasma serial dilutions (Garcia et al., 2012; Yang and Wilczynski, 2003). All plates were run concurrently and each sample was assayed in triplicate. Sample recovery rates were used to correct for extraction efficiency. Overall mean recovery was 91.92% and no corrections were made for samples with greater than 100% recovery. Overall intra-assay coefficients of variation were 7.57% for testosterone and 9.45% for corticosterone. The assays have a sensitivity of 32 pg/ml for T and 150 pg/ml for CORT according to the manufacturer's analysis.

3.2.3 Experiment 2: AVT and no behavioral challenge

3.2.3.1 Injections

Twenty individually housed male anoles were randomly assigned to one of two injection conditions. Ten males received intraperitoneal (IP) injections of VEH (50 μl reptile ringers) and 10 were injected with AVT (15 μg AVT/50 μl reptile ringers). This dose of AVT (Phoenix Pharmaceuticals; Burlingame, CA), approximately 3 μg/g body mass, was adapted from the published effective intraperitoneal (IP) administered dose in roughskin newts (Coddington and Moore, 2003) and green treefrogs (Burmeister et al., 2001) and has been shown to elicit behavior changes in this species (Dunham and Wilczynski, 2014). Following injection animals were returned to their home cage for 30 minutes. Animals were then recaptured and sacrificed with an overdose of IP injected Nembutal. Trunk
blood samples were collected within 3 minutes of capture and plasma samples were stored at -20 until assay via enzyme-linked immunoassay (EIA).

3.2.3.2  Plasma collection and hormone assays

Blood was collected from each individual thirty minutes after each behavior encounter. Heparinized 28 gauge insulin syringes were used to draw blood from the caudal vein accessible near the base of the tail. Whole blood was centrifuged for five minutes at 4C. The plasma fraction was stored at -80 until processing via ELISA. See Experiment 1 above for ELISA assay procedures.

All plates were run concurrently and each sample was assayed in triplicate. Sample recovery rates were used to correct for extraction efficiency. Overall mean recovery was 91.27% and no corrections were made for samples with greater than 100% recovery. Overall intra-assay coefficients of variation were 3.55% for testosterone and 12.42% for corticosterone. The assays have a sensitivity of 32 pg/ml for T and 150 pg/ml for CORT according to the manufacturer’s analysis.

3.3  Results

3.3.1  Experiment 1: AVT dose effect on mirror aggression and courtship

3.3.1.1  Mirror aggression

A one-way repeated measures ANOVA was used to examine the effect of AVT dose on mirror aggression. There was no difference in the number of aggressive bouts with any injection dose ($F_3 = 0.31, p = 0.82$; see Figure 3.1).
3.3.1.2  

Courtship

A one-way repeated measures ANOVA was used to examine the effect of AVT dose on courtship behavior. There was no difference in the number of courtship bouts with any injection dose ($F_3 = 0.55, p = 0.66$; see Figure 3.2).

The hormonal status of male anoles (VEH, lAVT, mAVT, hAVT) did not affect the number of courtship displays performed by the untreated females paired with them (one-way ANOVA; $F_3 = 0.41, p = 0.75$; see Figure 3.3).

3.3.1.3  

Plasma testosterone

A two-way repeated measures ANOVA was conducted with behavior (courtship or aggression) and dose (Veh, lAVT, mAVT, hAVT) as factors (see Figure 3.4). There was no main effect of behavior type on circulating testosterone ($F_1 = 0.01, p = 0.92$). There was also not a main effect of dose on circulating testosterone ($F_3 = 0.11, p = 0.95$). There was not a significant interaction between behavior and dose ($F_3 = 0.54, p = 0.66$).

3.3.1.4  

Plasma corticosterone

To examine the effect of behavior and AVT dose on plasma corticosterone, a two-way repeated measures ANOVA was used (see Figure 3.5). There was not a main effect of behavior ($F_1 = 3.60, p = 0.08$) however a significant main effect of dose was observed ($F_3 = 19.82, p < 0.01$). Tukey tests were used to conduct multiple pair-wise comparisons. Injection with vasotocin at any dose resulted in a significant increase in circulating corticosterone over VEH (lAVT: $q = 9.38, p < .01$; mAVT: $q = 8.94, p < 0.01$; hAVT: $q = 8.54, p < 0.01$). Within the hAVT dose, corticosterone was increased significantly more in animals in the mirror aggression group than in the courtship group ($q = 3.17, p = 0.32$). This
difference was not observed at any other dose although a trend in the same direction was present with mAVT injection (VEH: q = 0.73, p = 0.61; lAVT: q = 1.89, p = 0.19; mAVT: q = 2.74, p = 0.06).

### 3.3.2 Experiment 2: AVT and no behavior challenge

#### 3.3.2.1 Plasma testosterone

A t-test revealed no significant difference in the level of circulating T between AVT and VEH injected animals ($t_{17} = -2.05, p = 0.06$; see Figure 3.6).

#### 3.3.2.2 Plasma corticosterone

Animals injected with AVT had significantly higher circulating CORT than those injected with VEH (Mann-Whitney U = 100.00, $p < 0.001$; see Figure 3.7).

#### 3.3.2.3 Steroid hormone correlations

A Pearson product-moment correlation (PPMC) revealed a trend toward an overall relationship between plasma corticosterone and testosterone (natural log transformed; $r_{17} = 0.43, p = 0.06$; see Figure 3.8A) when data were collapsed across treatment condition (VEH and AVT). This suggests that overall, animals with high levels of corticosterone tend to have high levels of circulating testosterone. However when I examined the correlation between corticosterone and testosterone by treatment condition, I did not observe a significant relationship in either the VEH (natural log transformed; $r_7 = -0.11, p = 0.78$; see Figure 3.8B) or AVT (natural log transformed; $r_8 = 0.34, p = 0.34$; see Figure 3.8C) treated groups.
3.4 Discussion

The goal of these experiments was to understand the effect of AVT on steroid endocrinology and the behavioral impact of increasing AVT doses. Previously I have demonstrated that treatment with approximately 3 µg/g body weight resulted in a significant increase in circulating corticosteroids in animals exposed to a social behavior inducing stimulus (mirror, conspecific male, female; Dunham and Wilczynski, 2014). The number of aggressive displays to a mirror was also reduced however, no effect of AVT on corticosterone was observed at that dose. In the present study I demonstrate that AVT at three different doses (1 µg/g, 3 µg/g, 10 µg/g) significantly increases CORT and at the highest dose, AVT increases CORT more in aggressing than courting animals. I did not observe any changes in social behavior at any dose of AVT. I also found that a social behavior stimulus is not necessary for AVT-induced increases in CORT.

In the current experiment, I did not observe a change in mirror aggression at any dose of AVT. The mAVT group received the same dose that resulted in reduced aggression in our previous experiment (Dunham and Wilczynski, 2014). While I do not have a clear explanation for this discrepancy in results, it should be noted that the overall number of aggressive display bouts observed in the control group during this reproductive season was less than half what had been observed in the previous year (Current: VEH M = 12.14 bouts vs. Previous: VEH M = 30.67 bouts). This variability in behavior could be due to many factors including environmental conditions in the field prior to capture, a change in the testing room from previous experiments, and the use of a repeated measures design where animals received four injections and behavior experiences over the course of the experiment. In addition, other researchers have suggested that consistent behavior effects
are harder to achieve when AVT is administered IP as opposed to intracerebroventricularly (Salek et al., 2002). It is also possible that AVT does not in fact have an effect on aggressive behavior as our previous study suggested. Any conclusions, however, should be drawn with caution given the relatively low levels of behavior overall.

I also did not observe an effect of AVT on courtship behavior at any dose. This corroborates our previous results. In green anole lizards, there is currently no evidence to suggest that AVT contributes to courtship behavior. Unlike our previous experiment where females paired with AVT treated males performed more courtship bouts than those paired with VEH treated males, I did not observe any effect of male hormone condition on female behavior. It is possible that the generally low levels of behavior among this cohort of animals masked any such effects.

In addition to the behavioral dose response, I also examined the endocrine effects of different AVT concentrations. While there was no effect on testosterone concentrations, AVT at all doses resulted in a significant increase in circulating corticosterone for animals in both the mirror aggression and courtship conditions. There were no differences in corticosterone conditions by dose within each behavior group suggesting that a dose of AVT as low as 1 µg/g body weight injected systemically results in a ceiling effect for corticosterone release. I did observe an interaction between the type of social behavior (aggression vs courtship) at the hAVT dose with CORT increasing significantly more in animals presented with a mirror than with a female. This result suggests that the impact of AVT on CORT release may differ depending on the type of stimulus (perceived male intruder vs. female conspecific). It is important to note that corticosterone concentrations were not different between aggression and courting animals in blood samples drawn
following VEH injection suggesting that the aggressive experience itself cannot account for the differences in circulating CORT with AVT.

Finally, I examined the effect of AVT injection on testosterone and corticosterone in single housed males without a social behavior challenge. There was not a significant effect of AVT on circulating T although there was a trend for higher T following AVT treatment (p = 0.06). AVT treatment did result in a significant increase in circulating CORT in animals without a behavior challenge. The level of CORT observed following AVT treatment was similar to what has been observed in animals following AVT and social behavior tests suggesting that AVT has a potent effect on CORT levels with or without social behavior. This result is corroborated by evidence from birds (Nephew et al., 2005). I also examined the relationship between testosterone and corticosterone in these animals. When data were collapsed across treatment condition (VEH and AVT) there was a trend toward a positive correlation between CORT and T levels (p = 0.06). However, this relationship was not observed when data were considered by treatment (VEH or AVT only). These data suggest that AVT effects on corticosterone do not necessarily lead to changes in testosterone.

Overall, these results demonstrate the potent ability of AVT to stimulate the release of CORT in green anole lizards. Regardless of dose and behavior, experience (mirror aggression, courtship, or no behavior) animals given exogenous AVT experience a significant increase in circulating CORT thirty minutes after an injection. In contrast, AVT treatment generally has little effect on circulating testosterone. I also found that social context might play a role in influencing the magnitude of the AVT – CORT response.
3.5 Acknowledgements

Thank you to Deborah Lutterschmidt, Javonte Carter, Skylar McHenry, and Sheena Palmer for their technical assistance and David Sinkiewicz and Bryce Chung for their helpful discussions during the preparation of this work.

3.6 Funding

Supported by the National Science Foundation [IBN 0751573 to W.W.] and the Georgia State University Brains & Behavior Area of Focus.
Figure 3.1 Effect of AVT doses on aggressive behavior bouts
There was no significant effect of AVT at any dose on the number of aggressive behavior bouts to a mirror. White bars indicate VEH injection and grey bars represent increasing doses of AVT. Data are presented as mean ± SEM and group sizes are indicated in parentheses.
Figure 3.2 Effect of AVT dose on courtship behavior bouts
There was no significant effect of AVT at any dose on the number of courtship behavior bouts. White bars represent VEH control injection and the grey bars indicate increasing doses of AVT. Data are presented as mean ± SEM and group sizes are indicated in parentheses.
Figure 3.3 Female courtship behavior to AVT treated males
There was no significant effect of male treatment condition on the number of courtship behaviors performed by untreated females. White bars represent VEH control injected males and the grey bars indicate increasing doses of AVT. Data are presented as mean ± SEM and group sizes are indicated in parentheses.
Figure 3.4 Effect of AVT dose on testosterone in courting and aggressing animals
At no dose of AVT is plasma testosterone significantly affected, regardless of the behavior challenge experienced. There was not a significant main effect of either behavior type or AVT dose. White bars represent animals in the courtship behavior condition and grey bars represent mirror aggression animals. Data are presented as mean ± SEM and group sizes are indicated in parentheses.
Figure 3.5 Effect of AVT dose on corticosterone in courting and aggressing males
AVT at any dose causes a significant increase in circulating corticosterone in both courting and aggressing males. At both the mid- and high-AVT doses, AVT increases circulating corticosterone more in aggressing animals than in courting individuals. This difference in circulating corticosterone between aggressing and courting animals is not observed in the VEH treated control groups. White bars indicate courting animals and grey bars denote the mirror aggression group. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses. Asterisks represent statistical significance (p < 0.05). Different letters indicate significant differences between groups (p < 0.05).
Figure 3.6 Effect of AVT on plasma testosterone without a behavior challenge
There was no significant effect of AVT treatment on circulating plasma testosterone in animals without a behavior challenge. White and grey bars represent VEH- and AVT-injected animals, respectively. Data are presented as mean ± SEM and group sizes are indicated in parentheses.
Figure 3.7 Effect of AVT on plasma corticosterone without a behavioral challenge
AVT treatment significantly increases circulating corticosterone in animals with no behavioral challenge. White and grey bars represent VEH- and AVT-injected animals, respectively. Data are presented as mean ± SEM and group sizes are indicated in parentheses. Asterisk indicates statistical significance (p < 0.05).
Figure 3.8 Correlation between plasma testosterone and corticosterone
There was not a significant relationship between corticosterone and testosterone in animals without a behavior challenge. This was true for (A) data collapsed across drug condition (VEH and AVT), (B) VEH treated animals only, and (C) AVT treated animals. Hormone concentrations are natural log normalized. VEH treated animals are represented by open circles, AVT treated animals are shown in closed circles.
4 THE EFFECT OF METYRAPONE AND ARGinine VASOTOCIN ON CORTICOSTERONE AND AGGRESSIVE BEHAVIOR IN GREEN ANOLE LIZARDS (*Anolis carolinensis*)

4.1 Introduction

Interactions between hormone systems play an important role in the expression of social behavior. Steroids such as testosterone (T) and corticosterone (CORT) can potently influence the expression and intensity of courtship and territorial aggression-related behaviors, both individually and through reciprocal interactions (DeNardo and Licht, 1993; Leary et al., 2006; Moore and Miller, 1984; Rose and Moore, 2002; Trainor and Nelson, 2012; Wade, 2005; Wingfield et al., 1998). In addition to the steroid hormones, neurohypophysial hormones such as arginine vasotocin (AVT) have been associated with the regulation of social behavior in a variety of non-mammalian species including fish, birds, and non-avian reptiles (Balment et al., 2006; Dunham and Wilczynski, 2014; Goodson and Bass, 2001; Rose and Moore, 2002).

I found that exogenous administration of AVT in green anole lizards resulted in fewer aggressive displays over the course of a 30-minute mirror aggression trial (Dunham and Wilczynski, 2014). This was in line with the behavioral effects of AVT that have been reported in other non-mammalian territorial species (Goodson, 1998a, b; Semsar et al., 2001). In addition to the behavior effects, however, I also found that AVT injection resulted in a significant increase in circulating corticosterone (CORT). While this is not necessarily surprising given the well-established role of AVT in stimulating the hypothalamic-pituitary-adrenal (HPA) axis (Gibbs, 1986), it does complicate the question of AVT’s role in social
behavior regulation. Does AVT affect CORT levels in the absence of a social stimulus? Does AVT contribute independently to the production of social behavior or does it suppress aggressive behavior primarily through activation of the HPA axis to stimulate CORT release?

These questions are not insignificant considering that most studies on the social behavior effects of AVT/AVP do not report circulating steroid concentrations following treatment and that CORT is known to have potent effects on social behavior. For example, brown anoles implanted with CORT show fewer aggressive displays when tested one week later (Tokarz, 1987). Similarly, green anoles that become subordinate maintain an elevated CORT level for a longer period of time than do dominant animals (Summers et al., 2003). There is strong evidence for interactions between AVT/AVP and steroid hormones, providing a potential mechanism through which behavioral variability and context-dependent responses could be achieved. For example, AVT/AVP cells are androgen sensitive and castration reduces immunoreactive cell number (Boyd, 1997; Kabelik et al., 2008; Miller et al., 1992; Wang and De Vries, 1993).

There is, however, evidence suggesting that the overall behavioral actions of AVT/AVP and CORT may be in opposition. For example, AVT stimulates courtship behavior while CORT suppresses it, and priming the system with one can block the effects of the other (Burmeister et al., 2001; Coddington and Moore, 2003). There are physiologically important interactions between AVT/AVP and steroid hormones and these interactions are likely to be critical influences on the expression of social behavior.

Therefore, the aim of this study was to determine 1) if AVT increases CORT in the absence of a social stimulus and 2) if AVT treatment affects aggressive behavior.
independently via effects on the brain or if the suppression of behavior by AVT is due primarily to its activation of the HPA axis and stimulation of CORT release. To address question 2, I employed the CORT synthesis inhibitor metyrapone.

4.2 Methods

4.2.1 Animals

Forty-eight male anole lizards were obtained from Charles Sullivan, Inc. (Nashville, TN) and were housed individually in half of a 10 gallon glass aquarium (24x12x18) on a 14L:10D light cycle with an ambient daytime temperature of 26-28°C. Each enclosure contained artificial leaves, a water dish, and an elevated perch. Animals were fed calcium gut-loaded crickets (Ghann’s Cricket Farm; Augusta, GA) three times weekly and water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University.

4.2.2 Mirror aggression

Individually housed male anole lizards were used for the mirror aggression tests. Previous studies have demonstrated that anoles will reliably and robustly aggress toward their reflected image (Baxter Jr et al., 2001; Farrell and Wilczynski, 2006). This method of eliciting behavior allows us to examine how AVT affects aggressive displays when the animal’s own behavior output matches the visual input it observes and a relative social status is not achieved.

Animals were allowed to acclimate to the laboratory conditions for at least one week prior to any behavior test. Each animal was randomly assigned to one of four
treatment conditions (n=12 per group): VEH/VEH, VEH/AVT, MET/VEH, MET/AVT. The 
VEH (50 μl reptile ringers), AVT (15 μg AVT/50 μl reptile ringers), and MET (250 μg 
MET/50 μl reptile ringers) injections were administered IP. The dose of MET (2-methyl-
1,2-di-3-pyridyl-1-propanone) given here, approximately 50 μg/g body mass, was used 
previously in green anole lizards (Yang and Wilczynski, 2003). In addition, MET 
ammodation in another non-avian reptile, the desert iguana, has been shown to 
significantly suppress circulating CORT within the time course of our experiment 
(Scholnick et al., 1997). Thirty minutes after the pretreatment injection (VEH or MET), the 
second treatment injection was given (VEH or AVT). Animals were allowed 30 min to 
recover from the second injection, after which time a two-way mirror (Reflection Products, 
Inc.; Roswell, GA) was placed in front of the home cage of a single-housed male. Behavior 
displays were captured by a video camera placed directly behind the mirror (Sony 
Handycam) and interactions with the mirror were filmed for 30 minutes. All behavioral 
tests were conducted between 10:30am and 1:30pm on the day of testing. Males were then 
sacrificed via an overdose with IP injected Nembutal. Trunk blood was collected within 3 
minutes of capture and stored at -20°C until processing via ELISA.

4.2.3 Behavior definition and scoring

Aggressive display behaviors have been thoroughly characterized for this species 
(Crews, 1975; DeCourcy and Jenssen, 1994; Garcia et al., 2014; Greenberg and Crews, 
1990b; Korzan et al., 2000; Lovern and Jenssen, 2003; Yang et al., 2001). Males engaging in 
aggressive displays perform pushups and head bobs, often in conjunction with extension of 
the red dewlap throat fan. Pushups occur when the body is moved up and down by flexing 
of the animal’s forelimbs. Head bobs, in contrast, involved movement of the head only.
These behaviors are typically accompanied by lateral compression of the body and nuchal crest extension; causing the challenging male to appear larger overall. Aggressive bouts can further escalate to include jaw sparring and biting, as well as the formation of a dark “eye spot” posterior to the animal’s orbit. At the conclusion of an aggressive interaction, the ‘loser’ will often develop a brown body color indicative of a subordinate status while the ‘winner’ or dominant animal will remain green.

The taped 30-minute trials were scored using Stopwatch + software (www.cbn-atl.org) by individuals blind to the injection condition. The latency to initiate behavior as well as the number of behavioral bouts was recorded. A single bout is defined as a rapid burst of display-associated behaviors (often including 5-10 instances of head-bobbing and extension of the red dewlap throat fan) followed by a brief pause. Quantification of behavioral bouts was chosen over individual behavior number as discrete display-associated behaviors (dewlap extension and head bob) are rarely performed in isolation but rather occur in highly stereotyped patterns (DeCourcy and Jenssen, 1994; Jenssen et al., 2012; Lovern and Jenssen, 2003; Orrell and Jenssen, 2003), suggesting that bout number provides a more salient measure of the overall level of social signaling. To account for temporal differences in behavior due to metabolism of drug treatments, I separated the 30 min behavior test into three 10-minute segments. Treated males that failed to perform at least one bout of behavior were considered non-responsive and excluded from the final analysis.

4.2.4 Hormone analysis

The plasma samples from all individuals were spiked with approximately 2000 cpm of [3H]-CORT to allow for measurement of extraction efficiency. Samples were diluted 1:25,
extracted with 3 ml of ether, and dried at 37°C under a nitrogen stream. The extracted samples were reconstituted with assay buffer (1:25 to 1:50 for testosterone; 1:300 to 1:750 for corticosterone) and analyzed via ELISA (Caymen Chemical; Ann Arbor, MI) for both T and CORT according to the manufacturer’s instructions. These assays have been previously validated for Anolis carolinensis by our lab and others using pooled plasma serial dilutions (Garcia et al., 2012; Yang and Wilczynski, 2003). All plates were run concurrently and each sample was assayed in triplicate. Sample recovery rates were used to correct for extraction efficiency. Overall mean recovery was 77.3% and no corrections were made for samples with greater than 100% recovery. The assays have a sensitivity of 32 pg/ml for T and 150 pg/ml for CORT according to the manufacturer’s analysis.

4.3 Results

4.3.1 Mirror aggression

When I examined the effects of pretreatment and treatment injections on the total number of aggressive displays to a mirror during the 30 minute trial, I did not observe an overall significant effect of either pretreatment ($F_{1,35} = 0.06, p = 0.81$) or treatment ($F_{1,35} = 3.52, p = 0.07$) on behavior (Figure 4.1). Due to the length of the protocol from first injection until the conclusion of the mirror aggression test, I elected to examine behavior on a finer time scale in order to account for any issues of drug metabolism. To accomplish this, I divided the 30-minute trial into 3 bins and examined the number of responses observed within each bin (See Figure 4.2). Within the first bin, representing the first 10 minutes of the trial, there was no significant effect of either pretreatment ($F_{1,35} = 3.83, p =
or treatment ($F_{1,35} = 1.02, p = 0.32$) on the number of bouts. Similarly, no differences were observed during the second bin (minutes 10-20 of the total trial; pretreatment: $F_{1,35} = 0.15, p = 0.70$; treatment: $F_{1,35} = 0.20, p = 0.66$). There were differences observed in the number of aggressive displays produced during the final 10 minutes of the trial, bin 3. During the final 10 minutes, a main effect of treatment on aggressive bouts was observed ($F_{1,35} = 6.12, p = 0.02$). Tukey tests to examine multiple pairwise comparisons of group differences showed that animals injected with AVT perform significantly fewer displays to the mirror during the final 10 minutes of the trial than those treated with VEH, regardless of their pretreatment injection (VEH or MET; $q = 3.11, p = 0.04$).

### 4.3.2 Endocrine effects

#### 4.3.2.1 Testosterone

A two-way ANOVA with pretreatment (VEH or MET; $F_{\text{ranks}} = 0.004, p = 0.94$) and treatment (VEH or AVT; $F_{\text{ranks}} = 0.60, p = 0.45$) as factors revealed no effect of either pretreatment or treatment drugs on circulating testosterone (Figure 4.3).

#### 4.3.2.2 Corticosterone

A two-way ANOVA revealed main effects of pretreatment ($F_{\text{ranks}} = 7.95, p = 0.008$) and treatment ($F_{\text{ranks}} = 71.41, p < 0.001$) on circulating CORT (Figure 4.4). Within the pretreatment groups (VEH or MET), those animals receiving an injection of the CORT synthesis inhibitor MET had significantly lower CORT than VEH injected controls regardless of treatment injection ($q_{\text{ranks}} = 3.99, p = 0.008$). Within treatment (VEH or AVT), AVT led to a significant increase in circulating CORT as compared to VEH ($q_{\text{ranks}} = 11.95, p <
There was not a significant interaction between pretreatment and treatment ($F_{\text{ranks}} = 0.02, p = 0.52$).

To isolate group differences in circulating CORT, Tukey tests were used to examine pairwise multiple comparisons. Of those individuals pretreated with VEH, animals subsequently injected with AVT showed a significant increase in CORT as compared to those treated with VEH (two-tailed; $q_{\text{ranks}} = 8.19, p < 0.001$). Similarly, among individuals pretreated with MET, animals subsequently injected with AVT also showed a significant increase in CORT as compared to those receiving VEH just prior to behavior (two-tailed; $q_{\text{ranks}} = 8.72, p < 0.001$). Among those animals receiving treatment with VEH, pretreatment with MET significantly lowered circulating CORT suggesting that our dose of MET was capable of reducing CORT synthesis (two-tailed; $q_{\text{ranks}} = 2.93, p = 0.046$). Among those treated with AVT, there was no difference between pretreatment drug condition (two-tailed; $q_{\text{ranks}} = 2.71, p = 0.06$).

The relationship between circulating CORT and aggressive behavior was examined through a number of PPMC analyses (CORT data were not normally distributed and were natural log transformed prior to PPMC). When treatment groups are collapsed, no correlation was observed between CORT and the number of aggressive display bouts ($r_{39} = -0.15, p = 0.36$; Figure 4.5A). When each treatment group was considered individually, no significant correlations were observed for MET/AVT ($r_{8} = 0.11, p = 0.76$; Figure 4.5B), MET/VEH ($r_{8} = 0.39, p = 0.27$; Figure 4.5C), VEH/AVT ($r_{8} = -0.41, p = 0.23$; Figure 4.5D), and VEH/VEH ($r_{7} = 0.18, p = 0.69$; Figure 4.5E).
4.4 Discussion

The goal of this study was to begin to untangle the complicated relationship between corticosterone and arginine vasotocin in the regulation of social behavior. Previously, I have demonstrated that AVT significantly reduces aggressive responding in the territorial green anole lizard. This result was confounded, however, by a dramatic AVT induced spike in circulating CORT (Dunham and Wilczynski, 2014) which occurred regardless of the type of behavior (aggression vs. courtship) and the manner in which it was stimulated (mirror vs. live pairing). In the present study, I demonstrate that AVT reduces aggression even when the CORT response is significantly reduced by treatment with a CORT synthesis blocker.

In this study, AVT treatment following VEH injection failed to significantly affect overall aggressive behavior although there was a trend toward an effect (P = 0.07). Due to the length of the protocol (two injections and recovery periods), I decided to analyze behavior during shorter 10-minute segments of the trial in order to account for any effects of drug washout. When looked at in this way, I did find a significant effect of AVT treatment on behavior during the final 10-minutes of the trial. Animals treated with AVT showed less aggressive responding in the final 10-minutes. The reason for this time dependent effect is unclear although it may point to a role for AVT in the motivational aspects of aggressive behavior. Perhaps these animals simply stop responding to a perceived intruder faster than animals with normal levels of AVT, thus reducing their overall number of behavioral bouts. The literature does suggest a role for AVT/AVP in social behavior motivation (Semsar et al., 2001). It is interesting to note that among animals injected with AVT, a consistent trend is observed where behavior starts at a low level, increases slightly, and
then falls again over the course of the trial (See Figure 4). This pattern is not observed among animals treated only with VEH, who tend to show a stable level of behavior throughout the duration of the stimulus.

Our results demonstrated that the dose of metyrapone administered in this study was sufficient to blunt the CORT response to an aggressive stimulus. When I consider the control group receiving two subsequent VEH treatments with the group injected first with MET and second with VEH, I see a significant reduction in CORT measured at the conclusion of the 30-minute aggression trial, suggesting that MET-treated animals were unable to fully mount a typical CORT response. However, I did observe significant increases in CORT among all animals treated with AVT, even in individuals pretreated with the dose of MET I used. There are several possible reasons for the incomplete CORT blockage. The first is that the dose of MET given here is sufficient to blunt CORT responses but that its blockage of 11β-hydroxylase is incomplete, thereby providing a mechanism where AVT can still increase CORT through its normal HPA axis stimulation of ACTH. With a higher dose of MET, I might observe a more complete blockage of CORT even with AVT treatment. It is also possible that AVT is stimulating CORT from a site other than the adrenal glands. In other species, very low levels of central CORT synthesis have been observed in the hippocampus where they are thought to play a role in regulating synaptic plasticity (Higo et al., 2011). This CORT however, is derived from the same enzymatic pathway as in the adrenals, requiring 11β-hydroxylase. Metyrapone is able to cross the blood brain barrier in pigs and humans (Otte et al., 2003). The permeability of the blood brain barrier in green anole lizards has been studied (Kenny and Shivers, 1974; Shivers, 1979; Shivers and Harris, 1984) although the ability of metyrapone to pass has not been
established, nor is it known if there is central CORT synthesis. Given the level of the increase I observed in circulating hormone levels, a 7.5 fold increase in CORT among those individuals treated with MET followed by AVT compared to MET only, this does not seem to be a likely explanation. Ultimately, the results here limit our ability to evaluate the distinct contribution of AVT to aggressive behavior, although I believe there are still important conclusions that can be drawn, especially considering that no correlation between circulating corticosterone and the number of aggressive behavior bouts was observed either when groups were collapsed across conditions or when considered within treatment groups (Figure 4.5). This suggests that the differences observed in behavior are not in fact driven primarily by CORT.

Overall, these data suggest an independent effect of AVT on aggressive behavior beyond its effect on CORT. AVT animals treated with metyrapone, and with a somewhat reduced CORT response still show a decrease in aggression. In addition, no evidence for a correlation between CORT and behavior bouts was observed. AVT has a strong effect on CORT release, but the data tend not to support the idea that AVT's behavioral effects depend on its elevation of CORT.

4.5 Acknowledgements

Thank you to Deborah Lutterschmidt, Hillary Doyle, Sheena Palmer, and Anandhi Martin for their technical assistance and to David Sinkiewicz and Bryce Chung for their helpful discussions during the preparation of this work.
4.6 Funding

Supported by the National Science Foundation [IBN 0751573 to W.W.] and the Georgia State University Brains & Behavior Area of Focus.

Figure 4.1 Effect of AVT and MET on total aggressive behavior bouts
There was no effect of drug treatment on the total number of aggressive behavior bouts although a trend was observed where animals receiving AVT performed fewer overall bouts than those injected with VEH. White and grey bars indicate VEH- and MET-pretreatment, respectively. Hashed bars indicate AVT-treatment. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses.
Figure 4.2 Effect of AVT and MET on aggressive behavior bouts by 10-minute bin

In the final 10-minutes of the interaction, animals injected with AVT performed significantly fewer bouts than those injected with VEH. Bars represent the mean number of aggressive display bouts per 10-minutes of trial. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses.
Figure 4.3 Effect of AVT and MET on plasma testosterone
There was no effect of drug treatment on circulating plasma testosterone. White and grey bars indicate VEH- and MET-pretreatment, respectively. Hashed bars indicate AVT-treatment. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses.
Figure 4.4 Effect of AVT and MET on plasma corticosterone

Pretreatment with MET significantly reduced circulating corticosterone in animals subsequently treated with VEH, compared to the VEH only controls (A vs B). Treatment with AVT significantly increased CORT, regardless of the pretreatment injection (A & B vs C). Within the VEH pretreatment groups, AVT treatment significantly increased CORT (#). Within the MET pretreatment groups, AVT treatment significantly increased CORT (*). Within AVT treatment, there was a non-significant trend ($p = 0.06$) for lower CORT in those pretreated with MET. White and grey bars indicate VEH- and MET-pretreatment, respectively. Hashed bars indicate AVT-treatment. Data are presented as mean ± SEM and group sample sizes are indicated in parentheses. Significance indicated at $p < 0.05$. 
Figure 4.5 Correlation between corticosterone and aggressive behavior bouts

There is not a significant correlation between corticosterone and aggressive behavior when data are collapsed across treatment condition (A). There are also no significant correlations when data are considered by treatment group (B-E).
5 THE EFFECT OF AGGRESSIVE BEHAVIOR EXPERIENCE ON AVT CELLS IN THE BRAIN OF GREEN ANOLE LIZARDS (*ANOLIS CAROLINENSIS*)

5.1 Introduction

The social behavior neural network comprises a number of brain regions that are functionally connected and implicated in the control of a variety of behaviors including courtship and affiliation, chemical and vocal communication, and aggression (Albers, 2012; Albers, 2014; Goodson, 2005; O’Connell and Hofmann, 2012). Evidence suggests that interactions between these regions contribute to variations in behavioral responses. Neuropeptides - their levels, release, and receptor distribution – are likely candidates for coordinating the activity of regions within the network. Individual differences in the neuropeptide arginine vasotocin (AVT) and arginine vasopressin (AVP) may represent one source of variation in social behavior (Albers, 2014; Goodson and Bass, 2001).

Arginine vasopressin is a neuropeptide, synthesized primarily in the hypothalamus, and found in peptidergic neurons within many of the social behavior neural network brain regions including the periventricular nucleus (PVN), the anterior hypothalamus (AH), the supraoptic nucleus (SON), and the preoptic area (POA) with AVT positive fibers extending both rostrally and caudally into extrahypothalmic regions (for review, see Goodson and Bass, 2001). AVT has been implicated in social behavior in a wide variety of species including fish, amphibians, birds, and reptiles (for reviews, see Albers, 2012; Balment et al., 2006; Caldwell et al., 2008; Goodson and Bass, 2001; Rose and Moore, 2002). While significant roles for AVT in affiliative and courtship behaviors have been
observed (for reviews, see: Caldwell et al., 2008; Insel, 1997, 2010; Keverne and Curley, 2004), it is AVT's effect on the expression of aggressive behavior that highlights the importance of this peptide in social behavior diversity. In the territorial violet eared waxbill, for example, septal infusions of AVT reduced overt aggression while these same treatments increased aggression in the closely related colonial zebra finch (Goodson, 1998b; Goodson and Adkins-Regan, 1999). Similarly, when territorial and non-territorial morphs of the same species are compared, AVT can show differential effects on aggression (Semsar et al., 2001). Recently, our lab has also identified a role for AVT in territorial aggression in green anole lizards (Dunham and Wilczynski, 2014). As has been observed in other naturally territorial species, I found that exogenous administration of AVT was associated with a significant reduction in the number of aggressive displays to a mirror.

Growing evidence suggests that both natural and experience driven differences in the components and activity of the AVT/AVP system – including peptide mRNA expression, cell number and type, patterns of cellular activity, and receptor density – correlate with the observed variability in behavioral effects. For example, the number and type of ir-positive cells (gigantocellular versus parvocellular) within the preoptic area of the multiband butterflyfish have been associated with increased and decreased aggression respectively (Dewan and Tricas, 2011) and the density of AVT-ir fibers correlate negatively with aggression in the beaugregory damselfish (Santangelo and Bass, 2010). In addition, evidence from the brown anole shows that the pattern of cellular activation in AVT cell populations tracks the type (male-male vs male-female), frequency, and intensity of behavior in a region specific manner within the social behavior neural network suggesting an important role for AVT in encoding social stimuli (Kabelik et al., 2013).
In addition, social experience may also play a role in shaping the AVT system. Social status as been correlated with changes in AVT cells in a number of species (Delville et al., 1998; Larson et al., 2006; Hattori and Wilczynski, 2009). In green anoles, chronic maintenance of a subordinate social status (10 day pairing) resulted in a decrease in the number of AVT-ir cells in the POA as compared to both dominant and single housed males (no-status; Hattori and Wilczynski, 2009). These results point to the ability of social status to shape the AVT system in the brain although it is important to consider that achieving a relative social status also results in a cascade of other changes, both short and long lived. For example, dominant anoles have a dramatic increase in testosterone one hour after winning but show a return to baseline after one week whereas testosterone remains suppressed in subordinate animals after one week (Greenberg and Crews, 1990b). Testosterone changes that follow from the formation of a social status hierarchy may affect AVT cell number given the steroid sensitivity of AVT cell number (Kabelik et al., 2008). In order to examine the contribution of aggressive behavior experience alone on AVT cell number in green anole lizards I utilized mirror-induced aggression, a paradigm in which males display to their own reflection (Baxter Jr et al., 2001; Farrell and Wilczynski, 2006). This procedure does not lead to changes in circulating androgens (Dunham and Wilczynski, 2014) and uncouples the physical experience of engaging in aggression from the effects of chronic social status.
5.2 Methods

5.2.1 Animals

Eighty male anole lizards were obtained from Charles Sullivan, Inc. (Nashville, TN) in May, 2012 and were housed individually in half of a 10 gallon glass aquarium (24x12x18) on a 14L:10D light cycle with an ambient daytime temperature of 26-28°C. Each enclosure contained artificial leaves, a water dish, and an elevated perch. Animals were fed calcium gut-loaded crickets (Ghann's Cricket Farm; Augusta, GA) three times weekly and water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University.

5.2.2 Repeated aggression

Individually housed male anole lizards were used for these mirror aggression tests. Previous studies have demonstrated that anoles will reliably and robustly aggress toward their reflected image (Baxter Jr et al., 2001; Farrell and Wilczynski, 2006). This method of eliciting behavior allows us to examine how aggressive displays may affect the AVT system when the animal's own behavior output matches the visual input it observes and a relative social status is not achieved.

Animals were randomly assigned to one of four groups for the number of exposures to a stimulus: 1(4hr), 1(24hr), 5 days, 10 days. Animals in both the 1(4hr) and 1(24hr) groups were exposed to the stimulus one time and then sacrificed either 4 hours or 24 hours after the single presentation. For the 5 day and 10 day groups, animals were exposed to the stimulus 20-minutes per day for that number of consecutive days and sacrificed 4 hours after the final exposure day. Within those groups, half of the animals
were assigned the aggression-producing stimulus – reflective surface of the mirror – and half were assigned the neutral stimulus – back of the mirror. Animals were allowed to acclimate to the laboratory setting for at least one week prior to any behavioral testing and all tests were conducted between 10:30 AM and 1:30 PM. On each day of testing, a video camera (Sony Handycam) was placed directly in front of an animal’s home cage. The video camera was left there for 15 minutes in order to allow the animal to acclimate to its presence. Following this period of time, the mirror stimulus was placed in the back of the home cage with either the reflective or the non-reflective surface facing the animal, depending on the condition. The mirror was present for 20 minutes, during which behavior was recorded. With the exception of the 1(24hr) group, animals were sacrificed 4 hours after their final stimulus presentation. Those in the 1(24hr) group were sacrificed 24 hours after a single exposure to the stimulus. Lizards were sacrificed via an overdose of IP injected Nembutal. Trunk blood was collected within 3 minutes of capture and stored at -20°C until analysis. Brains were fixed for 18 hours in 4% paraformaldehyde and sectioned at 20µm using a Leica CM3050 cryostat. Sections were thaw mounted to Superfrost/Plus slides (Fisher) until immunohistochemical (IHC) processing.

5.2.3 Behavior definition and scoring

Aggressive display behaviors have been thoroughly characterized for this species (Crews, 1975; DeCourcy and Jenssen, 1994; Garcia et al., 2014; Greenberg and Crews, 1990b; Korzan et al., 2000; Lovern and Jenssen, 2003; Yang et al., 2001). Males engaging in aggressive displays perform pushups and head bobs, often in conjunction with extension of the red dewlap throat fan. Pushups occur when the body is moved up and down by flexing of the animal’s forelimbs. Head bobs, in contrast, involved movement of the head only.
These behaviors are typically accompanied by lateral compression of the body and nuchal crest extension; causing the challenging male to appear larger overall. Aggressive bouts can further escalate to include jaw sparring and biting, as well as the formation of a dark “eye spot” posterior to the animal’s orbit. At the conclusion of an aggressive interaction, the ‘loser’ will often develop a brown body color indicative of a subordinate status while the ‘winner’ or dominant animal will remain green.

The taped 20-minute trials were scored using Stopwatch + software (www.cbn-atl.org). The latency to initiate behavior as well as the number of behavioral bouts was recorded. A single bout is defined as a rapid burst of display-associated behaviors (often including 5-10 instances of head-bobbing and extension of the red dewlap throat fan) followed by a brief pause. Quantification of behavioral bouts was chosen over individual behavior number as discrete display-associated behaviors (dewlap extension and head bob) are rarely performed in isolation but rather occur in highly stereotyped patterns (DeCourcy and Jenssen, 1994; Jenssen et al., 2012; Lovern and Jenssen, 2003; Orrell and Jenssen, 2003), suggesting that bout number provides a more salient measure of the overall level of social signaling.

5.2.4 Hormone analysis

The plasma samples from all individuals were spiked with approximately 2000 cpm of [3H]-CORT to allow for measurement of extraction efficiency. Samples were diluted 1:25, extracted with 3 ml of ether, and dried at 37°C under a nitrogen stream. The extracted samples were reconstituted with assay buffer (1:25 to 1:50 for testosterone; 1:300 to 1:500 for corticosterone) and analyzed via ELISA (Caymen Chemical; Ann Arbor, MI) for both T and CORT according to the manufacturer’s instructions. These assays have been previously
validated for *Anolis carolinensis* by our lab and others using pooled plasma serial dilutions (Garcia et al., 2012; Yang and Wilczynski, 2003). All plates were run concurrently and each sample was assayed in triplicate. Sample recovery rates were used to correct for extraction efficiency. Overall mean recovery was 64.5% and no corrections were made for samples with greater than 100% recovery. Overall intra-assay coefficients of variation were 4.30% for testosterone and 10.17% for corticosterone. The assays have a sensitivity of 32 pg/ml for T and 150 pg/ml for CORT according to the manufacturer’s analysis.

### 5.2.5 Immunohistochemistry

Following cryostat sectioning of tissue, slides containing anole brains were removed from storage at -20°C and allowed to warm to room temperature. I outlined the slides using a PAP pen (info) to create a hydrophobic barrier that improves moisture coverage over the sections. Slides were then soaked in five changes of 0.1M PBS for five minutes each (5 x 5min). Following rehydration of the tissue, I quenched endogenous peroxidases with 1% hydrogen peroxide in methanol for thirty minutes. Slides were rinsed again in 0.1M PBS (4 x 5min), followed by one five minute bath in 0.1M PBS with 0.3% Triton X (PBS-T). I then removed slides one by one from the washing dishes and placed them flat in cell culture dishes. Working four slides at a time, I applied 400µl of PBS-T containing 10% goat serum and 10% avidin (Vector Labs, SP-2001) and allowed the tissue to incubate at room temperature for one hour to block the tissue. The slides were gently inverted to remove excess blocking solution and were then incubated in 300µl of 1:3000 rabbit anti-AVT antibody (graciously donated by Dr. Matthew Grober, GSU) diluted in PBS-T containing 10% goat serum and 10% biotin (Vector Labs, SP-2001). Strips of Parafilm were cut to size and gently laid over each slide to aid in retention of the antibody solution of the slide.
surface. After coverslips were placed, the cell culture dishes holding the slides were placed into a humid chamber and incubated for 48 hours at 4°C.

Following incubation, coverslips were gently removed by soaking slides in a PBS bath. Slides were then washed (4 x 5min in PBS, 1 x 5min in PBS-T), returned to their cell culture dishes, and incubated with 400µl of goat anti-rabbit biotinylated secondary antibody (Vector Labs, PK-6101) diluted 1:400 in PBS-T and incubated for one hour. Slides were washed (4 x 5min in PBS, 1 x 5min in PBS-T) and placed back into cell culture dishes where they were incubated with 400µl avidin-horseradish peroxidase conjugate (Vector Labs, PK-6101) for one hour. Slides were washed once again.

To visualize the signal, slides were stained using 1.25% DAB until sufficient color development was achieved (approximately 15 minutes) and the reaction was terminated by soaking slides in three changes of nanopure water for five minutes each. Tissue was then dehydrated in an ethanol series (One minute each in 75%, 85%, 95%, and 100%; two minutes in fresh 100% EtOH). Tissue was cleared in two changes of CitriSolv (Fisher) for one and five minutes. Slides were then coverslipped using Protocol Permount mounting media (Fisher) and glass coverslips (Fisher). Slides were allowed to dry completely before quantification.

5.2.6 Cell quantification

Following IHC for AVT, immunoreactive cells were quantified using a microscope (Olympus BX41). Our regions of interest included the preoptic area (POA), the paraventricular nucleus (PPN), the periventricular nucleus/anterior hypothalamus (PVN/AH), and the supraoptic nucleus (SON). Regions were defined using published schematic atlases for green anole lizards (Greenberg, 1982; Lopez et al., 1992; Naik et al.,
1981). The first section in which AVT-ir positive cells was noted and individual cells were hand counted by an observer blind to the experimental condition of each individual. Total ir-cell count was considered in addition to the number of cells observed in each region.

5.3 Results

5.3.1 Arginine vasotocin cell quantification

5.3.1.1 Total cells

A two-way ANOVA with number of exposures ($F_{3,47} = 0.87, p = 0.43$) and stimulus ($F_{1,47} > 0.01, p = 0.95$) as factors revealed no main effects of the number of aggressive exposures or the type of stimulus presented on the total number of AVT ir-positive cells in the brain. There was not a significant interaction between exposures and stimulus ($F_{3,47} = 1.03, p = 0.34$) on total AVT cell number (Figure 5.3).

5.3.1.2 Preoptic area

A two-way ANOVA with time ($F_{3,50} = 3.42, p = 0.02$) and stimulus ($F_{1,50} = 0.28, p = 0.60$) as factors revealed a main effect of the number of exposures on the number of AVT ir-positive cells. Overall, animals with 5 consecutive exposures to a stimulus had more AVT ir-positive cells than those sacrificed 4 hours after a single exposure, regardless of which stimulus was presented (Tukey test, $q = 4.51, p = 0.01$). In addition to this main effect, there was also a significant interaction effect between the number of aggressive exposures and the type of stimulus presented on the number of AVT ir-positive cells in the POA ($F_{3,50} = 3.42, p = 0.02$; Figure 5.4).
To isolate group differences for the interaction effect, Tukey tests were used to examine pairwise comparisons. Among individuals sacrificed four hours after a single stimulus exposure, there was no significant difference between those who were shown the mirror back and mirror front \( (q = 1.27, p = 0.37) \). There was also no significant difference in AVT cell number among animals sacrificed one day following a single exposure to the mirror or mirror back \( (q = 1.05, p = 0.46) \). When I considered animals exposed for five consecutive days to the stimulus, I found a significant effect \( (q = 3.28, p = 0.03) \) where animals interacting with the reflective surface of the mirror had significantly more AVT ir-positive cells than those who were presented with the back of the mirror (mirror front: \( M = 161.75, \text{SEM} = 21.02 \); mirror back: \( M = 98.83, \text{SEM} = 17.16 \)). After 10 consecutive days of exposure, there was no difference in the number of AVT positive cells regardless of the type of stimulus presented \( (q = 2.68, p = 0.06) \).

Among those animals that were presented with the aggression inducing stimulus, individuals who were exposed to the mirror on 5 consecutive days had significantly more AVT ir-positive cells than those sacrificed 4 hours after one exposure \( (q = 5.30, p > 0.01) \) and those exposed for 10 consecutive days \( (q = 4.55, p = 0.01) \). There were no other significant differences among groups presented the mirror. There were also no differences in AVT cell number among individuals who observed the back of the mirror (neutral stimulus), regardless of the number of exposure days.

5.3.1.3 Periventricular preoptic nucleus

A two-way ANOVA with number of exposures \( (F_{3,49} = 0.26, p = 0.99) \) and stimulus \( (F_{1,49} = 0.04, p = 0.84) \) as factors revealed neither main effects of the number of aggressive exposures or the type of stimulus presented nor an interaction between exposures and
stimulus \( (F_{3,49} = 0.90; p = 0.45) \) on the number of AVT ir-positive cells in the PPN (Figure 5.5).

5.3.1.4 Paraventricular nucleus/Anterior hypothalamus

A two-way ANOVA with number of exposures \( (F_{3,48} = 1.34, p = 0.27) \) and stimulus \( (F_{1,48} = 0.90, p = 0.35) \) as factors revealed neither main effects of the number of aggressive exposures or the type of stimulus presented nor an interaction between exposures and stimulus \( (F_{3,48} = 0.26, p = 0.85) \) on the number of AVT ir-positive cells in the PVN/AH (Figure 5.6).

5.3.1.5 Supraoptic nucleus

A two-way ANOVA with number of exposures \( (F_{3,48} = 1.12, p = 0.35) \) and stimulus \( (F_{1,48} = 0.84, p = 0.37) \) as factors revealed no main effects of either the number of aggressive exposures or the type of stimulus presented on the number of AVT ir-positive cells in the SON. There was not a significant interaction between the number of exposures and the stimulus presented \( (F_{3,48} = 1.03, p = 0.39; \text{Figure 5.7}) \).

5.3.2 Endocrine effects

5.3.2.1 Testosterone

A two-way ANOVA with number of exposures \( (4\text{hr, 1d, 5d, 10d}; F_{3,51} = 0.332, p = 0.80) \) and stimulus \( \text{(Back of Mirror, Mirror; } F_{1,51} > 0.01, p = 0.99) \) as factors revealed no main effects of the number of aggressive exposures or the type of stimulus presented on the level of circulating testosterone. The interaction between exposure number and stimulus was not significant \( (F_{3,51} = 0.412, p = 0.75; \text{See Figure 5.8}) \).
5.3.2.2 **Corticosterone**

There was not a main effect of either the number of stimulus exposures ($F_{(3,58)} = 2.41, p = 0.08$) or stimulus type ($F_{(1,58)} = 0.27, p = 0.60$) on the level of circulating corticosterone. The interaction between exposure number and stimulus type was not significant ($F_{(3,58)} = 2.51, p = 0.07$; See Figure 5.9).

5.3.3 **Correlation between steroid hormones and AVT-ir cell number**

5.3.3.1 **Testosterone and AVT-ir cell number**

A Pearson-product moment correlation revealed no significant relationship between circulating T and the total number of AVT-ir cells ($r_{44} = -0.08; p = 0.58$; Figure 5.10A). No correlation was observed for cells in the preoptic area ($r_{45} = 0.15; p = 0.31$; Figure 5.10B), the periventricular preoptic nucleus ($r_{44} = 0.11; p = 0.48$; Figure 5.10C), or the supraoptic nucleus ($r_{44} = 0.003; p = 0.98$; Figure 5.10E). A significant negative correlation was observed for cells within the paraventricular nucleus/anterior hypothalamus ($r_{42} = -0.31; p = 0.04$; Figure 5.10D). This suggests that as testosterone levels increase, there are fewer AVT-ir cells observed in this region.

5.3.3.2 **Corticosterone and AVT-ir cell number**

No significant correlation was observed between circulating corticosterone and the total number of AVT-ir cells ($r_{50} = 0.23; p = 0.09$; Figure 5.11A). When the relationship between corticosterone and AVT-ir cell number was considered by region, no significant correlations were observed in the POA ($r_{52} = 0.23; p = 0.09$; Figure 5.11B), the PPN ($r_{50} = 0.17; p = 0.22$; Figure 5.11C), the PVN/AH ($r_{50} = -0.03; p = 0.84$; Figure 5.11D), or the SON ($r_{48} = 0.08; p = 0.57$; Figure 5.11E).
5.4 Discussion

In the current study, I demonstrate a region specific effect of aggressive behavior on AVT immunoreactivity. Within the preoptic area, five days of exposure to an aggression-inducing stimulus resulted in a significant increase in AVT cell number when compared to neutral stimulus controls and there is a non-significant trend for this increase after ten exposures. No differences were observed in any other brain region and these results are not dependent on differences in circulating steroid hormones.

Our results fit in with previous studies demonstrating the importance of the POA to aggressive behavior in green anole lizards. While the methods are not directly comparable (long term pairs vs. repeated mirror exposure), our lab has previously demonstrated that subordinate animals in a 10-day stable social hierarchy had significantly fewer AVT-ir cells in the POA than dominants or single-housed (no status) males (Hattori and Wilczynski, 2009). This result appears to have been driven by a specific reduction in cell number within the subordinate group given that single-housed control males and dominant males showed similar levels. A similar region-specific change in androgen receptor expression has also been found in this species after dominant/subordinate status was adopted following aggressive interactions. Dominant animals showed a significant increase in androgen receptor mRNA within the POA, but not other hypothalamic or limbic regions, both 2-hours after winning an interaction and after 3-days of pair housing in a stable hierarchy as compared to subordinates (Hattori and Wilczynski, 2014).

Our results also revealed a significant difference in the number of AVT cells depending on the number of times the mirror was observed. Animals sacrificed at both
four hours after a single exposure and after ten daily exposures had fewer AVT-ir cells than animals exposed to the mirror five times. This suggests that the number of ir-positive cells increases with repeated exposure to a point and then begins to approach baseline as the number of exposures increase. One possible explanation for this finding could be habituation. Perhaps animals perceive the mirror differently after five exposures which results in a change in the experience and thus its effect on AVT immunoreactivity. Previous work in our lab has demonstrated that anoles habituate to a video stimulus after five consecutive presentations (Yang and Wilczynski, 2003). We are currently investigating whether a similar pattern occurs in the mirror-aggression paradigm.

It should be also be noted that given the limitations of immunohistochemistry, it is impossible to determine if our results point to an increase in AVT synthesis with repeated aggression or to a reduction in AVT release, however several factors would point to the later explanation. First, I observed no differences in circulating steroid hormones in any groups, regardless of the number of exposure days or the type of stimulus observed. Our previous work has demonstrated that high circulating AVT led to a significant increase in circulating corticosterone (Dunham and Wilczynski, 2014). That I did not observe elevated corticosterone in the group with the greatest number of AVT-ir cells might point to a decreased release in AVT among that group. In addition, given our previous finding that anoles with increased exogenous AVT show reduced aggression to a mirror, it is possible that these results point not to an increase in the production of AVT but to a decrease in the release. Changes in immunoreactive cell number as an indication of altered peptide release and not increased peptide production has been posited (Lutterschmidt and Wilczynski,
Analysis of behavioral changes that coincide with the AVT-ir cell number changes in the POA would help to resolve this.

Overall, our results point to a reciprocal role of aggressive experience on the AVT system. AVT can impact the amount of aggressive behavior produced by an individual in anoles (Dunham and Wilczynski, 2014). At the same time, aggressive experience, even without the context of a social hierarchy, can alter AVT levels within the POA and this effect appears to be independent of steroid hormone levels. An effect on the POA is particularly significant as this region is a part of the social behavior neural network implicated in male aggression, and it is the site where changes in social status resulted in a change in AVT cell number and in androgen receptor expression (Hattori and Wilczynski, 2009; Hattori and Wilczynski, 2014). This interplay between AVT hormone and aggressive experience may be an important component in contributing to individual variability in behavior.

5.5 Acknowledgements

Thank you to Dr. Matthew Grober for providing the AVT antibody for the IHC assays. Thank you also to Deborah Lutterschmidt, Sheena Palmer, Anandhi Martin, and Reagan Norris for their technical assistance and to David Sinkiewicz and Bryce Chung for their helpful discussions during the preparation of this manuscript.

5.6 Funding

Supported by the National Science Foundation [IBN 0751573 to W.W.] and the Georgia State University Brains & Behavior Area of Focus.
Figure 5.1 Location of AVT-ir cell populations in *Anolis carolinensis*
Whole brain schematic adapted from Greenberg, 1982. Brain slice schematics adapted from Lopez et al., 1992. A) The rostral-most populations of AVT-ir cells are observed in the POA, PPN, and SON. B) AVT-ir cells continue to be detected through the POA, PPN, and SON. C) The caudal-most populations of AVT-ir cells are observed in the PVN/AH. Scale bar represents 100 μm. 10X magnification.

Figure 5.2 20X magnification of AVT immunoreactive cells
AVT-ir cell bodies and numerous vesiculated fibers are observed throughout the POA, PPN, PVN/AH, SON of the green anole brain. Scale bar represents 100 μm. 20X magnification.
Figure 5.3 Effect of aggressive behavior on total AVT-ir cell number
The number of exposures to an aggression-inducing stimulus (mirror) had no effect on the total number of AVT-ir cells observed in the brain. White bars represent back of mirror and grey bars indicate front of mirror presentation. Error bars represent standard error of the mean and group sample sizes are indicated in parentheses.
Figure 5.4 Effect of aggressive behavior on AVT-ir cell number in the POA
Animals presented with an aggression-inducing stimulus on five consecutive days had significantly more AVT-ir cells in the preoptic area than those presented the back of the mirror. Among those individuals observing the mirror, animals with only one exposure (sacrificed four hours later) and animals with ten consecutive exposures had fewer AVT-ir cells than those observing the mirror for five days. White bars represent back of mirror and grey bars indicate front of mirror presentation. Error bars represent standard error of the mean and group sample sizes are indicated in parentheses. The asterisk indicates statistical significance (p < 0.05). Different letters indicate statistical significance between groups (p < 0.05).
Figure 5.5 Effect of aggressive behavior on AVT-ir cell number in the PPN
The number of exposures to an aggression-inducing stimulus (mirror) had no effect on the number of AVT-ir cells observed in the supraoptic nucleus. White bars represent back of mirror and grey bars indicate front of mirror presentation. Error bars represent standard error of the mean and group sample sizes are indicated in parentheses.
Figure 5.6 Effect of aggressive behavior on AVT-ir cell number in the PVN/AH
The number of exposures to an aggression-inducing stimulus (mirror) had no effect on the number of AVT-ir cells observed in the periventricular nucleus/anterior hypothalamus. White bars represent back of mirror and grey bars indicate front of mirror presentation. Error bars represent standard error of the mean and group sample sizes are indicated in parentheses.
Figure 5.7 Effect of aggressive behavior on AVT-ir cell number in the SON
The number of exposures to an aggression-inducing stimulus (mirror) had no effect on the
number of AVT-ir cells observed in the supraoptic nucleus. White bars represent back of
mirror and grey bars indicate front of mirror presentation. Error bars represent standard
error of the mean and group sample sizes are indicated in parentheses.
Figure 5.8 Effect of aggression behavior on plasma testosterone

The number of exposures to an aggression-inducing stimulus (mirror) had no effect on circulating plasma testosterone concentrations. White bars represent back of mirror and grey bars indicate front of mirror presentation. Error bars represent standard error of the mean and group sample sizes are indicated in parentheses.
Figure 5.9 Effect of aggressive behavior on plasma corticosterone
The number of exposures to an aggression-inducing stimulus (mirror) had no effect on circulating plasma corticosterone concentrations. White bars represent back of mirror and grey bars indicate front of mirror presentation. Error bars represent standard error of the mean and group sample sizes are indicated in parentheses.
Figure 5.10 Correlation between plasma testosterone and AVT-ir cell number
There was not a significant correlation between circulating testosterone and AVT-ir cell number for (A) total cell number and regional cell number in (B) preoptic area, (C) periventricular preoptic nucleus, or (E) supraoptic nucleus. There was a significant negative correlation between T and AVT-ir cell number in the paraventricular nucleus/anterior hypothalamus. Asterisk indicates statistical significance (p < 0.05).
Figure 5.11 Correlation between plasma corticosterone and AVT-ir cell number

There was not a significant correlation between circulating testosterone and AVT-ir cell number for (A) total cell number and regional cell number in (B) preoptic area, (C) periventricular preoptic nucleus, (D) paraventricular nucleus/anterior hypothalamus, or (E) supraoptic nucleus.
6 GENERAL DISCUSSION AND CONCLUSIONS

This dissertation was aimed at understanding the role of arginine vasotocin in the regulation of social behavior, its interactions with steroid hormone endocrinology, and to consider the reciprocal effects that social behavior experience, specifically aggressive behavior, might have on the AVT system in the brain using a reptile, the anole lizard, an often used model for studying aggression and social behavior. A summary of the findings is presented below followed by a discussion of the general conclusions that emerged across experiments and the possible future directions for this research.

6.1 Summary

In Chapter 2, the impact of exogenous AVT on aggressive (mirror and paired) and courtship display behaviors was investigated. Vasotocin treatment resulted in a robust reduction in the number of aggressive display bouts performed to an animal’s mirrored reflection however, no effect was observed on aggression during a more naturalistic live pairing test. Decreased aggression with AVT treatment has been described in other territorial animals (see Goodson, 1998a, b; Semsar et al., 2001 among others). No effects of AVT treatment were observed on male courtship displays however, un-manipulated females paired with AVT treated males showed an increase in the number of displays. Regardless of the behavior challenge experienced, treatment with AVT led to significant increases in circulating CORT, which has also been shown to impact aggressive behavior in reptiles (DeNardo and Licht, 1993; Tokarz, 1987). Findings from this chapter suggest a possible role for AVT in aggressive behavior and demonstrate the potent ability of AVT to increase CORT. AVT influences on aggression could be direct and independent of other
hormone systems, or AVT could be working on behavior indirectly through, or in conjunction with CORT effects.

In Chapter 3, the behavioral and endocrine effects of different AVT doses on social behavior were considered further. In addition, the impact of AVT on steroid hormones in single-housed, isolated animals was examined. No effects of AVT on mirror aggression or courtship behavior were observed in this experiment. AVT-induced CORT increases were observed for every dose of AVT administration. An interesting interaction between circulating CORT and social behavior experience was observed. Animals injected with a high dose of AVT and then exposed to an aggression-inducing experience experienced a greater increase in CORT than animals exposed to a conspecific female for a courtship opportunity. Importantly, no differences in circulating CORT were observed between aggressing and courting animals treated with VEH. This result suggests that the impact of AVT on CORT release may differ depending on the type of stimulus encountered by a male (perceived male intruder vs. female conspecific). In addition, I demonstrate that AVT increased CORT independent of a social behavior experience. In isolated animals, treatment with AVT led to a significant increase in CORT at levels comparable to those observed with AVT plus social behavior. AVT is clearly capable of inducing robust corticosterone synthesis in this species. These results reiterate the importance of considering the impact of AVT on CORT when examining its function as a social behavior hormone.

In Chapter 4, the goal was to untangle the AVT and CORT relationship that emerged from the results reported in the previous chapters in order to determine the contribution of AVT to social behavior independent of its glucocorticoid effects. The CORT synthesis
inhibitor metyrapone (MET) was administered prior to AVT in an attempt to block CORT increases. Regardless of MET treatment, no significant effects of AVT on mirror aggression were observed on the total number of behavior bouts completed during the trial although there was a trend for overall lower aggression. In the last 10 minutes of the trial, however, AVT treatment did reduce aggressive responding in both VEH and MET pretreated groups. I found that the dose of MET administered was effective at inhibiting the CORT response in animals subsequently treated with VEH, but that it was incompletely effective in animals treated with AVT. AVT was able to increase CORT significantly in all animals, regardless of pretreatment condition. A non-significant trend for slightly lower CORT in the MET/AVT than the VEH/AVT group was observed (P = 0.06) suggesting that MET did blunt the normal CORT response typically observed with AVT treatment. Furthermore, I did not observe any correlations between circulating corticosterone and the number of aggressive behavior bouts: 1) when groups were collapsed across conditions or 2) when considered within treatment groups, suggesting that differences in the number of aggressive behavior bouts are not in fact driven primarily by CORT but might be explained by a glucocorticoid independent AVT effect. The results in this chapter show a strong effect of AVT on CORT release, but do not provide support for the idea that AVT’s behavioral effects depend on its elevation of CORT.

In Chapter 5, I departed from looking at direct effects of AVT on behavior and steroid hormone endocrinology and instead examined the ability of aggressive behavior experience (and not social status) to affect the AVT system in nuclei of the social behavior neural network. Others have reported social status dependent changes on AVT-ir cells in anoles (Hattori and Wilczynski, 2009) and here I find that similar changes can be induced
by aggressive behavior experience independent of social status. Animals exposed to an aggression-inducing stimulus (mirror) for five consecutive days had significantly more AVT-ir cells in the preoptic area than animals exposed the same number of times to a neutral stimulus (nonreflective mirror back). Among animals exposed to the mirror, AVT-ir cell number was significantly higher in those exposed for five days as compared to animals sacrificed four hours after a single exposure and those exposed for ten consecutive days. This suggests that the number of ir-positive cells increases with repeated exposure to a point and then begins to approach baseline as the number of exposures increase. In general, AVT-ir cell number was not correlated with either T or CORT levels, except in the PVN/AH, where a negative correlation between circulating T and AVT-ir cell number was observed. Overall, our results point to a reciprocal role of aggressive experience on the AVT system.

6.2 Synthesis and Future Directions

6.2.1 AVT and social behavior

6.2.1.1 Aggression

Arginine vasotocin certainly seems to have an inhibitory effect on aggressive behavior in male green anole lizards. While not always significant statistically, if I look across the experiments described in Chapters 2, 3, and 4, an obvious trend emerges in which AVT tends to reduce aggressive responding both in mirror aggression and in more ecologically relevant, but less controlled, paired aggression encounters. This result fits in with findings from other non-mammalian species exhibiting territorial aggression. It is
likely that the results observed in these experiments would be more consistent and potentially more robust if I were to administer intracerebroventricular injections of AVT as opposed to our IP method (Salek et al., 2002). Technical constraints with the anatomy of green anole lizards have made this prospect difficult but it would be worth attempting in future experiments to strengthen the effects I report here and to demonstrate more explicitly that AVT influences aggressive behavior through a central mechanism and not primarily through its peripheral actions (blood pressure, water balance, etc.).

The mechanism through which AVT inhibits aggression is not clear from our data. Hypotheses have been posited from other species including alterations to sensorimotor information processing (Rose and Moore, 2002) as well as influences on social behavior motivation (Semsar et al., 2001). Our results from Chapter 4 could suggest that AVT is working on motivation. When the 30-minute behavior trial was parsed out into shorter segments, the reduction in behavior was observed primarily in the final 10 minutes. Perhaps these animals simply stop responding to a perceived intruder faster than animals with normal levels of AVT, thus reducing their overall number of behavioral bouts. Future studies aimed specifically at this question are needed to determine how AVT influences aggression in this species.

6.2.1.2 Courtship

Arginine vasotocin does not appear to have a substantial effect on courtship behavior in this species. AVT effects on courtship display behavior have been described for fish (Salek et al., 2002; Santangelo and Bass, 2010; Semsar et al., 2001), frogs (Boyd, 1994; Burmeister et al., 2001; Kime et al., 2007; Marler et al., 1995; Propper and Dixon, 1997; Ten Eyck, 2005; Tito et al., 1999), and newts (Moore and Miller, 1983; Moore and Zoeller, 1979)
but directed birdsong does not seem to be affected (Goodson and Adkins-Regan, 1999; Goodson et al., 2004). The lack of an effect I observed might represent a difference between reptiles and some other vertebrates, but more work is required.

In Chapter 2, I did find that untreated females display more toward AVT treated males however, I did not observe this phenomenon in Chapter 3 when overall levels of behavior were reduced in all animals. This possible effect should be studied further as it suggests that there might be some hidden aspect of male signaling that is affected by AVT. This phenomenon needs to be examined to determine what, if anything AVT is doing in courtship behavior and for understanding more about reptile social communication.

### 6.2.2 AVT and corticosterone

On its own, AVP has been described as a weak secretogogue of CORT (Vale et al., 1983) but when present in conjunction with CRF, maximal activation of the HPA axis is achieved (DeBold et al., 1984; Favrod-Coune et al., 1993; Gillies et al., 1982). While I did not measure CRF in our animals, AVT is certainly capable of inducing substantial increases in CORT within 30 minutes of injection. This observation held both for animals in social behavior tests (Chapters 2, 3, and 4) and for single housed individuals treated with AVT (Chapter 3). As a result, however, interpretation of AVT effects on social behavior can be difficult. Given that CORT itself can influence aggressive behavior both in an inhibitory and stimulatory way (Summers et al., 2005b), the behavioral contribution of AVT independent of glucocorticoids could not always be determined with certainty. The results from Chapter 4 attempted to resolve this question by inhibition of 11β – hydroxylase, the enzyme that catalyzes 11 – deoxycorticosterone into CORT. Although metyrapone was only marginally successful at reducing CORT in animals treated with AVT, I believe that the data
do point to an independent role for AVT. When considered overall and within treatment groups, no correlations between CORT and behavior were observed suggesting that the differences in aggressive display number (overall trend and significant decrease during the final 10 minutes) were associated not with CORT but with one of our other treatments. Given that MET/VEH animals did not differ from VEH/VEH animals in their level of behavior, the likely culprit seems to be AVT.

6.2.3 AVT and social behavior: Reciprocal interactions in the social behavior neural network

A region specific change in AVT-ir cell number was observed. Here, I found that animals given more experience with aggression (five exposures) have increased AVT-ir cells in the POA as compared to those not stimulated to be aggressive. This difference was true only for the POA and not for total AVT-ir cell number or for any other brain region containing AVT cells. Similar results have been reported in several other species where social status has been associated with changes in POA AVT-ir cell number (Hattori and Wilczynski, 2009; Larson et al., 2006). Specifically, when dominant and subordinate anoles were considered, changes in AVT-ir cell number were most robust among subordinate animals (Hattori and Wilczynski, 2009). Subordinates had fewer AVT-ir cells in the POA than dominant males, single housed males, and males housed with a female. Like our results, these changes were specific to the POA and were not observed in other brain nuclei. It should be noted that immunohistochemistry assays do not allow us to infer whether changes in cell number are related to increased synthesis or decreased release of peptides (Lutterschmidt and Wilczynski, 2012; Marler et al., 1999) and looking at receptors in future studies would also be informative. Regardless, these results suggest that among
the relevant nuclei in the social behavior neural network, the POA is particularly sensitive to aggression.

Generally, the data presented in Chapter 5 represent, to me, an exciting direction for questions regarding experience-mediated changes in the brain. Becoming a dominant or subordinate animal leads to a plethora of changes from both transient and persistent variations in endocrinology to alterations of future behavior responses. When considering the effects of social status on the brain, however, it is often not feasible to differentiate the specific contributions of status from behavior experience itself. Green anole lizards are an excellent model for assessing this given that aggression can be elicited either with through introduction of a live male conspecific or through presentation of a mirror stimulus in which behavior output matches visual input and no relative social status is achieved, allowing us hone in on the influence of aggressive behavior experience itself. Is it social status and the process of becoming a dominant or subordinate individual that influences the brain, or is it simply engaging in social behaviors that is important? The results from our current study would suggest that the individual’s behavior itself is a critical component.

In future studies, I would like to use this experimental design to consider how aggressive behavior experience influences other aspects of the AVT system including receptor distribution and number (V1a and V1b which are both implicated in social behavior function - Albers et al., 2006; Phelps and Young, 2003; Wersinger et al., 2002). While the distribution of AVT-ir cells has been described for *Anolis carolinensis* (Propper et al., 1992), virtually nothing is known about the receptor distribution or contributions of these receptors to behavior in a reptile. A description of vasotocin receptors in any
reptilian species would fill in an evolutionary gap in the literature (Albers, 2014). Including both mirror aggression animals as well as live pairs would allow analysis of both behavior experience and status dependent effects on the brain.

6.3 Conclusions

The experiments presented here demonstrate that AVT likely does contribute to the levels of social behavior in male *Anolis carolinensis* but only in the context of territorial aggression. Corticosterone is potently regulated by AVT but the effects of AVT on aggressive behavior appear to be independent of its effects on CORT. Finally, aggressive behavior experience affects AVT-ir specifically in the POA, a brain region critical to social behavior expression. The data presented here contribute to the already rich body of research on AVT/AVP with the examination of social behavior effects in reptiles, showing that AVT’s effects are conserved across vertebrates including this vertebrate group. They also underscore the importance of considering hormone interactions when interpreting behavior data. More work is needed to determine the mechanism of AVT action on reptile social behavior, most notably the characterization and neural distribution of AVT receptors.
REFERENCES


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