

RESEARCH ARTICLE

Arginine vasotocin, steroid hormones and social behavior in the green anole lizard (*Anolis carolinensis*)

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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 i.p. with either reptile Ringer solution (vehicle) 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 vehicle-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.

KEY WORDS: Arginine vasotocin, Aggression, *Anolis carolinensis*, Corticosterone, Courtship

INTRODUCTION

Decades of research have established that arginine vasopressin (AVP; found in mammals) and arginine vasotocin (AVT; non-mammalian homolog 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 Goodson and Bass, 2001; Rose and Moore, 2002; Balment et al., 2006; Caldwell et al., 2008; Albers, 2012). 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 versus gregarious

(Goodson, 1998a; Goodson, 1998b; Goodson and Adkins-Regan, 1999; Goodson and Evans, 2004; Lema and Nevitt, 2004)], the behavioral phenotype of the individual [e.g. territorial versus non-territorial morphs (Semsar et al., 2001); dominant versus subordinate (Goodson et al., 2009a)], and the specific context of the interaction [e.g. sexually naïve versus experienced (Winslow et al., 1993); territory defence versus 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 granatinus*) (Goodson, 1998a; Goodson, 1998b). 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, co-localization of the immediate early gene *Fos* with AVT/AVP cells in several hypothalamic regions of both mammalian and aves brains correlates negatively with measures of aggression (Goodson and Evans, 2004; Goodson and Kabelik, 2009; Ho et al., 2010; Goodson et al., 2012), 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 Zoeller, 1979; Moore and Miller, 1983). Courtship display behaviors such as attending behavior in fish (Semsar et al., 2001; Salek et al., 2002; Santangelo and Bass, 2010) and aspects of calling in several species of frogs including frequency and acoustic characteristics (Marler et al., 1995; Burmeister et al., 2001; Kime et al., 2007) 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

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List of abbreviations

AVP	arginine vasopressin
AVT	arginine vasotocin
ELISA	enzyme-linked immunosorbent assay
HPA axis	hypothalamic–pituitary–adrenal axis
i.p.	intraperitoneal
-ir	immunoreactive

performed was observed (Goodson et al., 2009b). Similar evidence has emerged in the brown anole lizard in which the number of ‘social engagement behaviors’ towards a female was positively correlated with co-localization of AVT peptide and expression of the immediate early gene *Fos* in several behaviorally significant brain regions (pre-optic 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 can potentially influence the expression and intensity of courtship and territorial aggression-related behaviors, both individually and through reciprocal interactions (Moore and Miller, 1984; Denardo and Licht, 1993; Wingfield et al., 1998; Rose and Moore, 2002; Wade, 2005; Leary et al., 2006; Trainor and Nelson, 2012). In the anole brain, AVT immunoreactivity has been described in several regions known to influence social behaviour, including the supra-optic nucleus, pre-optic area and anterior hypothalamus, some of which are also steroid sensitive (Propper et al., 1992; Hattori and Wilczynski, 2009). 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 [in rodents (Miller et al., 1992; Wang and De Vries, 1993; Boyd, 1997; Kabelik et al., 2008) and reptiles (Kabelik et al., 2008)].

It is well established that AVT can promote the release of corticosterone from the adrenal gland 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 corticosterone may be in opposition. For example, AVT stimulates courtship behavior while corticosterone 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 crucial 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 reptile. 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* Voigt 1832). 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 reliance of the green anole 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 (Goodson and Bass, 2001; Caldwell et al., 2008; Albers, 2012).

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).

RESULTS**Experiment 1: mirror aggression**

A Mann–Whitney test revealed that injection of a 15 µg/50 µl dose of arginine vasotocin resulted in a significant decrease in the overall number of aggressive display bouts (two-tailed; vehicle: $N=6$, mean \pm s.e.m.=30.67 \pm 7.55 bouts; AVT: $N=10$, 4.60 \pm 1.23 bouts; $U=4.50$, $P<0.01$) compared with Ringer-injected controls (see Fig. 1A). There was no effect of AVT on the latency to initiate the first aggressive bout (two-tailed; vehicle: $N=6$, mean \pm s.e.m.=363.57 \pm 130.22 s; AVT: $N=10$, 429.21 \pm 97.24 s; $t_{14}=-0.41$, $P=0.69$). Vasotocin injection did not alter circulating levels of total testosterone (two-tailed; vehicle: $N=5$, mean \pm s.e.m.=670.44 \pm 68.37 pg ml⁻¹; AVT: $N=10$, 645.19 \pm 67.16 pg ml⁻¹; $t_{13}=0.24$, $P=0.82$; Fig. 1B) but led to a significant increase in total circulating corticosterone (two-tailed; vehicle: $N=6$, mean \pm s.e.m.=55.84 \pm 14.58 ng ml⁻¹; AVT: $N=10$, 282.75 \pm 35.36 ng ml⁻¹; $U=55.00$, $P<0.01$; Fig. 1C) compared with control animals. Overall, corticosterone was negatively correlated with the number of behavior bouts ($r=-0.60$, $P=0.02$; Fig. 1D).

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; vehicle: $N=10$, mean \pm s.e.m.=57.8 \pm 10.62 bouts; AVT: $N=10$, 36.9 \pm 8.42 bouts; $t_9=1.56$, $P=0.15$; Fig. 2A). 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 vehicle-injected partner (two-tailed; $\chi^2=1.8$, $P=0.18$). Within pairs, there was no difference in the latency to first aggressive display bout (two-tailed; vehicle: $N=10$, mean \pm s.e.m.=57.36 \pm 24.23 s; AVT: $N=10$, 67.50 \pm 19.06 s; $t_9=-0.35$, $P=0.73$). AVT treatment did not affect plasma testosterone (two-tailed; vehicle: $N=10$, mean \pm s.e.m.=660.14 \pm 139.18 pg ml⁻¹; AVT: $N=10$, 794.41 \pm 108.00 pg ml⁻¹; $t_{18}=-0.762$, $P=0.46$; Fig. 2B) but individuals injected with AVT had significantly higher circulating corticosterone (two-tailed; vehicle: $N=10$, mean \pm s.e.m.=24.11 \pm 3.88 ng ml⁻¹; AVT: $N=10$, 339.35 \pm 31.75 ng ml⁻¹; $U=100.00$, $P<0.01$; Fig. 2C). Overall, corticosterone was negatively correlated with the number of aggressive display bouts ($r=-0.47$, $P=0.04$; Fig. 2D).

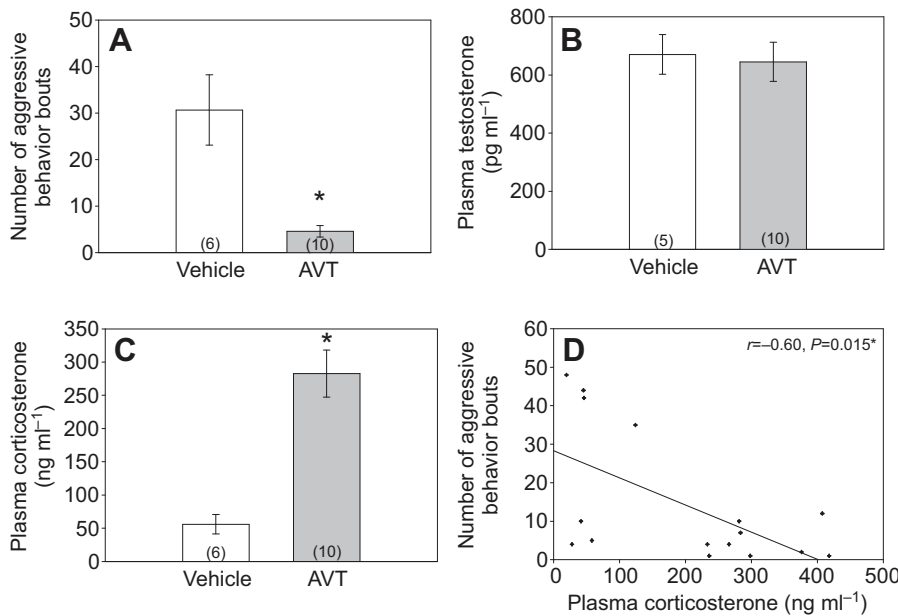


Fig. 1. Effect of arginine vasotocin (AVT) on *Anolis carolinensis* aggression and steroid hormone concentrations during mirror presentation. (A) During mirror-stimulated aggression, males treated with AVT perform significantly fewer aggressive display bouts in 30 min than vehicle-injected males. (B) AVT did not affect circulating testosterone. (C) AVT treatment results in significantly more circulating plasma corticosterone following mirror aggression. (D) Corticosterone was negatively correlated with the overall number of aggressive display bouts towards the mirror. Error bars represent s.e.m. Asterisks indicate statistical significance ($P < 0.05$).

Experiment 3: courtship

Courtship display behavior was not significantly affected by injection with 15 $\mu\text{g}/50 \mu\text{l}$ AVT compared with control animals (two-tailed; vehicle: $N=10$, mean \pm s.e.m. = 14.1 ± 3.95 bouts; AVT: $N=7$, 17.71 ± 5.93 bouts; $U=30.50$, $P=0.70$; Fig. 3A). There was also no effect of AVT on the latency to initiate the first courtship bout (two-tailed; vehicle: $N=10$, mean \pm s.e.m. = 131.56 ± 37.18 s; AVT: $N=7$, 142.53 ± 65.84 s; $t_{15} = -0.16$, $P=0.88$). In courting males, vasotocin injection did not affect testosterone (two-tailed; vehicle: $N=6$, mean \pm s.e.m. = 1234.63 ± 626.56 pg ml^{-1} ; AVT: $N=7$, 1119.09 ± 255.52 pg ml^{-1} ; $t_{11} = 0.18$, $P=0.86$; Fig. 3B) but did lead to a significant increase in circulating corticosterone (two-tailed; vehicle: $N=10$, mean \pm s.e.m. = 152.09 ± 61.62 ng ml^{-1} ; AVT: $N=7$, 612.52 ± 63.37 ng ml^{-1} ; $U=5.00$, $P < 0.01$; Fig. 3C). Overall, corticosterone was not correlated with the number of courtship displays observed ($r=0.16$, $P=0.54$; Fig. 3D).

We also analysed the behavior of the untreated females used as stimulus animals for the vehicle- and AVT-treated males. Females

paired with AVT-treated males performed significantly more courtship displays than those paired with vehicle-treated males (two-tailed; vehicle paired: $N=10$, mean \pm s.e.m. = 5 ± 2.80 bouts; AVT paired: $N=7$, 14.29 ± 1.74 bouts; $U=9.00$, $P=0.01$; Fig. 4).

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 also appears to be the case in the green anole. Treatment of territorial species or morphs with AVT leads to a reduction in aggression in a variety of species (Goodson, 1998a; Goodson, 1998b; Goodson and Adkins-Regan, 1999; Semsar et al., 2001; Goodson and Evans, 2004; Lema and Nevitt, 2004). In green anole males, which form territories and dominant-subordinate relationships, AVT 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.

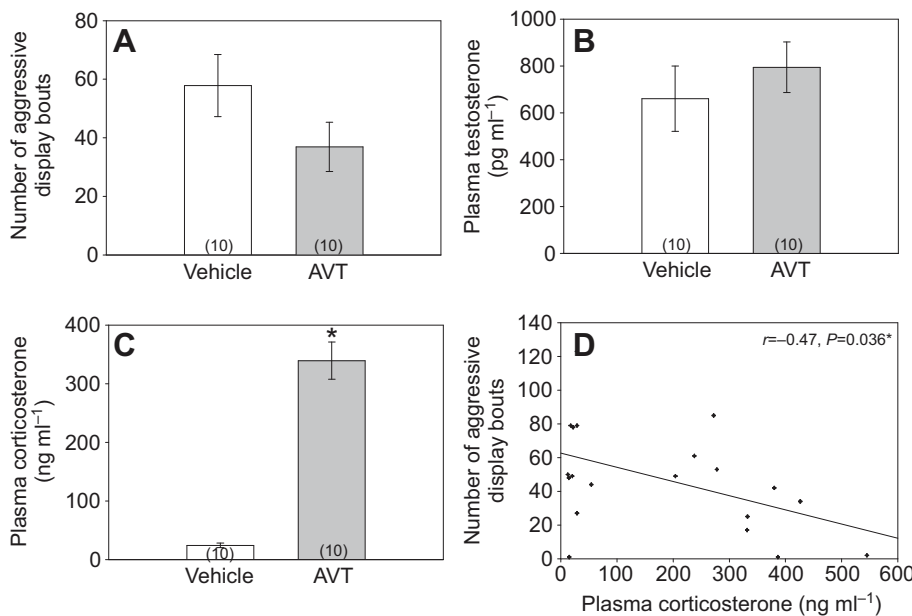


Fig. 2. Effect of AVT on aggression and steroid hormone concentrations in size-matched pairs. (A) Within pairs, AVT-injected animals did not behave significantly differently from their vehicle-injected partners. (B) There was no effect of AVT treatment on circulating testosterone. (C) Individuals injected with AVT showed a significant increase in plasma corticosterone following a paired aggressive interaction with a vehicle-injected partner. (D) Overall, corticosterone levels were negatively correlated with the number of observed display bouts. Error bars represent s.e.m. Asterisks indicate statistical significance ($P < 0.05$).

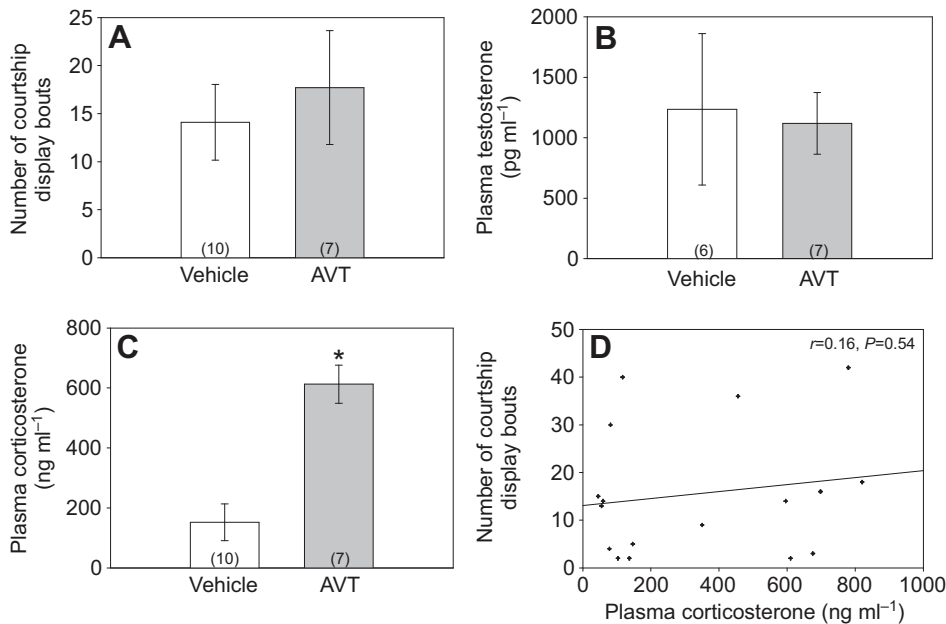


Fig. 3. Effect of AVT on courtship and steroid hormone concentrations. (A) AVT injection did not affect the number of courtship displays to a female conspecific. (B) There was no effect of AVT treatment on plasma testosterone. (C) Individuals injected with AVT had significantly higher circulating corticosterone than vehicle-injected controls. (D) There was no correlation between corticosterone and the number of observed courtship displays. Error bars represent s.e.m. Asterisks represent statistically significant differences ($P < 0.05$).

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 vehicle-treated individuals. While this does not reach statistical significance (two-tailed χ^2 test), it suggests to us a trend for individuals with high circulating AVT to be less aggressive overall.

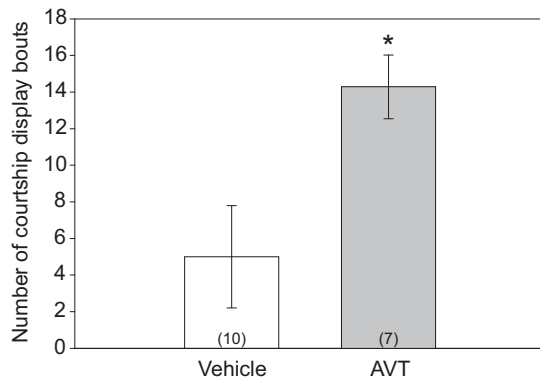


Fig. 4. Female courtship behavior towards AVT- and vehicle-treated males. Untreated females paired with an AVT-treated male perform significantly more bouts of courtship displays than untreated females paired with vehicle-injected control males. Error bars represent s.e.m. The asterisk represents statistical significance ($P < 0.05$).

A previous study examining the relationship between social status and AVT-ir cell populations in *A. carolinensis* demonstrated that subordinate males had significantly fewer ir-positive AVT cells in the pre-optic area compared with dominant males, singly housed males, and a male 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 probably 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. We did, however, 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 vehicle-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 probably 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 (López and Martín, 2005; Kopena et al., 2011; Labra, 2011), 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 corticosterone system, especially considering its potent behavioral effects. Here, we demonstrate that AVT treatment results in a significant increase in circulating corticosterone. This increase in corticosterone 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 corticosterone 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 corticosterone. Corticosterone 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 corticosterone 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 impacts behavior independently via effects on the brain or if the observed effects work primarily through its activation of the HPA axis and stimulation of corticosterone release. There is some indication, in mammals, that a small but measurable portion of peripherally administered radiolabelled-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 is probably crossing over in some amount.

To summarize, 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 corticosterone under all conditions. Further investigations are needed to determine if this elevation contributes to changes in aggression seen after AVT treatment.

MATERIALS AND METHODS

Animals

Seventy-six male and 24 female anole lizards *A. carolinensis* were obtained from Charles Sullivan, Inc. (Nashville, TN, USA) and were housed individually in half of a 10 gallon (45.5 litre) glass aquarium on a 14 h:10 h light:dark 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, USA) 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.

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 Ringer) or vehicle (50 µl reptile Ringer). This dose of AVT (Phoenix Pharmaceuticals, Burlingame, CA, USA), ~3 µg g⁻¹ body mass, was adapted from the published effective intraperitoneal (i.p.) 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 min. All behavioral tests were conducted between 10:30 and 13:30 h on the day of testing. Males were then killed via an overdose with i.p. injected Nembutal. Trunk blood was collected within 3 min of capture and stored at –20°C until processing via enzyme-linked immunosorbent assay (ELISA).

Behavior definition and scoring

Aggressive and courtship display behaviors have been thoroughly characterized for this species (Crews, 1975; Greenberg and Crews, 1990; Decourcy and Jenssen, 1994; Korzan et al., 2000; Yang et al., 2001; Lovern and Jenssen, 2003; Garcia et al., 2014). Males engaging in aggressive displays perform push-ups and head bobs, often in conjunction with extension of the red dewlap throat fan. Push-ups 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 push-ups, 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 min 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 five to 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; Lovern and Jenssen, 2003; Orrell and Jenssen, 2003; Jenssen et al., 2012), suggesting that bout number provides a more salient measure of the overall level of social signalling. 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 vehicle and AVT) that failed to perform at least one bout of behavior were considered non-responsive and excluded from the final analysis.

Hormone analysis

The plasma samples from all individuals were spiked with ~2000 c.p.m. of [³H]-corticosterone to allow for measurement of extraction efficiency. Samples were diluted 1:25 and 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 1:100 for testosterone; 1:300 to 1:750 for corticosterone) and analysed via ELISA (Cayman Chemical Company, Ann Arbor, MI, USA) for both testosterone and corticosterone according to the manufacturer's instructions. These assays have been previously validated for *A. carolinensis* by our laboratory and others using pooled plasma serial dilutions (Yang and Wilczynski, 2003; Garcia et al., 2012). 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⁻¹ for testosterone and 150 pg ml⁻¹ for corticosterone 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 ~2 to 20 ng ml⁻¹ in breeding condition animals (Husak et al., 2007; Husak et al., 2009). The levels reported here were, however, consistent across all of our experimental groups.

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 i.p. injection of the assigned drug, a two-way mirror (Reflection Products, Inc., Roswell, GA, USA) 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.

Experiment 2: paired aggression

In order to determine whether 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 vehicle. Fifteen minutes post-injection, both males (one AVT- and one vehicle-injected) were introduced to a neutral cage and the interaction was recorded for 30 min. 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.

Experiment 3: courtship

Twenty-four individually housed male anole lizards were injected with AVT or vehicle. Fifteen minutes post-i.p. 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 were, however, 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 min, 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.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.A.D. and W.W. contributed to the conception and design of the experiments as well as the interpretation of results and the preparation and editing of the manuscript. L.A.D. was primarily responsible for the execution of the experiments.

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