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Neuroendocrine Mechanisms Mediating Pheromonal Modulation of Behavior in Terrestrial Salamanders

Corina Wack

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NEUROENDOCRINE MECHANISMS MEDIATING PHEROMONAL
MODULATION OF BEHAVIOR IN TERRESTRIAL SALAMANDERS

A Dissertation

Bayer School of Natural and Environmental Sciences

Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By

Corina Lee Wack

August 2011

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Corina Lee Wack

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NEUROENDOCRINE MECHANISMS MEDIATING PHEROMONAL
MODULATION OF BEHAVIOR IN TERRESTRIAL SALAMANDERS

By

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ABSTRACT

NEUROENDOCRINE MECHANISMS MEDIATING PHEROMONAL MODULATION OF BEHAVIOR IN TERRESTRIAL SALAMANDERS

By

Corina Lee Wack

August 2011

Dissertation supervised by Sarah K. Woodley

Pheromones are chemosensory cues released by an individual to cause a behavioral or physiological change in a conspecific. These changes can range from increasing a female's receptivity to altering hormone secretions in the body. The red-legged salamander (*Plethodon shermani*) is an emerging non-mammalian model for understanding the evolution of chemical communication due to their well-characterized pheromones. Plethodontid salamanders secrete pheromones from their submandibular gland, called mental gland pheromones. Previous studies showed that mental gland pheromones increased receptivity in females during courtship and also increased corticosterone (CORT) concentrations in males. CORT is a metabolic hormone that mediates vertebrate stress responses. To further understand the neuroendocrine mechanisms involved in behavioral and physiological responses to pheromones, I

conducted several experiments. First, I investigated whether pheromones altered levels of two neuromodulators in the brain, gonadotropin-releasing hormone (GnRH) and arginine vasotocin (AVT) in *P. shermani*. GnRH is both a neuromodulator and hormone, and has strong effects on reproductive behavior. Additionally, AVT has broad behavioral effects in vertebrates, particularly in newts. I found that application of mental gland pheromones altered the number of GnRH-immunoreactive neurons, but had no effect on the number of AVT-immunoreactive neurons. Second, I examined the effects of AVT and AVP antagonist on courtship and mating in dusky salamanders (*Desmognathus spp.*). There was no effect of AVT or antagonist on reproductive behaviors in dusky salamanders. Finally, I investigated the role of increased CORT concentrations in male *P. shermani*. I first validated a non-invasive method to transdermally deliver CORT through a dermal patch. I then used dermal patches to exogenously elevate plasma CORT and examine the effects of CORT on behavior (activity and chemoinvestigation) and metabolism. CORT had no effect on behavior, but increased metabolic rates in male red-legged salamanders. Together these studies provide insight into the mechanisms by which pheromones work to alter behaviors and physiological functions in vertebrates.

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Chapter 1

Introduction

SPECIFIC AIMS

Pheromones are chemosensory signals that are required for normal sexual behavior in many vertebrates, including salamanders. Pheromones are released by an individual to cause a physiological or behavioral change in a conspecific. Pheromones induce behavioral changes by directly manipulating neuromodulators in areas of the brain that control behavior (Fig. 1.1). Pheromonal information is transmitted from the vomeronasal organ (VNO), which is the neuroepithelium that primarily detects chemosensory signals. The main olfactory epithelium (MOE) also detects pheromones. From the VNO and/or MOE, pheromonal signals are transmitted to and received by brain areas of the vomeronasal projection pathway, which include many areas that control reproductive behavior (Moffatt, 2003). Pheromones also manipulate behaviors indirectly by altering hormone concentrations (Fig. 1.1).

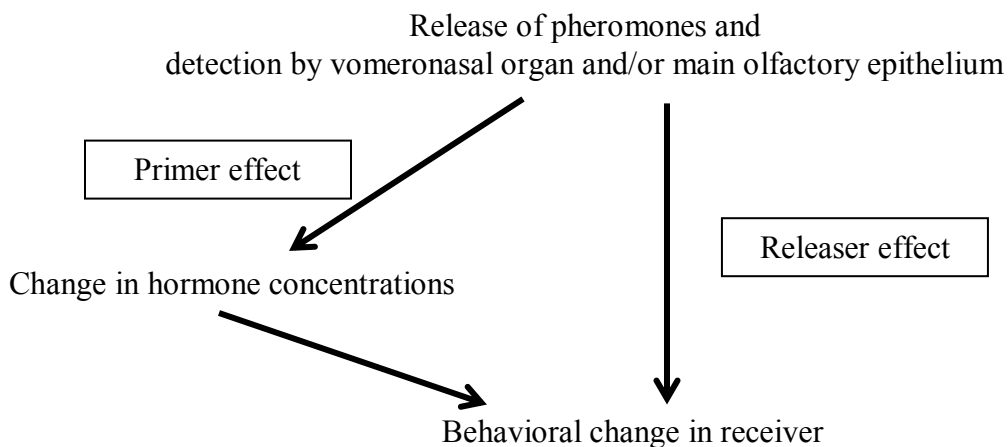


Figure 1.1. Diagram of how pheromones alter behaviors in vertebrates.

To understand the interactions between pheromones, neuroendocrine system and behavior, I used plethodontid salamanders as a model. Plethodontid salamanders are emerging non-mammalian models for understanding the evolution and nature of chemical communication due to their characterized courtship pheromones. Males secrete pheromones from their submandibular gland, the mental gland, which are delivered to females during courtship and increase female receptivity (Rollmann et al., 1999). The mechanism employed by mental gland pheromones to manipulate behaviors is still unknown. I conducted a series of experiments to further understand how mental gland pheromones interact with neuromodulators and the endocrine system to alter behavior. Below I list the specific aims of my dissertation research and a brief background of each aim. I also state the main results found from my research.

AIM 1: Effects of pheromones on gonadotropin releasing hormone (GnRH) and arginine vasotocin (AVT) systems in the brain.

Two neuromodulators, gonadotropin-releasing hormone (GnRH) and arginine vasotocin (AVT) alter behaviors and interact with social signals in vertebrates. Exposure to auditory social signals increased the number of GnRH neurons in the brain of frogs (Burmeister and Wilczynski, 2005). Additionally, application of pheromones increased responsiveness of AVT neurons in the brain of roughskin newts (Thompson et al., 2008). I exposed mental gland pheromones to male and female red-legged salamanders and measured the changes in numbers of GnRH-immunoreactive (-ir) and AVT-ir neurons. I found a significant gender by pheromone treatment interaction in the number of GnRH-ir neurons. However, there was no effect of pheromone treatment on the number of AVT-ir

neurons. These data suggest that mental gland pheromones may manipulate the GnRH system rather than the AVT system to manipulate behaviors in plethodontid salamanders.

Aim 2: Effects of pheromones on arginine vasotocin (AVT) system in the brain and the effects of AVT on courtship and mating

I investigated the role that AVT plays in altering reproductive behaviors in plethodontid salamanders. AVT and its mammalian homologue, arginine vasopressin (AVP), act synergistically with sex steroid hormones to induce a variety of different social and reproductive behaviors in amphibians, birds, and mammals (Moore and Rose, 2002). To explain the widespread effects of AVT/AVP on social behavior in vertebrates, the sensorimotor hypothesis proposed that AVT/AVP acts on specific sensory and/or motor pathways to increase neuronal responsiveness to species-specific social cues (Moore and Rose, 2002). The sensorimotor hypothesis was based on studies conducted on the sensorimotor processing of tactile information in newts (Rose and Moore, 2002). In *Plethodon shermani*, AVT cells are located in brain regions associated with the vomeronasal projection pathway, (Hollis et al., 2005) and thus it is reasonable that AVT/AVP may interact with pheromonal processing. I injected Desmognathine salamanders with AVT and an antagonist, but these injections had no effect on courtship and mating. Roughskin newts have been used as a model to generalize the effects of AVT on behavior, but I could not replicate these results in plethodontid salamanders. Therefore, generalizations of AVT's role in behavior, particularly those related to salamander behavior, should be made with caution.

Aim 3: The role of increased plasma corticosterone (CORT) concentrations in male red-legged salamanders.

Sex steroid hormones, estrogens and androgens, are important regulators of reproduction and reproductive behavior in vertebrates (Norris, 2006). Additionally, the glucocorticoid hormone, corticosterone (CORT), is a metabolic hormone that manipulates behavior in vertebrates. A study performed by a previous master's student in the Woodley lab and later replicated by myself showed that application of mental gland pheromones had no effect on estradiol or testosterone in female or male red-legged salamanders (*P. shermani*), respectively. However, these pheromones increased plasma CORT concentrations in males but not females (Schubert et al., 2009). This is the first time that a pheromone has been shown to alter steroid hormone concentrations in an amphibian. The goal of my research was to understand the functional role of increased plasma CORT in male *P. shermani* after application of mental gland pheromones.

To manipulate plasma CORT, I validated a method first developed in lizards and later used in birds to non-invasively deliver exogenous CORT (Knapp and Moore, 1997). This method uses small dermal patches to deliver CORT transdermally in salamanders. I validated this method by showing dose responses and the time course of plasma CORT elevation after application of CORT patches. I also used CORT patches to increase plasma CORT concentrations acutely and repeatedly over several days. CORT patches elevated plasma CORT in the physiological range and in a physiologically relevant time period.

Since behavior may be altered through pheromonal increase of CORT concentrations, I utilized CORT patches to investigate the role of CORT in altering

behavior in male red-legged salamanders. I measured locomotor activity and chemoinvestigation after application of CORT patches. I found no consistent effects of CORT or a glucocorticoid receptor blocker on behavior in male red-legged salamanders. These results suggest that CORT has no effect on acute expression of locomotor activity or chemoinvestigation in males. Therefore, pheromones may not be utilizing manipulations of CORT to alter behavior.

CORT is a metabolic hormone and also is secreted in response to stressors. CORT is involved in increasing intermediary metabolism by acting on several metabolic pathways to increase glucose concentrations. The increase in intermediary metabolism may be translated to changes in whole-animal metabolism. I investigated the effects of CORT and mental gland pheromones on metabolic rates in male *P. shermani*. Application of CORT patches increased oxygen consumption in males within four hours after CORT patches were removed. These CORT patches increased plasma CORT concentrations to physiologically relevant concentrations. Few other studies have shown physiological elevations in CORT increasing metabolic rates in vertebrates. Furthermore, this type of study has never been performed in an amphibian. These results suggest that acute increases in CORT are energetically costly.

STUDY ORGANISM

Plethodontid salamanders are emerging, non-mammalian models of chemosensation and stress physiology. They are distinguished by being lungless and having nasolabial grooves. Plethodontid salamanders are found throughout the Appalachian Mountains from New York to South Carolina (Bruce et al., 2000). I chose

three species of plethodontid salamanders to investigate: the red-legged salamander (*P. shermani*), Allegheny dusky salamander (*Desmognathus ochrophaeus*), and the Southern dusky salamander (*D. ocoee*). *P. shermani* inhabit terrestrial habitats under leaf-litter and logs. *D. ochrophaeus* and *D. ocoee* are semi-terrestrial and inhabit moister habitats near streams and creeks. Male *P. shermani* breed each fall for several months, but *D. ochrophaeus* and *D. ocoee* breed in the spring and fall. Males and females engage in a mating behavior, called tail straddling walk, in which a receptive female straddles the tail of a male prior to sperm deposition and insemination. During this time the male delivers courtship pheromones to the female from his submandibular “mental” gland (Arnold, 1976). A male will deposit a spermatophore and will guide the female over the spermatophore for her to collect it into her cloaca. The female will store this sperm, where it will be used later to fertilize eggs during ovulation and oviposition (Bruce et al., 2000).

Plethodontid salamanders are an ideal model for studies in chemical communication for several reasons. First, behavioral and neuroanatomical studies are facilitated by simple stereotyped behaviors and relatively simple brains (Roth et al., 1993). Furthermore, a brain atlas from *P. shermani* has been developed, which has been used to identify areas of neural activation (Laberge, 2008; Laberge and Roth, 2005). Additionally, the distribution of AVT neurons has been described in *P. shermani* (Hollis et al., 2005). Second, unlike lab mice and rats, plethodontid salamanders are a non-domesticated model, so studies from plethodontid salamanders may be more applicable to free-living vertebrates in general. The comparative information also will be useful to determine whether results obtained from mammals are conserved or restricted to that

group alone. Finally, conducting comparative research on various groups of animals, we can understand how conserved biological mechanisms are among vertebrates.

An advantage to using *P. shermani* to study chemosensation is the fact that several pheromones have been molecularly and biochemically characterized. These pheromones are found in the submandibular gland (mental gland) of male *P. shermani* (PMF; Feldhoff et al., 1999; Houck et al., 2007; PMF; Rollmann et al., 1999). These pheromones are variable among individuals within a species and have experienced positive selection, most likely due to their important function in reproduction (Palmer et al., 2005; Watts et al., 2004). Mental gland pheromones experimentally increased receptivity in females by decreasing the duration of courtship it required to achieve insemination (Houck et al., 2007; Rollmann et al., 1999). Additionally, these pheromones stimulated neurons of the VNO (Schubert et al., 2006; Schubert et al., 2008; Wirsig-Wiechmann et al., 2002; Wirsig-Wiechmann et al., 2006). In addition to its role in increasing female receptivity, male mental gland pheromones may have additional functions in males (Schubert et al., 2009).

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Chapter 2

Pheromonal modulation of gonadotropin-releasing hormone and arginine vasotocin neurons in a terrestrial salamander

ABSTRACT

Pheromones are released by an individual to cause a specific behavioral or physiological change in a conspecific. To achieve a behavioral outcome, pheromones may alter neuromodulators in the brain, such as gonadotropin-releasing hormone (GnRH) and arginine vasotocin (AVT). GnRH acts both as a neuromodulator as well as a hormone that leads to the eventual secretion of estrogens and androgens. AVT mediates a broad range of behaviors in vertebrates, for example, amplexic clasping behavior in male roughskin newts. To investigate the interactions of pheromones and neuromodulators, male and female red-legged salamanders (*Plethodon shermani*) were treated with mental gland pheromones. In the rostral part of the brain, the effect of pheromones on the number of GnRH-immunoreactive (ir) neurons depended on gender, with treatment increasing the number of GnRH-ir neurons in females. Additionally, females had more GnRH-ir neurons in the caudal portion of the brain compared to males. There was no effect of pheromone treatment on AVT-ir neurons in any region of the brain. However, females had more AVT-ir neurons in the dorsal pallium compared to males, and reproductive males and females had more AVT-ir neurons in the posterior preoptic area compared to non-reproductive females. These data suggest that pheromones may interact with the GnRH system, but not the AVT system, to alter behavior.

INTRODUCTION

Pheromones are non-volatile substances released by an individual to cause a specific change in a conspecific (Karlson and Luscher, 1959). Pheromones transmit information regarding an individual's reproductive status, identity, and sex (Halpern and Martinez-Marcos, 2003). Releaser pheromones elicit a change in the recipient's behavior, whereas primer pheromones affect the receiver's physiology, such as causing release of hormones (Wyatt, 2003). Pheromones are detected primarily through a specialized epithelium called the vomeronasal organ (VNO), although the main olfactory epithelium may also detect pheromones. The VNO projects axons to the accessory olfactory bulb, which projects to other areas of the vomeronasal projection pathway in the brain, such as the preoptic area and the hypothalamus (Mombaerts, 2004). Pheromones may alter neuromodulators within areas of the vomeronasal projection pathway, such as gonadotropin-releasing hormone (GnRH) and arginine vasotocin (AVT).

GnRH is a hormone secreted from two different populations of cells in the brain: the GnRH-I and GnRH-II systems. The GnRH-I system is further subdivided into two subpopulations, the terminal nerve GnRH-I (TN-GnRH-I) system and the hypophysiotropic GnRH-I system. The terminal nerve is a ganglionated nerve that extends from the olfactory epithelium to the caudal telencephalon along the base of the brain and lies within the meninges (Demski, 1984; Wirsig-Wiechmann, 2000). The TN-GnRH-I neurons are found in the terminal nerve, medial septum, and but rarely found past the preoptic area (Muske et al., 1994; Muske and Moore, 1994). The hypophysiotropic GnRH-I system is located in the medial septum, preoptic area, and

hypothalamic region of the brain (Tsai, 2011). The GnRH-II system is located primarily in the caudal regions of the brain, near the midbrain (Tsai, 2011).

The hypophysiotropic GnRH-I system is part of the hypothalamo-pituitary-gonadal (HPG) axis. GnRH release from hypothalamic cells causes secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. LH and FSH act on the gonads to trigger secretion of gonadal steroids, including testosterone and estradiol (Norris, 2006). Most hypophysiotropic GnRH neurons are located in the preoptic area. GnRH fibers from the preoptic area terminate in the median eminence where GnRH is released into the hypophysial portal system. GnRH travels through the portal system to act on the anterior pituitary to cause release of LH and FSH (Tsai, 2011).

GnRH secreting cells are also located in the terminal nerve, a cranial nerve that lies along the base of the brain. (Demski, 1984; Wirsig-Wiechmann, 2000). In the caudal telencephalon, the terminal nerve branches into two pathways: one extends into the brain dorsocaudally to the medial septal area and the other remains at the ventral part of the brain and extends toward the preoptic area. The latter pathway is contiguous with the hypophysiotropic GnRH-I system, with no obvious morphological difference between these two subpopulations (Muske et al., 1994; Muske and Moore, 1987; Muske and Moore, 1988; Muske and Moore, 1994; Schmidt et al., 1988).

In many vertebrates, hypophysiotropic GnRH cells are activated by exposure to pheromones, causing an increase in GnRH release. Electrical stimulation of the VNO activated GnRH neurons of the medial preoptic area in hamsters, as indicated by double-labeling of the neuronal activation marker, c-fos, and GnRH (Meredith and Fewell,

2001). Lesions of the VNO decreased hormone concentrations of the HPG axis. Exposure to female urine induced LH release in male mice with an intact VNO, but not in males with a lesioned VNO (Coquelin et al., 1984). Additionally, there was a suppression of androgen surges in male hamsters with lesioned VNOs (Pfeiffer and Johnston, 1994). In male hamsters, pre-exposure to female vaginal fluid or injections of GnRH lead to a full complement of male sexual behavior (Westberry and Meredith, 2003). These data show that pheromones initiate certain reproductive behaviors via interactions with GnRH.

GnRH from the terminal nerve may affect chemosensory processing by acting directly on the olfactory sensory neurons. In the prairie vole, the terminal nerve projected directly to the VNO, which expressed GnRH receptors (Wirsig-Wiechmann and Wiechmann, 2001). Eisthen et al. (2000) used voltage clamp techniques to determine that GnRH excited olfactory sensory neurons in mudpuppies. In axolotl salamanders, GnRH decreased electrical field potentials in the olfactory epithelium when concurrently applied with odorants (Park and Eisthen, 2003). In an electrophysiological study of teleosts, terminal nerve GnRH neurons had rhythmic, spontaneous discharges that were not dependent upon efferent inputs, suggesting that GnRH released from the terminal nerve are neuromodulators (Oka and Matsushima, 1993). Together these data show that the GnRH-I system (both hypophysiotropic and terminal nerve populations) is altered by olfactory and pheromonal stimuli.

Another neuromodulator in the brain that interacts with pheromones is arginine vasopressin (AVP) and its non-mammalian homologue, AVT. AVT/AVP neurons and/or fibers are located in the preoptic area, hypothalamus, amygdala, bed nucleus of the stria terminalis, lateral septum, and nucleus accumbens in many tetrapod vertebrates in which

its distribution has been described (Goodson and Bass, 2001). Many of these areas are also components of the vomeronasal projection pathway. In the red-legged salamander, AVT neurons are located in the areas mentioned above except the hypothalamus and nucleus accumbens (Hollis et al., 2005).

To understand AVT's effects on behavior, the sensorimotor hypothesis was established, which states that AVT increases sensitivity to sensory stimuli in the medulla to induce a motor response to cause a species-specific behavioral change (Rose and Moore, 2002). Much of the work to understand the interaction of sensory stimuli and AVT has been done in male roughskin newts in which tactile stimulation of the male's abdominal area induces a clasping response, which is mediated by AVT (Moore and Miller, 1983). Limited evidence indicates that AVT may also interact with pheromonal information. Pheromone application increased firing of neurons in the medulla in roughskin newts, but this effect was blocked by an AVP antagonist (Thompson et al., 2008). These data suggest that AVT may modulate sensory information, including pheromonal information, to induce specific behavioral responses.

Sex differences in AVT distribution have been found in several brain regions in the newt, *T. granulosa*. Males had more AVT-immunoreactive (-ir) cells in the bed nucleus of the stria terminalis, dorsolateral amygdala, and anterior preoptic area, which are all areas involved in sexual behavior (Moore et al., 2000). Females had more AVT-ir cells in the dorsal and ventromedial hypothalamus during the breeding season and bed nucleus of the decussation of the fasciculus lateralis telencephali and in the nucleus visceralis superior during the non-breeding season, which are brain regions involved in female-typical behavior (Moore et al., 2000).

Plethodon shermani is an emerging model in understanding the chemosensory processing in a non-mammalian vertebrate. Male *P. shermani* secrete courtship pheromones from submandibular (mental) glands. Mental gland pheromones have been experimentally delivered to females during courtship and found to increase female receptivity (Rollmann et al., 1999). Due to the effects of mental gland pheromones on female receptive behavior, I hypothesized that application of mental gland pheromones would alter the number of GnRH-ir and AVT-ir neurons in the brain differently between males and females. Specifically, I predicted that (1) after exposure to pheromones, reproductive females would have more GnRH-ir neurons throughout the brain compared to males and females with vehicle delivered, (2) after exposure to pheromones, reproductive females would have more AVT-ir neurons compared to males and non-reproductive females.

METHODS

Two experiments were conducted to examine the effects of pheromones on GnRH and AVT neurons in the brain of *P. shermani*. In the first experiment, mental gland extract (MGE) or PBS vehicle were applied to the nares of reproductive male and females and the number of GnRH-ir of neurons was counted. In the second experiment, MGE or PBS vehicle was applied to the nares of reproductive males and females and non-reproductive females and the number of AVT-ir neurons was counted.

Animals

Plethodon shermani (red-legged salamanders) were caught in August 2007 and August 2008 at a single location in North Carolina (Wayah Bald, Macon County, NC, 83° 39' 30" N longitude; 35° 19' 49" W latitude) with appropriate permits from North Carolina Wildlife Commission and US Forest Service. Females were determined as reproductive by having large developed follicles that were visible by palpation. Non-reproductive females had no noticeable follicles. Males were determined as being in reproductive condition by having a mental gland. Animals were maintained at 16°C on a 14:10 light:dark cycle in 16 x 16 x 5 cm plastic boxes with a moistened paper towel substrate and refugia. They were fed wax moth larvae ("wax worm"). All procedures were approved by the Institutional Animal Care and Use Committee of Duquesne University.

Preparation of pheromone and application

Pheromone consisted of extract from male mental glands (MGE) (Schubert et al., 2006; Wirsig-Wiechmann et al., 2002). To obtain MGE, males were captured and mental glands were removed (for details see Wirsig-Wiechmann et al., 2002; procedure approved by Oregon State University ACUP to Dr. Lynne Houck). Mental glands from multiple males were pooled and pheromone was extracted with acetylcholine-chloride and resuspended in PBS. Pheromone concentration was standardized at 1 µg of total protein extracted from the mental gland per µl. MGE was provided to us by Drs Rick and Pam Feldhoff and Lynn Houck. This concentration elevated plasma CORT concentrations (Schubert et al., 2009) and activated vomeronasal sensory neurons in male

P. shermani (Schubert et al., 2006; Wirsig-Wiechmann et al., 2002). A volume of 5 μ l of pheromone or vehicle (PBS) was pipetted onto the nares every 5 minutes for 45 minutes for a total of 10 applications, as described in Schubert et al. (2009). Males used to prepare MGE were not used as test subjects.

Brain tissue processing

Two hours after last pheromone application, animals were euthanized by rapid decapitation. The brains were exposed by removing skin and cartilage over the brains and lower jaws. The heads were placed in 4% paraformaldehyde. After 2-3 hours, brains were removed from the skull and returned to 4% paraformaldehyde, and left on a shaker overnight. Brains were cryoprotected at room temperature in 30% sucrose in PBS for 24 hours. The next day, brains were placed in 3:1 solution of 30% sucrose:OCT overnight on a shaker. Brains were then embedded in OCT and snap frozen in liquid nitrogen. Brain tissue were maintained at -20°C until they were cut in 20 μm sections on a cryostat and thaw mounted on superfrost plus slides coated with polylysine. Slides were kept at -80°C until GnRH or AVT neurons were visualized using standard immunocytochemical procedures.

Immunocytochemistry

Immunocytochemistry (ICC) was performed on every third section using standard procedures. Briefly, sections were washed with PBS and 0.5% H_2O_2 . They were then placed in a pre-incubation solution containing PBS, 0.2% Triton-X, 1% normal goat serum and 0.04% sodium azide. (Note that sodium azide was only used in preincubation

solution and primary antibody solution.) Sections were incubated overnight at room temperature in anti-mammalian GnRH antibody generated in rabbits (gift from Dr. Pei-San Tsai) at a 1:1000 dilution for GnRH ICC. For AVT ICC, sections were incubated in anti-AVT antibody generated in rabbits (gift from Dr. Matthew Grober) at a dilution of 1:2000.

The following day sections were washed with PBS and incubated with biotinylated goat-anti-rabbit IgG. Sections were then washed again and incubated with Vectastain ABC reagent. Sections were washed and then immunoreactive cells were visualized with diaminobenzidine as the chromagen. Sections were washed and dehydrated with an ascending series of ethanol and coverslipped with permount.

Various procedures were conducted to validate the use of the GnRH and AVT antibodies in red-legged salamanders. First, specificity was tested by removing the primary GnRH or AVT antibodies from the ICC. Second, the AVT antibody was pre-absorbed by incubating AVT primary antibody with AVT overnight at a concentration of 5 µg/ml. Preabsorption or the absence of the primary antibody resulted in a lack of immunoreactivity. Results of the no GnRH primary antiserum control can be seen in figure 2.1 and the AVT preabsorption control in figure 2.2. Results for the no AVT primary antiserum control are not shown, because staining was so light for these controls that photomicrographs could not be captured.

GnRH and AVT Analysis

All processed sections were examined using an Olympus Brightfield microscope at 200x magnification. Anatomy of the *P. shermani* brain was determined using Laberge

and Roth (2005). Distribution of AVT neurons were previously determined by Lowry et al. and Hollis et al. (2005). All cells were counted throughout the brain in every third section. Numbers presented were counted from these sections of the brain, thus they represent approximately one third of the neurons. GnRH-ir neurons were separated in two populations. Cells rostral to the preoptic area were determined as terminal nerve GnRH. Cells caudal to the preoptic area were determined as primarily hypophysiotropic GnRH.

For GnRH neurons, intensity of antibody staining and areas of cells were also measured. Intensity of antibody staining was measured using Image Pro Plus software. The protocol described in Xavier et al. (2005) was utilized to determine optical densities. Briefly, grayscale images were taken of all the neurons with the computer software. A background image was taken with no slide on the microscope. This background image was subtracted for each neuron for which optical density was measured. To measure cell area, a line was drawn around each circle and the Image Pro Plus software calculated a cell area. Intensities and cell areas were not quantified for AVT-ir neurons.

When data did not meet assumptions of normality (Kolmogorov-Smirnov, $P < 0.05$) or homogeneity of variance (Levene's test, $P < 0.05$), data was square-root transformed. For data that did not meet assumptions of normality (Kolmogorov-Smirnov, $P < 0.05$) or homogeneity of variance (Levene's test, $P < 0.05$) even with transformations, a non-parametric Kruskal-Wallis test was performed. For normally distributed data, differences among treatments and gender in the number GnRH-ir and AVT-ir neurons were analyzed by a two-way ANOVA. For GnRH-ir neurons, the two populations, rostral and caudal brain, were analyzed separately. For AVT-ir data, each brain region in which AVT-ir neurons were located was analyzed separately. Tukey post hoc tests were

performed after significant main or interaction effects were found in the ANOVA analyses. All analyses were performed using SPSS, version 12.0.

RESULTS

GnRH-ir neurons

Males and females had similar numbers of GnRH-ir neurons in the rostral brain (Fig 2.3; $F_{1,35}=0.32$, $P=0.57$), but females had more GnRH-ir neurons in the caudal brain (Fig. 2.4; $F_{1,35}=4.28$, $P=0.046$). There was no effect of pheromone delivery in the rostral brain (Fig. 2.3; $F_{1,35}=1.82$, $P=0.19$), but animals that received pheromone had marginally fewer GnRH-ir neurons in the caudal brain (Fig. 2.4; $F_{1,35}=3.23$, $P=0.081$). Additionally, there was a sex*treatment interaction in the rostral brain (Fig. 2.3; $F_{1,35}=4.54$, $P=0.04$), but significant effects of treatment within each sex were not detected by post hoc tests. Representative micrographs of GnRH-ir neurons are shown in Figure 2.5.

Males and females had similar staining densities of GnRH neurons in the rostral brain ($F_{1,34}=0.14$, $P=0.71$) and the caudal brain ($F_{1,34}=1.15$, $P=0.29$). Nor was there an effect of pheromone on staining densities of GnRH neurons in the rostral brain ($F_{1,34}=0.86$, $P=0.77$) or the caudal brain ($F_{1,34}=0.26$, $P=0.62$). Additionally, males and females had similar GnRH-ir cell areas in the rostral brain ($F_{1,31}=0.13$, $P=0.73$) and caudal brain ($F_{1,31}=0.15$, $P=0.701$). Pheromone treatment also had no effect on GnRH-ir cell area in the rostral brain ($F_{1,31}=1.02$, $P=0.32$) and caudal brain ($F_{1,31}=0.62$, $P=0.44$). All data regarding cell area and staining densities are shown in table 2.1. The size of the brain was not a significant covariate in any of the above analyses.

AVT-ir neurons

AVT-ir neurons were found throughout the brain. Table 2.2 compares the distribution of AVT-ir neurons in red-legged salamanders with the distribution of AVT-ir neurons in the rough skin newt, *Taricha granulosa* (Lowry et al., 1997) and the distribution of cells positive for AVT transcripts in *P. shermani* (Hollis et al., 2005). In the dorsal pallium, reproductive females had more AVT-ir neurons than did males, but not compared to non-reproductive females (Fig. 2.6; $F_{2,37}=4.87$, $P=0.014$). Application of MGE did not have an effect on the number of AVT neurons in the dorsal pallium (Fig. 2.6; $F_{1,37}=1.43$, $P=0.24$). Gender altered the number of AVT-ir neurons in the posterior preoptic area (Fig. 2.7; $F_{2,37}=3.83$, $P=0.03$), although differences among gender groups could not be distinguished via post hoc analyses. MGE treatment did not alter the number of AVT-ir neurons in the posterior preoptic area (Fig. 2.7; $F_{1,37}=0.05$, $P=0.83$). Representative micrographs of AVT-ir neurons in the dorsal pallium and posterior preoptic area are shown in figures 2.8 and 2.9, respectively.

Gender of the salamander did not alter the number of AVT-ir neurons in any other area of the brain, including the medial pallium, lateral pallium, lateral cellular prominence, ventral cellular prominence, medial septum, bed nucleus of the stria terminalis, bed nucleus of the decussation of the lateral forebrain bundle, ventral thalamus, dorsal hypothalamus, ventral hypothalamus, ventral preoptic area, anterior preoptic area, magnocellular preoptic area. Nor did MGE treatment alter the number of AVT-ir neurons in medial pallium, lateral pallium, lateral cellular prominence, ventral cellular prominence, medial septum, bed nucleus of the stria terminalis, bed nucleus of the decussation of the lateral forebrain bundle, ventral thalamus, dorsal hypothalamus,

ventral hypothalamus, ventral preoptic area, anterior preoptic area, magnocellular preoptic area. Data are listed in table 2.3.

DISCUSSION

The current study presented a significant pheromone treatment by gender interaction in the number of GnRH-ir neurons of the rostral brain. A trend indicated that pheromone application altered the number of GnRH-ir neurons in the rostral portion of the brain, which primarily is comprised of terminal nerve GnRH-secreting neurons. However, pheromone application had no effect on the number of AVT-ir neurons in any portion of the brain in which they were found. Sex differences were also found in the GnRH and AVT systems. Females had more GnRH-ir neurons in the rostral region of the GnRH-I system, which comprises the hypophysiotropic GnRH system. Females also had more AVT-ir neurons in the dorsal pallium. Additionally, reproductive males and females had more AVT-ir neurons in the posterior preoptic area compared to non-reproductive females.

Multiple molecular forms of GnRH have been detected in vertebrates. The primary forms include mammalian GnRH, chicken GnRH, and salmon GnRH (Moore et al., 1987). The GnRH-I system consists of a single form of GnRH, either mammalian GnRH or the ranid frog GnRH (Tsai, 2011). The GnRH-II system contains primarily the chicken GnRH form. To date, the primary form present in the GnRH-I system of most amphibian species has been determined as mammalian GnRH (Licht et al., 1994; Sherwood et al., 1986; Somoza et al., 1996). An antibody that detects mammalian GnRH was used in the current study. Thus, the scope of the current study was limited to the

detection of the GnRH-I system, which consists of the terminal nerve and hypophysiotropic GnRH populations.

A significant gender by pheromone treatment interaction was found in the current study. Although no statistically significant differences among groups were discerned via post hoc pairwise comparisons, pheromones tended to increase the number GnRH-ir in neurons in females compared to males in the rostral brain. When females had pheromone delivered, the number of GnRH-ir neurons was increased compared to when vehicle was delivered. However, the number of GnRH-ir neurons detected in males tended to show minimal change with treatment of pheromone. A difference among these groups may have been detected if every other section was counted rather than every third section. Amphibian GnRH cell bodies are large and few and number, so at least half of the brain should be counted in the future.

The significant gender by pheromone treatment interaction was found in the rostral portion of the brain, which contains primarily GnRH-secreting neurons of the terminal nerve. The function of the terminal nerve is largely unknown, but its close association with the main olfactory epithelium suggests that it may act to modulate olfactory and chemosensory information. The fibers of terminal nerve GnRH terminate near the main olfactory epithelium in mudpuppies (Eisthen et al., 2000). Application of GnRH to the olfactory epithelium modulated responses of the olfactory epithelium after exposure to odorants (Park and Eisthen, 2003; Zhang and Delay, 2007). Thus, it is possible that processing of pheromonal stimuli is interacting with terminal nerve GnRH.

Even though the ultimate role of terminal nerve GnRH is unknown, there are data that suggest GnRH neurons of the terminal nerve may be involved in vertebrate

reproduction. Lesions of the terminal nerve decreased or ablated certain reproductive behaviors in gourami fish and hamsters (Wirsig and Leonard, 1987; Yamamoto et al., 1997). Alternatively, social cues altered the terminal nerve GnRH-I system. Male green treefrogs exposed to chorusing frogs had increased number of GnRH neurons compared to male frogs that listened to control tones. These GnRH neurons were located in the medial septum and preoptic area region of the brain, which are part of the terminal nerve GnRH-I system. Additionally, androgen concentrations also increased in these males (Burmeister and Wilczynski, 2005). Courtship increased GnRH-ir neurons of the accessory olfactory bulb, terminal nerve, and tenia tecta in female musk shrews; however, these numbers declined immediately after ejaculation (Dellovade et al., 1995). Similar results were seen in female roughskin newts, *T. granulosa*, where GnRH concentrations increased in the anterior telencephalon at the beginning of courtship, but began to decline by sperm transfer. Courted females also had increased concentrations of estradiol than uncourted females and this persisted for 24 hours after mating (Propper and Moore, 1991). The GnRH concentrations of the preoptic area and hypothalamus, which comprises the hypophysiotropic GnRH-I system was not altered (Propper and Moore, 1991). Therefore, pheromonal information may be interacting with terminal nerve GnRH to alter reproductive behavior in female *P. shermani*.

There was no effect of pheromones on GnRH-secreting neurons in the caudal portion of the GnRH-I system. This portion of the brain contains primarily hypophysiotropic GnRH, which is involved in reproductive axis. Secretion of GnRH to the median eminence causes release of LH and FSH, which then act on the gonads to release the sex steroid hormones, androgens and estrogens (Norris, 2006). Any increase

in hypophysiotropic GnRH should result in increased estrogens and/or androgens. Concurrent with no effect on the hypophysiotropic GnRH, application of pheromone had no effect on plasma sex steroid hormones in another study performed in *P. shermani* (Schubert et al., 2009).

Female *P. shermani* had more neurons in the caudal, hypophysiotropic region of the GnRH-I system than males. It is unknown what the functional significance of this sex difference is. These animals were collected during the breeding season while in reproductive condition. It is possible that reproductive females have more hypophysiotropic GnRH during the breeding season because they have elevated estradiol levels (Norris, 2006; Woodley, 2007). Differences between reproductive and non-reproductive animals should be investigated to examine any differences in GnRH-ir neurons. In other species, males and females sometimes differ in the numbers of GnRH cells. In the musk shrew, males had significantly more GnRH neurons than females (Rissman and Li, 1998). In the halfspotted goby, females had more and larger terminal nerve GnRH cells during the peak and non-spawning periods than males (Maruska et al., 2007). Conversely, in the goldfish, there were no sex differences in terminal nerve GnRH neurons (Parhar et al., 2001).

Pheromones did not alter the number of AVT-ir neurons in the red-legged salamanders. This finding potentially contrasts with studies in male roughskin newts, in which olfactory stimuli increased firing of medullary neurons in male roughskin newts. This response was blocked when males were given an AVP antagonist, suggesting that AVT is mediating this pheromonal response (Thompson et al., 2008). AVT also induced clasping behavior in male roughskin newts (Moore and Miller, 1983). The brains of the

salamanders in the current study were processed two hours after last pheromone application. It is possible that an increase in AVT-ir neurons would be seen at another time point.

Sex differences in AVT-ir neurons were found in the brain of *P. shermani*, but not to the extent that is found in roughskin newts (Moore et al., 2000). Reproductive and non-reproductive female red-legged salamanders had more AVT-ir neurons than did males in the dorsal pallium. Reproductive males and females had more AVT-ir neurons than did non-reproductive females in the posterior preoptic area. In roughskin newts, breeding females had more AVT-ir neurons in the hypothalamus than males, and non-breeding females had more AVT-ir neurons in the bed nucleus of the decussation of the fasciculus lateralis telencephali and in the nucleus visceralis superior, nucleus isthmi region compared to males. In the bed nucleus of the stria terminalis, the amygdala, and the anterior preoptic area, male newts had more AVT-ir neurons than females (Moore et al., 2000). The hypothalamus, amygdala, and preoptic area are all areas that control reproductive behavior (Moore et al., 2000). The AVT system in roughskin newts is much more extensive occurring in 19 brain areas, whereas AVT-ir neurons were found in only 14 brain areas in *P. shermani* (Hollis et al., 2005; Lowry et al., 1997). A lack of strong sex differences in the brain of *P. shermani* suggest that AVT may not be as involved in sexually dimorphic behavioral functions in red-legged salamanders as it is in the roughskin newt.

In conclusion, there was a trend for pheromones to increase terminal nerve GnRH-ir in females but had no effect on AVT. Given these results, pheromones may interact with the TN-GnRH-I system to alter behavior in *P. shermani*. Conversely, AVT

may not have the strong behavioral effects in *P. shermani* as is seen in the roughskin newt. More comparative work needs to be done to examine the mechanisms by which pheromones induce changes in behavior and to examine differences in GnRH and AVT systems.

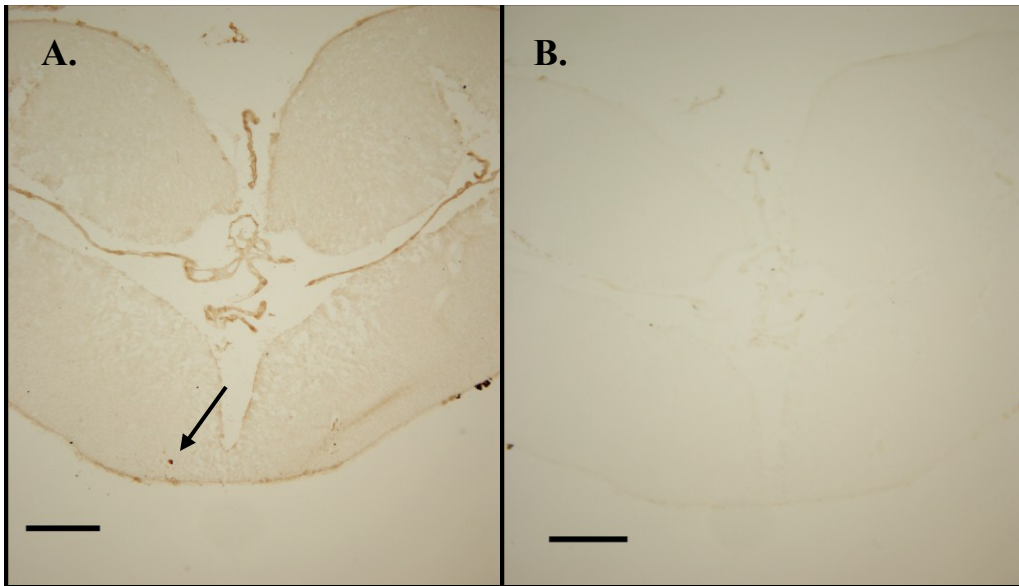


Figure 2.1. Photomicrographs from *P. shermani* brain sections. a) GnRH antibody staining as indicated by the arrow. b) An alternate brain section that was not treated with the primary antibody showing a lack of GnRH-ir neurons or fibers. Scale bars indicate 100 μ m. Brains were sectioned in the transverse plane.

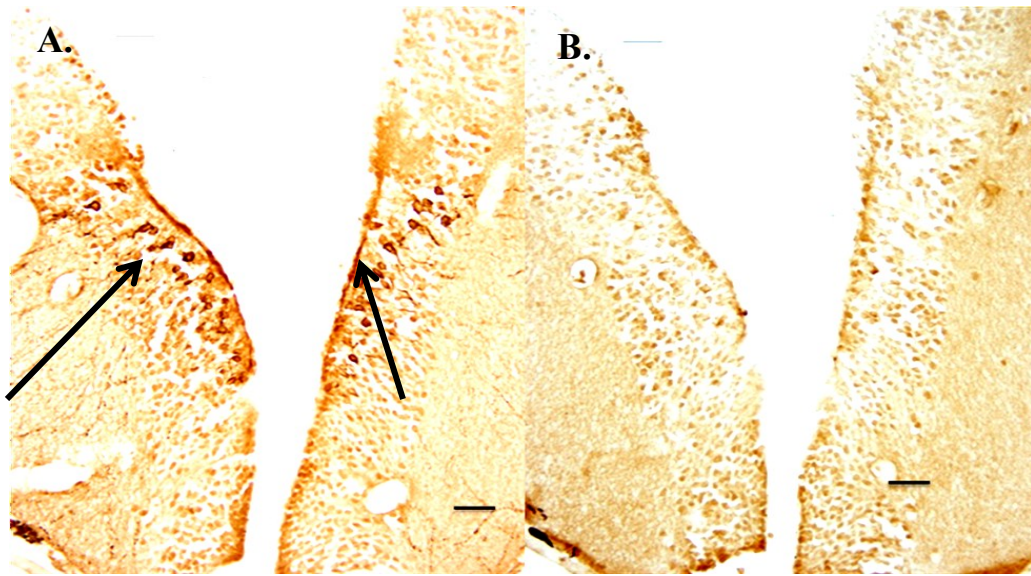


Figure 2.2. Photomicrograph of *P. shermani* brain section of the preoptic area with a) AVT-ir neurons as indicated by arrows. b) An alternate brain section processed with an AVT primary antibody preabsorbed with AVT peptide. Note the absence of AVT-ir neurons and fibers. Scale bar indicates 50 μm . Brains were sectioned in the transverse plane.

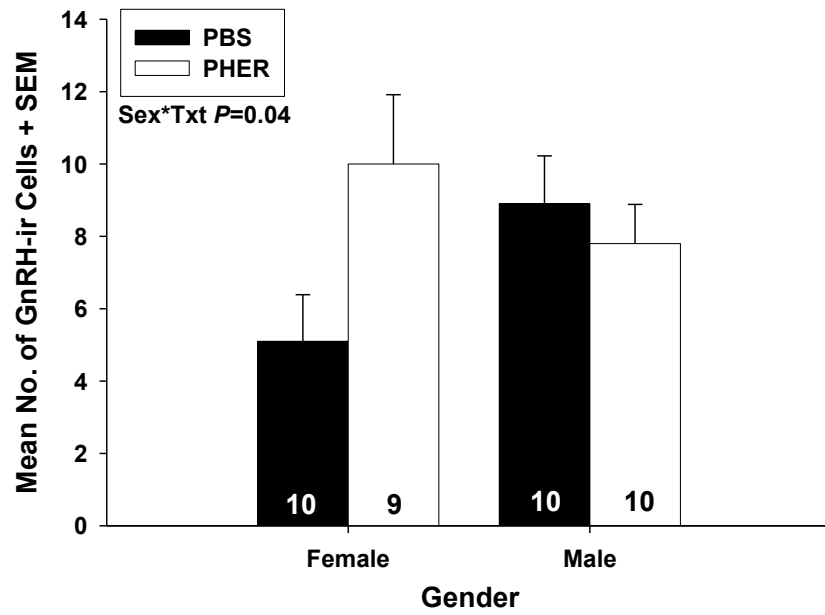


Figure 2.3. Mean number of GnRH-ir numbers in the rostral brain in male and female *P. shermani* treated with vehicle (PBS) or pheromone (PHER). Significant interaction effect listed in panel. Sample sizes are listed within each bar.

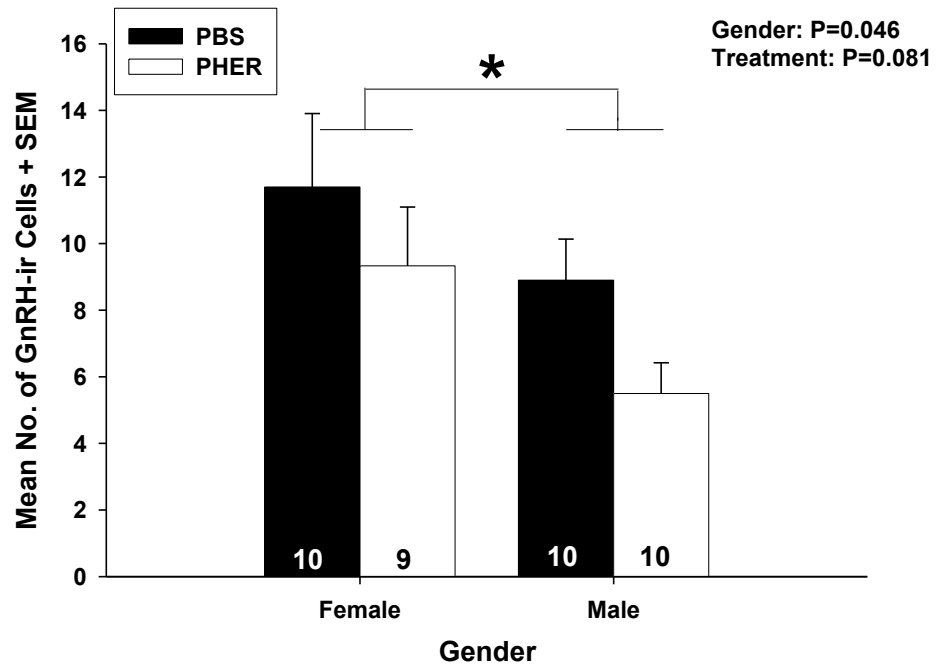


Figure 2.4. Mean number of GnRH-ir numbers in the caudal brain in male and female *P. shermani* delivered with vehicle (PBS) or pheromone (PHER). Asterisk denotes significant difference between genders. Main effects listed in panel. Sample sizes are listed within each bar.

Table 2.1. Cell area and optical density of GnRH-ir neurons located in the rostral and caudal brains of male and female *P. shermani*. Animals received either vehicle (PBS) or mental gland pheromone (PHER) treatment.

		Rostral Brain		Caudal Brain	
		PBS	PHER	PBS	PHER
Cell Area (micron²)	Female (n=9)	0.00058 ± 0.000044	0.00055 ± 0.000083	0.00053 ± 0.000033	0.00050 ± 0.000030
	Male (n=9)	0.00072 ± 0.000024	0.00049 ± 0.000042	0.00056 ± 0.000059	0.00051 ± 0.000057
Optical Density	Female (n=9)	0.089 ± 0.013	0.094 ± 0.011	0.11 ± 0.017	0.11 ± 0.012
	Male (n=9)	0.093 ± 0.012	0.082 ± 0.0049	0.10 ± 0.012	0.085 ± 0.010

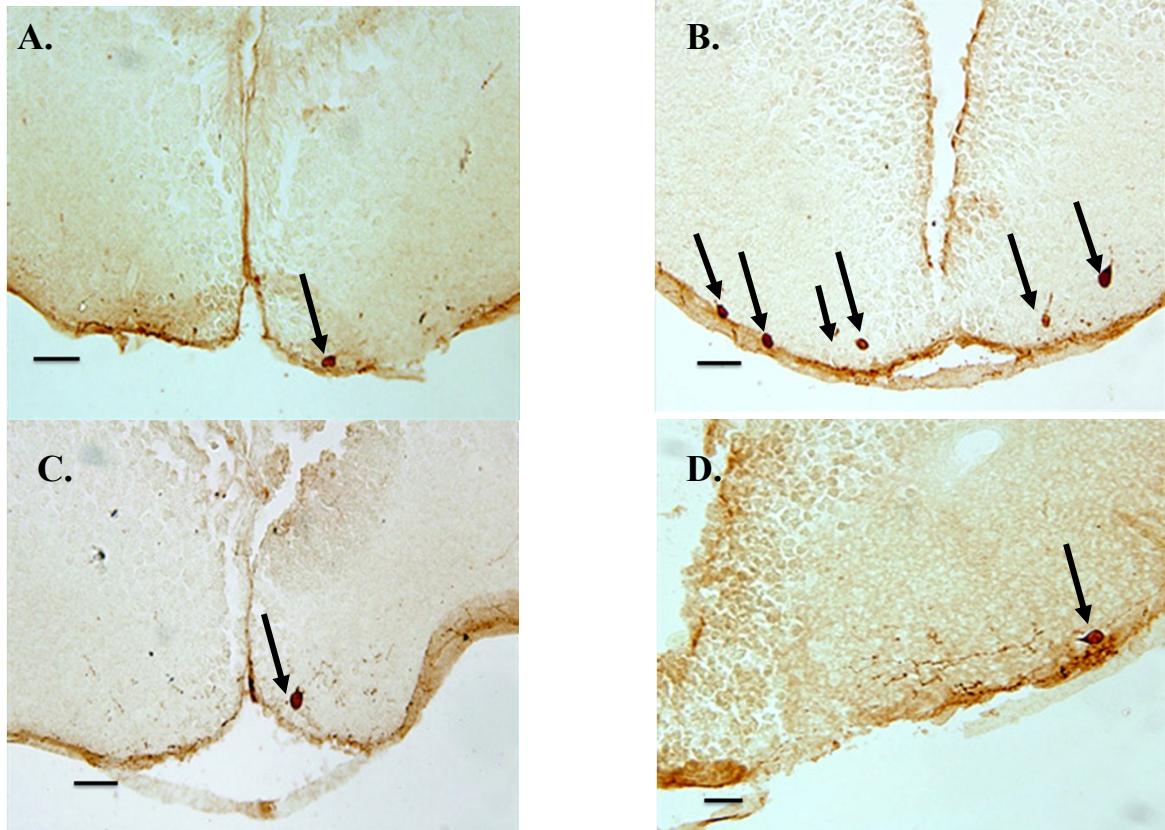


Figure 2.5. Representative photomicrographs of *Plethodon shermani* showing GnRH-ir neurons in a) female treated with a vehicle, b) female treated with pheromone, c) male treated with vehicle, d) male treated with pheromone. Scale bars indicate 50 μm . Arrows indicate GnRH-ir neurons. Brains were sectioned in the transverse plane.

Table 2.2. Brain regions in which AVT-ir neurons or transcripts were found in studies performed in roughskin newts (*T. granulosa*; Lowry et al., 1997) and in red-legged salamanders (*P. shermani*; Hollis et al., 2005) and the current study.

*Brain Regions	Lowry et al., 1997 (ir)	Hollis et al., 2005 (transcript expression)	Present Study (ir)
Dorsal pallium (V1*)	++	+	++
Medial pallium (V2)	++	++	++
Medial septum (V3)	++	+	+
Lateral pallium		+	+
Bed nucleus of the stria terminalis (V4)	++	+	+
Amygdala (V5)	+	+	-
Bed nucleus of the decussation of the lateral forebrain bundle (V6)	+	+	+
Anterior POA (V7)	++	+	++
Ventral Thalamus (V8)	+	+	+
Magnocellular POA (V9)	++	+	++
Posterior POA (V10)	++	+	++
Ventral POA (V11)	+	+	+
Dorsal hypothalamus (V12)	+	-	+
Ventral hypothalamus (V13)	+	-	+
Dorsolateral hypothalamus (V14)	+	-	-
Rostral ventromedial mesencephalon (V15)	+	-	-
Nucleus visceralis superior-nucleus isthmi region (V16)	++	+	-
Nucleus cerebella (V17)	+	-	-
Optic tectum		+	-
Inferior colliculus (V18)	+	-	-
Lateral auricle-area acusticolateralis continuum (V19)	+	-	-

*Populations of AVT-ir neurons indentified by Lowry et al., 1997 in *T. granulosa*.

Table 2.3. Number of AVT-ir neurons (mean \pm SEM) in various brain regions of male and female (reproductive and non-reproductive) *P. shermani* treated with vehicle (PBS) or pheromone (PHER). Numbers presented here were counted in every third brain section.

Brain Region	Male		Female		Non-rep. Female		
	PBS (n=7)	PHER (n=6)	PBS (n=5)	PHER (n=7)	PBS (n=7)	PHER (n=6)	
Medial Pallium	227.3 \pm 41.8	231.8 \pm 125.2	223 \pm 96.5	206.4 \pm 61.78	209 \pm 68.5	214 \pm 51.5	Gender: F _{2,37} =0.20, P=0.82 Treatment: F _{1,37} =0.01, P=0.93
Dorsal Pallium	26.1 \pm 25.9	27.5 \pm 8.2	58 \pm 35.1	60.6 \pm 28.2	32.3 \pm 17.7	56 \pm 42.2	Gender: F_{2,37}=4.87, P=0.014 Treatment: F _{1,37} =1.43, P=0.24
Lateral Pallium	1.0 \pm 2.2	1.0 \pm 1.6	0.4 \pm 0.6	0.4 \pm 0.8	0.7 \pm 1.1	0.5 \pm 0.6	Gender: χ^2 =0.36, P=0.84 Treatment: χ^2 =0.064, P=0.80
Bed Nucleus of the Stria Terminalis	0.4 \pm 0.8	0.3 \pm 0.5	0.4 \pm 0.6	1 \pm 1.6	0	0.2 \pm 0.4	Gender: χ^2 =5.61, P=0.061 Treatment: χ^2 =1.17, P=0.28
Bed Nucleus of the Decussation of the Lateral Forebrain Bundle	0	0	0	1.3 \pm 2.6	0.3 \pm 0.8	0	Gender: χ^2 =2.83, P=0.30 Treatment: χ^2 =0.39, P=0.53
Lateral Cellular Prominence	0.1 \pm 0.4	0.3 \pm 0.5	1.6 \pm 3.1	0	0.3 \pm 0.5	0.2 \pm 0.4	Gender: χ^2 =0.096, P=0.95 Treatment: χ^2 =0.71, P=0.40
Medial Septum	1.9 \pm 4.1	2.3 \pm 2.5	3 \pm 3.7	0.4 \pm 1.1	2.1 \pm 4.1	5.2 \pm 6.3	Gender: χ^2 =0.68, P=0.71 Treatment:

							$\chi^2=0.13$, P=0.72
Ventral Cellular Prominence	2.7 ± 3.5	3 ± 3.5	5.2 ± 5.3	0.7 ± 1.5	4.7 ± 6.0	0.5 ± 1.2	Gender: $\chi^2=0.53$, P=0.77 Treatment: $\chi^2=2.86$, P=0.091
Anterior Preoptic Area	31.8 ± 9	28.8 ± 6.1	35.4 ± 13.5	24.7 ± 6.3	38.9 ± 9.0	20.7 ± 6.7	Gender: $F_{2,37}=0.17$, P=0.85 Treatment: $F_{1,37}=2.33$, P=0.14
Ventral Thalamus	2.5 ± 2.31\	0.2 ± 0.2	1.2 ± 0.7	2.3 ± 1.8	0.3 ± 0.3	5.7 ± 2.7	Gender: $\chi^2=1.01$, P=0.60 Treatment: $\chi^2=0.64$, P=0.43
Magnocellular Preoptic Area	47.3 ± 17.2	30.6 ± 10.7	46 ± 20.6	38.4 ± 8.6	23 ± 7.2	34.8 ± 9.8	Gender: $F_{2,37}=0.59$, P=0.56 Treatment: $F_{1,37}=0.13$, P=0.72
Posterior Preoptic Area	22.5 ± 4.8	33 ± 9.6	48.4 ± 4.8	29.1 ± 6.9	17.3 ± 3.7	24.7 ± 9.2	Gender: $F_{2,37}=3.83$, P=0.03 Treatment: $F_{1,37}=0.05$, P=0.83
Ventral Preoptic Area	0.2 ± 0.2	0.3 ± 0.3	2.6 ± 2.4	0.3 ± 0.3	0	0	Gender: $\chi^2=3.58$, P=0.17 Treatment: $\chi^2=0.22$, P=0.64
Dorsal Hypothalamus	0.9 ± 1.5	2.3 ± 4.4	0	0.3 ± 0.5	0.6 ± 1.5	0.3 ± 1.5	Gender: $\chi^2=1.58$, P=0.45 Treatment: $\chi^2=0.40$, P=0.53
Ventral Hypothalamus	0	2.7 ± 6.5	0	0	0	0.2 ± 0.4	Gender: $\chi^2=0.39$, P=0.53 Treatment: $\chi^2=0.95$, P=0.62

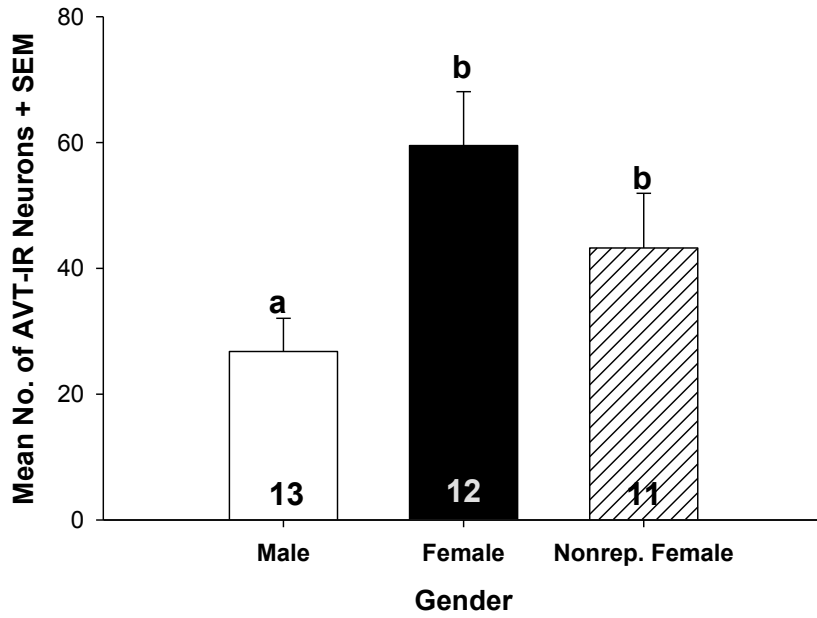


Figure 2.6. Mean number of AVT-ir numbers in the dorsal pallium in male, female, and non-reproductive female *P. shermani*. Letters denote significant differences among genders. Sample sizes are listed within each bar.

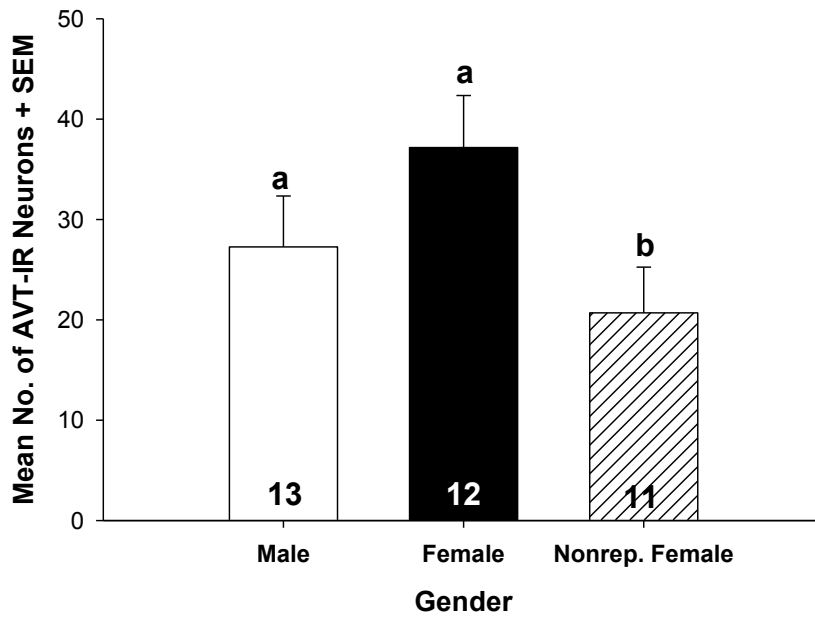


Figure 2.7. Mean number of AVT-ir numbers in the posterior preoptic area in male, female, and non-reproductive female *P. shermani*. Letters denote significant differences among genders. Sample sizes are listed within each bar.

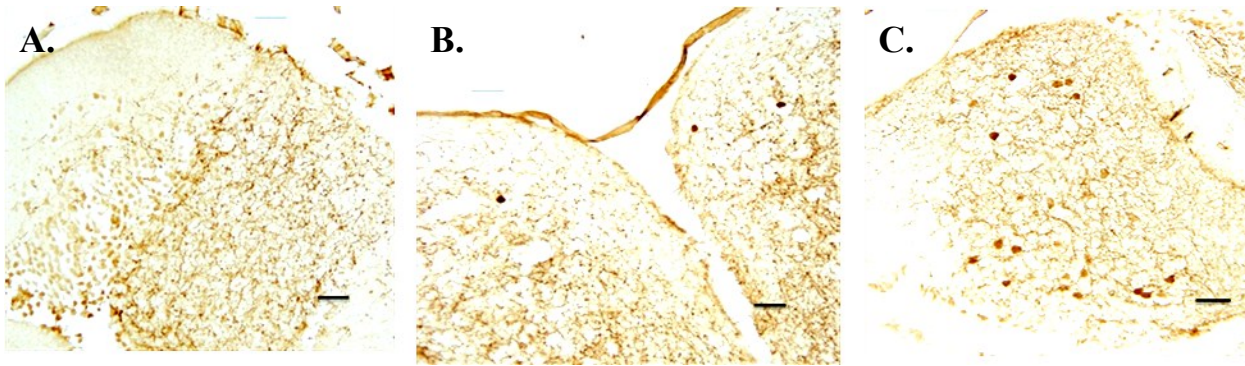


Figure 2.8. Representative photomicrographs from *P. shermani* with AVT-ir neurons in the dorsal pallium in a) male, b) female, c) and non-reproductive female. Scale bars indicate 50 μ m. Brains were sectioned in the transverse plane.

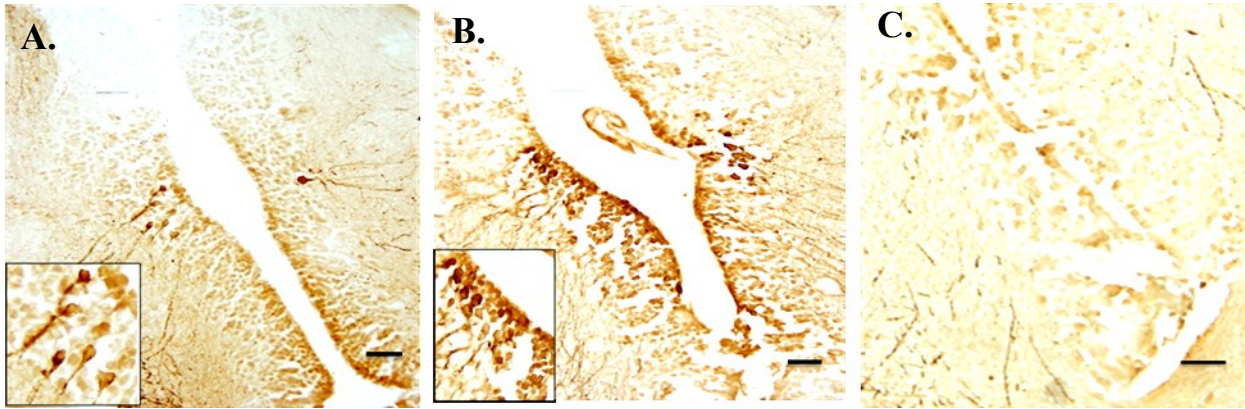


Figure 2.9. Representative photomicrographs from *P. shermani* with AVT-ir neurons in the posterior preoptic area in a) male, b)

41 female, c) and non-reproductive female. Scale bars indicate 50 μ m. Brains were sectioned in the transverse plane.

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Chapter 3

Effects of vasotocin and vasopressin antagonist on courtship and mating in *Desmognathine* salamanders

ABSTRACT

Arginine vasotocin (AVT) and its mammalian homologue, arginine vasopressin (AVP), alter various social behaviors in vertebrates, including newts. I investigated the role of injections of AVT and AVP antagonist on the expression of courtship and mating behavior in two species of *Desmognathine* salamanders, *Desmognathus ochrophaeus* and *D. osee*. I also quantified the sex differences in the number of AVT-immunoreactive (ir) neurons in the brain. I injected several doses of AVT and AVP antagonist and measured behaviors at 30 minutes, 2 hours and 4 hours after injection. There was no effect of AVT or AVP antagonist in latency time for a male to approach a female, engage in an advanced stage of courtship, or deposit a spermatophore. Additionally, the number of males that successfully mated was similar regardless of whether males were injected with AVT, AVP antagonist or vehicle. AVT-ir neurons were found in many brain areas involved in male reproductive behaviors. The only sex difference was that females had more AVT-ir neurons in the ventral preoptic area than did males. These data suggest that AVT may not be involved in sexually-dimorphic behaviors in two species of semi-

terrestrial Desmognathine salamanders as it is in newts, which are fully aquatic species. More studies need to be performed to understand the degree to which behavioral actions of AVT are conserved in amphibians and other vertebrates.

INTRODUCTION

Sex steroid hormones, such as testosterone and estradiol, are required for the expression of sexual behavior, but are not always sufficient. In addition to sex steroid hormones, various neuropeptides, such as arginine vasotocin (AVT) or its mammalian homologue, arginine vasopressin (AVP) (Goodson and Bass, 2001; Moore and Rose, 2002) are necessary. AVT/AVP are members of the nonapeptide family, which also includes oxytocin, mesotocin and isotocin. The best characterized role of AVP/AVT is water reabsorption by the kidney, but AVP/AVT also acts in the brain through the V1a receptor to affect social behavior in many vertebrates (Keverne, 2004). AVT/AVP have widespread behavioral effects in vertebrates including altering sexual behavior in fish, amphibians, birds, and mammals, scent marking in amphibians and mammals, parental behavior and mate choice in mammals, and aggressive behavior in birds and mammals (reviewed in Goodson and Bass, 2001). AVT/AVP generally affects male behavior more than female behavior, however this may reflect the relatively large number of studies examining male rather than female sexual behavior (Goodson and Bass, 2001).

Given the widespread behavioral effects of AVT in vertebrates, Rose and Moore (2002) proposed that AVP/AVT modulates vertebrate sensorimotor processing. That is, AVP modulates processing of sensory information, such as somatosensory or chemosensory inputs, to initiate a motor response. Much of the work to support the

sensorimotor hypothesis has been done in salamanders, specifically newts. In male roughskin newts (*Taricha granulosa*), AVT injections caused males to engage in amplexic clasping of females more often than males treated with the vehicle (Moore and Miller, 1983). Application of AVT increased responsiveness of medullary neurons to tactile stimulation of the cloaca, suggesting that AVT increased responsiveness of neurons to tactile stimuli (Moore and Rose, 2002). Additionally, AVT-treated male *T. granulosa* spent more time in proximity to female visual and olfactory stimuli compared to controls (Thompson and Moore, 2000). Application of pheromones in male *T. granulosa* initiated action potentials in the medulla, which controls motor responses. Responsiveness of the medulla to pheromones was blocked with an AVP antagonist (Thompson et al., 2008). Intraperitoneal (ip) injections of AVT in male Japanese newts (*Cynops pyrrhogaster*) increased incidence and frequency of courtship behavior and increased numbers of spermatophores deposited, which are gel-like substances on which a male deposits his sperm. Additionally, ip injections of an AVP V1a receptor antagonist blocked courtship behavior and decreased spermatophore deposition (Toyoda et al., 2003). These data suggest that AVT modulates processing of sensory stimuli, including chemosensory stimuli, to alter expression of reproductive behavior in amphibians.

AVT/AVP neurons are found in various regions throughout the brain, but are more extensive in urodeles than in other vertebrates. Using immunohistochemical and in situ hybridization techniques, Lowry et al. (1997) found 19 AVT populations from the rostral portion of the pallium to the tegmentum in *T. granulosa*. Mammals have AVT-ir neurons in only 11 of these 19 populations (Moore and Lowry, 1998). Additionally, in *T. granulosa*, there are strong sex differences in the number of AVT-immunoreactive (ir)

neurons in the brain. Males had greater numbers of AVT neurons than females in the bed nucleus of the stria terminalis, amygdala, and anterior preoptic area, and breeding females had more AVT-ir neurons than breeding males in dorsal and ventromedial hypothalamus. Each of these brain regions are important in controlling sexual behavior (Moore et al., 2000). The extensive distribution of AVT neurons and multiple sex differences found in the brain regions that control sexual behavior support the behavioral data in *T. granulosa* in which AVT modulates amplexic clasping in males.

To further understand AVT/AVP's effect on social behaviors in vertebrates, I investigated the role AVT plays in reproductive behavior of semi-terrestrial plethodontid salamanders. In accordance with the sensorimotor hypothesis, AVT should modulate the visual, tactile, and chemosensory information during courtship to enhance courtship and mating in Desmognathine salamanders. Our hypothesis was that AVT mediates the expression of male sexual behavior and is distributed in the brain in a sexually-dimorphic fashion in male *D. ochrophaeus* and *D. ocoee*. I predicted that injection with 1) an AVP V1a receptor antagonist would increase latency to courtship and decrease the incidence of mating and 2) AVT injections would decrease latency to courtship and increase the incidence of mating. I also predicted that I would see a male-biased sex difference in the numbers of AVT neurons in brain regions linked to male mating behavior.

METHODS

To understand the effects of AVT and AVP antagonist on courtship and mating in plethodontid salamanders, a series of 5 experiments were performed on two species of plethodontid salamanders, *Desmognathus ochrophaeus* and *D. ocoee* (Table 3.1). These

two species are closely-related species in the *D. ochrophaeus* complex and have similar reproductive patterns and ecological niches (Benner and Woodley, 2007; Tilley and Mahoney, 1996). *D. ochrophaeus* and *D. ocoee* are found in Pennsylvania and North Carolina, respectively, and are semi-terrestrial, inhabiting stream-side habitats in the Appalachian Mountains. Males and females breed during both spring and fall. Females mate with multiple males and store the sperm until ovulation and oviposition occur, which may be much later after mating. Females lay their eggs in late spring.

Two experiments used *D. ochrophaeus*, and three experiments used *D. ocoee*, based on availability (Table 3.1). Males were paired with females after injections of AVP antagonist or AVT, and courtship and mating was observed by digital recording or scan sampling. Courtship and mating were recorded by a time lapse digital camcorder 30 minutes after injections in *D. ochrophaeus* and *D. ocoee* during October 2008 and May 2009, respectively. Scan sampling was performed in the remaining experiments. Courtship and mating was observed at 4 hours after injection in *D. ochrophaeus* and *D. ocoee* during July 2009 and June 2009, respectively, and also at 2 hours after injection for *D. ocoee* during June 2009.

Table 3.1. Outline of experiments, which includes the species used, treatment, the time interval between treatment and pairing with females, and the method used to observe behaviors.

Species	Treatment: Injection with...	Time Interval	Behavioral Recording Method
<i>D. ochrophaeus</i>	0 and 20 µg AVP antagonist	30 min	Digital
<i>D. ocoee</i>	0, 10, and 50 µg AVP antagonist	30 min	Digital
<i>D. ocoee</i>	0 and 75 µg AVP antagonist	2 hrs	Scan sampling
<i>D. ocoee</i>	0 or 75 µg AVP antagonist	4 hrs	Scan sampling

<i>D. ochrophaeus</i>	10 and 50 µg/g AVT and 0 and 20 µg/g AVP antagonist	4 hrs	Scan sampling
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Animals

Ocoee salamanders (*D. ocoee*) were hand caught in Macon County, NC in August 2008 (83° 33' 9" N longitude; 35° 2' 22" W latitude). Allegheny dusky salamanders (*D. ochrophaeus*) were hand caught in Westmoreland County, PA (79° 13' 51" N longitude; 40° 9' 54" W latitude) during May, June, and September 2008 and October 2009. The mental glands of *D. ocoee* were removed for biochemical analysis of courtship pheromones (procedures conducted by Dr. Lynne Houck). Male *D. ochrophaeus* retained their mental glands. Animals were collected with appropriate permits from the North Carolina Wildlife Commission and Pennsylvania Fish Commission. Animals were housed individually in 16x16x5 cm plastic home boxes lined with moist paper towels under a 14:10 light:dark cycle at 16°C. They were fed wax worms every two weeks. All methods were approved by Duquesne University's Institutional Animal Care and Use Committee.

Behavioral Experiments

I injected males with either AVT or AVP antagonist depending upon their propensity to mate in pre-mating trials, and I observed their courtship and mating after pairing with females. Arginine vasotocin (Bachem) and arginine vasopressin V1a receptor antagonist (Manning compound; Bachem) were dissolved in amphibian ringers to make 5 mg/ml stock solutions. Different doses of arginine vasopressin (AVP) antagonist (10, 20, 50 and 75 µg) and arginine vasotocin (AVT) (10 and 50 µg/g body

weight) were prepared from the stock solutions. Depending on the experiment, males were paired with females at 30 minutes, 2 hours, and 4 hours after injections to examine the time course of action of AVT or AVP antagonist. Doses and time courses were chosen from previous studies by Moore and Zoeller (1979), Moore and Miller (1983), and Toyoda et al. (2003). Animals were either recorded with a digital recorder or visually scanned to measure the time it took males to engage in courtship and successfully mate with a female.

Mating Behavior

In plethodontid salamanders, males transfer sperm externally via a spermatophore. The spermatophore is placed on the substrate during courtship and is made up of a gelatinous material that is topped by a sperm mass. Courtship and mating consist of a series of stereotyped behaviors. Male courtship begins when a male approaches a potentially receptive female and applies a mental gland courtship pheromone to the female (Houck and Reagan, 1990; Rollmann et al., 2003). Next, the male positions himself in front of the female. The female then straddles the male's tail. While the pair is tail straddling position (TSP), the male and female moves forward in tandem. This stereotyped courtship behavior is an advanced stage of courtship and is called tail-straddling walk (TSW) (Arnold, 1976; Promislow, 1987). At some point during TSW, the male deposits a spermatophore and moves forward until the female's cloaca is above the spermatophore, at which point the female collects the sperm into her cloaca. Sperm is externally visible in a female's cloaca for several hours after

insemination (Verrell and Mabry, 2003). Spermatophores remain on the substrate for several hours after courtship.

Mating trials were performed before the experiment to determine initial mating propensity. Successful mating was determined by the presence of spermatophore and/or the presence of a sperm plug in the female's cloacal vent. Since AVT increased, and AVP antagonist decreased, amplexic clasping in newts (Moore and Miller, 1983; Toyoda et al., 2003), I injected males with AVT when initial mating propensity was less than 50% but injected males with AVP antagonist when mating propensity was above 50%.

On the day of an experiment, injections were administered intracoelomically on the lower side of the abdomen with a sterile 0.5 ml TB syringe fitted with a 25 gauge needle. Subjects were then placed in petri dishes (13.5 cm diameter) or testing chambers (4.5x4.5x3 cm), that were lined with paper towels moistened with *ddH₂O*. Males then were paired with females for 0.5, 2, or 4 hours after injections. Testing was done under dim light, and animals were visually isolated from one another. Depending on the experiment, injections were given between 14:30 hr and 18:30 hr, and males were paired with females between 17:00 hr and 19:00 hr. For all experiments, each animal received each treatment in a repeated measures design, except for the final experiment in *D.*

ochrophaeus when males received an injection of AVT, AVP antagonist or vehicle.

Mating and courtship was observed via a digital camcorder or visual scans. In the first two experiments, chambers were recorded using a time lapse digital camcorder that recorded for 2 seconds every 30 seconds for 10 hours. Viewing of digital recordings allowed for an accurate measurement of latency to touching and TSW. However, spermatophore deposition and female insemination could not be easily and reliably

discerned via digital recordings. In subsequent experiments, chambers were visually observed and behavior recorded using scan sampling methods (Martin and Bateson, 1993) every 5 to 15 minutes for 4-6 hours or until 75% of males had deposited a spermatophore. Using scan sampling methods, I could determine the time at which touching, TSW, and spermatophore deposition occurred within the 4 to 6 hours of observation. After visual observations, pairs were left in testing chambers until the next morning. The following morning, testing chambers were examined for spermatophore deposition, and females were examined for insemination.

AVT-immunoreactivity

Brain Tissue Processing

To determine sex differences in the distribution and number of AVT-ir neurons in the brains of *D. ochrophaeus*, adult animals of both sexes were collected from the field during October 2009. One week after capture, animals were euthanized by rapid decapitation. Heads were partially dissected by removing the skin and cartilage over the brains and were placed in 4% paraformaldehyde. After 2-3 hours, brains were removed from the heads and placed back in 4% paraformaldehyde and left on a shaker overnight. Brains were cryoprotected in 30% sucrose in PBS for 24 hours. After cryoprotection, brains were placed in 30% sucrose:OCT (3:1) overnight on a shaker. Brains then were embedded in OCT and snap frozen in liquid nitrogen. Brains were stored at -20°C until cut in 20 µm sections with a cryostat and thaw mounted on polylysine-coated slides. Slides were kept at -80°C until AVT neurons were visualized using standard immunocytochemical procedures.

AVT Immunocytochemistry

Immunocytochemistry (ICC) was performed at room temperature on every third brain section using standard procedures. Briefly, sections were washed with PBS and 0.5% H₂O₂ and placed in a pre-incubation solution containing PBS, 0.2% Triton-X, 1% normal goat serum and 0.04% sodium azide. Sections then were incubated overnight in anti-AVT antibody (gift from Dr. Matthew Grober, raised in rabbits) diluted 1:2000 in preincubation solution. The following day sections were washed with PBS and incubated with biotinylated goat-anti-rabbit IgG diluted 1:400 in PBS for 30 minutes. Sections were washed again and incubated with Vectastain ABC reagent for 1 hour. Sections were washed and immunoreactive cells were visualized using diaminobenzidine as the chromagen. Sections were washed and dehydrated with an ascending series of ethanol. Coverslips were affixed to slides using permount.

Various controls were conducted to test the immunocytochemical methods used on alternate sections. To check for background staining from the secondary antibody, the primary AVT antibody was removed from the procedure. To test for the specificity of antibody labeling, a preabsorption control was performed. The preabsorption control was conducted by incubating primary AVT antibody with 5 µg/ml concentration of AVT overnight. Each of these controls resulted in an absence of immunoreactivity. Results of the AVT preabsorption control is seen in figure 3.2. Results for the no AVT primary antiserum control are not shown, because staining was so light for these controls that photomicrographs could not be captured.

AVT Analysis

Sections were examined using an Olympus Brightfield microscope. All AVT-ir cells were counted throughout the brain. Distribution of AVT neurons were determined using salamander brain atlases (Hollis et al., 2005; Lowry et al., 1997) as guides.

Analysis

For behavioral data collected from digital recording, latency times were calculated to the nearest 30 seconds. For behavioral data collected from visual scans, latency times were calculated to the nearest 5 or 15 minutes depending on period of observation. For those animals that did not enter TSW or deposit a spermatophore, animals were given the maximum time of observation of 10 hours for digital recording or 4-6 hours for visual scans.

As necessary, data were log-transformed to meet assumptions of normality (Kolmogorov-Smirnov, $P < 0.05$) and homogeneity of variances (Levene, $P < 0.05$) and analyzed using repeated measures or one-way ANOVA. When data did not meet assumptions of parametric statistics, data were analyzed using nonparametric repeated measures Wilcoxon signed ranks tests or Friedman tests. Differences between males and females in the number of AVT-ir neurons were performed separately for each brain region in which AVT-ir neurons were located. All analyses were performed using SPSS, version 12.0.

RESULTS

The effects of AVT and AVP antagonist on courtship and mating

AVP antagonist injections (20 μg) had little effect on mating in male *D. ochrophaeus* when paired with females 30 minutes after injections. The latency time for males to engage in TSW (Fig. 3.2a; $F_{1,9} = 0.11$, $P=0.75$) and the number of females inseminated ($Z=-0.58$, $P=0.56$) were similar regardless whether males were injected with AVP antagonist or vehicle. However, fewer male *D. ochrophaeus* injected with 20 μg AVP antagonist deposited spermatophores when examined the morning after injections compared to those injected with vehicle (Fig. 3.2c; $Z=-2.24$, $P=0.025$).

In an effort to follow up on the initial result in *D. ochrophaeus*, I examined the effects of AVP antagonist on courtship and mating in male *D. ocoee*. Males were paired with females 30 minutes after injections. Latency time for males to engage in TSW after injection was similar regardless of when males were injected with 10 or 50 μg AVP antagonist or vehicle (Fig. 3.3a; $F_{2,32} = 1.65$, $P=0.21$). There were a similar number of spermatophores deposited (Fig. 3.3c; $Z=2.91$, $P=0.23$) and females inseminated ($Z=0.93$, $P=0.63$) when examined the morning after injections regardless of when males were injected with AVP antagonist or vehicle.

To determine whether AVP antagonist had delayed effects, I paired males with females 2 and 4 hours after injections. Males injected with 75 μg AVP antagonist and paired 2 hours after injections had a similar latency time for a male to approach a female ($Z=-0.97$, $P=0.33$), latency time to engage in TSW (Fig. 3.4a; $Z=-0.85$, $P=0.39$), and latency time to spermatophore deposition (Fig. 3.4a; $Z=-1.32$, $P=0.19$) compared to when these males were injected with vehicle. Additionally, these males deposited a similar

number of spermatophores (Fig. 3.4c; $Z=-0.71$, $P=0.48$) and inseminated a similar number of females ($Z=-0.33$, $P=0.74$) within 6 hours after pairing. When examined the next morning, there was still no difference among treatments in the number of spermatophores deposited (Fig. 3.4c; $Z=-0.82$, $P=0.41$) and the number of females inseminated ($Z=-0.38$, $P=0.71$).

Males injected with 75 μg of AVP antagonist and paired 4 hours after injections engaged in TSW sooner than males who were injected with vehicle (Fig. 3.5a; $F_{1,18} = 4.60$, $P=0.046$). Males injected with AVP antagonist had a similar latency for a male to approach a female ($F_{1,18} = 1.95$, $P=0.18$) and latency to spermatophore deposition (Fig. 3.5a; $F_{1,18} = 2.20$, $P=0.16$) compared to males injected with vehicle. Within the first six hours after pairing, percentage of males engaged in TSW ($Z=0.0$, $P=0.32$), deposited spermatophores (Fig. 3.5c; $Z=-0.61$, $P=0.54$) and inseminated females ($Z=-0.503$, $P=0.62$) were similar between males injected with AVP antagonist or vehicle. No additional males deposited a spermatophore when examined the next morning after visual scans were complete.

Male *D. ochrophaeus* injected with AVT and AVP antagonist did not differ from males injected with vehicle with regards to the latency times for a male to approach a female (Fig. 3.5a; $\chi^2=0.97$, $P=0.81$), for a male to engage in TSW (Fig. 3.6a; $\chi^2=0.54$, $P=0.91$), or to deposit a spermatophore (Fig. 3.6c; $\chi^2=0.54$, $P=0.91$). Additionally, there were no differences in mating success as measured by the incidence of spermatophore deposition (Fig. 3.6c; $\chi^2=0.47$, $P=0.93$) or insemination ($\chi^2=0.67$, $P=0.88$) within the first 5 hours after pairing. The incidence of spermatophore deposition (Fig. 3.6c; $\chi^2=1.67$,

P=0.64) and insemination ($\chi^2=2.79$, P=0.43) did not differ among treatments by the following morning.

Number of AVT-ir neurons in the brain

AVT-ir neurons were found throughout the brain in males and females, including the medial and dorsal pallium, septum, and anterior, magnocellular, ventral, and posterior preoptic area (Table 3.2). Females had more AVT-ir neurons in the ventral preoptic area than did males (Table 3.2; $F_{1,18}=7.92$, P=0.011). Males and females had similar number of AVT-ir neurons in the medial pallium ($F_{1,18}=1.17$, P=0.29), dorsal pallium ($F_{1,18}=0.021$, P=0.89), septum ($F_{1,18}=0.57$, P=0.46), anterior preoptic area ($F_{1,18}=0.12$, P=0.74), magnocellular preoptic area ($F_{1,18}=3.68$, P=0.071), posterior preoptic area ($F_{1,18}=0.010$, P=0.92). Representative photomicrographs of AVT staining in the preoptic area are shown in figure 3.7.

DISCUSSION

AVT/AVP has widespread effects on social behaviors in vertebrates, including newts. The sensorimotor hypothesis states that AVT modulates specific sensory information to initiate a motor response, which promotes species-specific social behaviors (Moore and Rose, 2002). I asked whether AVT/AVP had behavioral effects in two species of semi-terrestrial plethodontid salamanders. Specifically, I wanted to investigate the effect of AVT on courtship and mating, behaviors that involve tactile sensory exchange between males and females. After several experiments injecting animals with different doses of AVT and AVP antagonist and pairing males with females

at different times after injections, I was unable to find consistent effects on male courtship and mating. Out of five experiments, there were only two significant results: in one study 20 μ g AVP antagonist decreased spermatophore deposition when paired with females 30 minutes later, but in another study, AVP antagonist caused males to engage in TSW faster than when those males were injected with vehicle. The lack of consistent or robust results suggests that treatment with AVP antagonist did not affect male courtship and mating.

Our results contrast with results from similar studies done in newts (Moore and Zoeller, 1979; Toyoda et al., 2003). The lack of an effect of AVT/AVP antagonists on courtship and mating could be due to a number of variables, such as dose or the time interval between injections and testing; however, I do not believe this to be true. I used several doses of AVP antagonist and AVT in an attempt to find an effect on courtship and mating. Initial doses were similar to those given to Japanese and roughskin newts (Moore and Zoeller, 1979; Toyoda et al., 2003). When those doses did not replicate results seen in newts, I increased the dose of AVP antagonist and used low and high doses of AVT. There was still no effect on courtship and mating in males at increased doses. It is possible that these increased doses were still not high enough to see an effect in the *D. ochrophaeus* and *D. ocoee*. I also paired males with females at different times after injections: 0.5, 2, and 4 hours. The time intervals were similar to those used in newts (Moore and Miller, 1983; Moore and Zoeller, 1979; Toyoda et al., 2003), but again, it is possible that AVP antagonist and AVT needed more time to take an effect in Desmognathine salamanders.

Even though I could not find an effect of AVT on courtship and mating, it is possible that AVT modulates other sexual behaviors that were not measured in the current study. In addition to AVT's effects on sexual behavior, Toyoda et al. (2003) found that AVT increased pheromone secretion in Japanese newts. Abdominal glands that secrete the newt pheromone, sodefrin, were analyzed for sodefrin content after AVT injections. Animals injected with AVT had less sodefrin than animals injected with the vehicle, suggesting that AVT is involved in pheromone secretion (Toyoda et al., 2003). Additionally, animals treated with AVP antagonist had decreased contractions of the abdominal glands compared to animals treated with saline (Toyoda et al., 2003). The mental gland of Desmognathine salamanders secrete a courtship pheromone that increases female receptivity during courtship (Houck and Reagan, 1990). AVT may act on the mental gland in plethodontid salamanders to increase secretion of this courtship pheromone. If AVT increases secretion of pheromones, I would expect increased mating success in animals injected with AVT and decreased mating success in males injected with AVP antagonist. In the current study, male *D. ocoee* had their mental glands removed prior to the experiment, so courtship pheromone secretion was not possible. However, male *D. ochrophaeus* retained their mental gland in both experiments in which this species was used. In the initial experiment, male *D. ochrophaeus* deposited fewer spermatophores when they were injected with AVP antagonist compared to controls. However, in the subsequent experiment, this result could not be replicated. Unlike the initial experiment, the propensity to mate was low. The experiments in the current study should be replicated using males with and without mental glands of the same species.

Additionally, future experiments should examine the content of the male's mental gland after injections of AVT or AVP antagonist.

In addition to AVT's effects on sexual behavior, AVT also has a wide variety of effects on other social behaviors in other vertebrates. For example, AVT modulated aggressive behavior in lizards, birds, and fish (Backström and Winberg, 2009; Goodson et al., 2009; Hattori and Wilczynski, 2010). To date there are no data published examining AVT's effect on aggressive behavior in salamanders. Additionally, injections of AVT has been shown to increase locomotion in bullfrogs compared to vehicle injected frogs (Boyd, 1991). AVT injections are also involved in mate attraction by altering parameters of calling in male frogs (reviewed in Boyd, 1997). For example, AVT increased calling behavior in male bullfrogs (Schmidt and Kenmitz, 1989). In tungara frogs, AVT treatment influenced males' motivation to call and their ability to call (Kime et al., 2010). These behaviors could have important roles in the ability for males to find and attract mates. Since AVT has diverse roles in modulating behaviors in vertebrates, other behaviors must be investigated in plethodontid salamanders to determine whether AVT has behavioral effects in plethodontid salamanders.

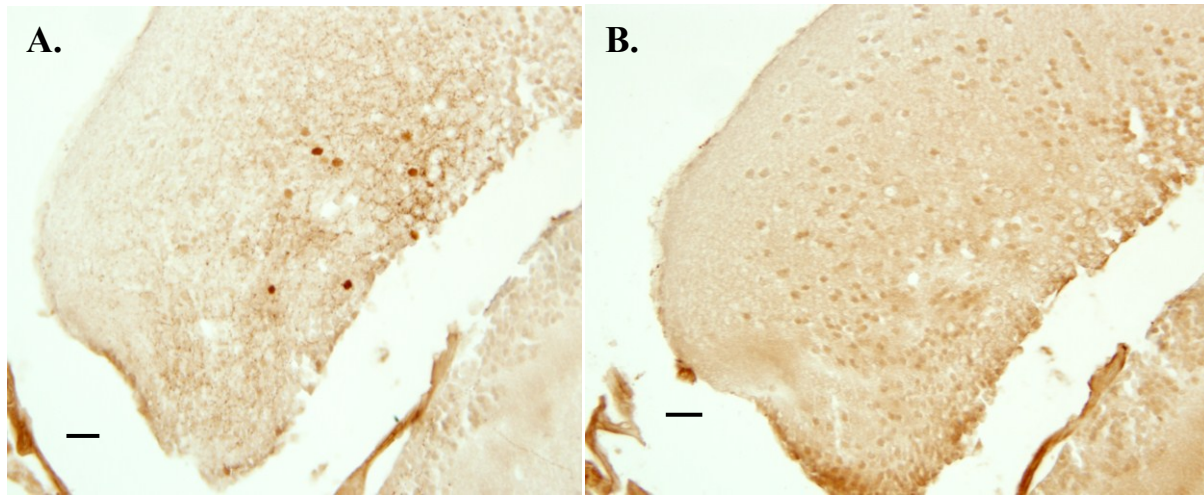
The lack of strong sex differences in the number of AVT-ir neurons in the brain of *D. ochrophaeus* supports the behavioral data in the current study. Studies performed in *T. granulosa* demonstrated a strong effect of AVT on mating in males (Moore and Zoeller, 1979) as well as sexually dimorphic distribution of AVT in the brain regions that control sexual behavior (Moore et al., 2000). In Desmognathine salamanders, a sex difference was found in only one brain region, the ventral preoptic area, with female *D. ochrophaeus* having more AVT-ir neurons than did males. The preoptic area is an area of

the brain that regulates sexual behavior in vertebrates, including salamanders (Malacarne and Giacoma, 1980). Increased numbers of AVT-ir neurons of the ventral POA in females may indicate that AVT has a stronger behavioral effect in female *D. ochrophaeus* compared to males. Future studies should examine female sexual behavior of plethodontid salamanders. However, it is likely that there will not be a strong effect of AVT on mating and courtship in Desmognathine salamanders due to the lack of the many strong sex differences in areas of the brain that control sexual behavior in *D. ochrophaeus* compared to *T. granulosa*.

The lack of an effect on courtship and mating in Desmognathine salamanders in the current study compared to other studies in newts may be related to phylogenetic differences in these two groups of salamanders. AVP/AVT has diverse and evolutionarily divergent behavioral roles in vertebrates (reviewed in Goodson and Bass, 2001). In mammals, AVP promoted parental behavior in monogamous voles, but had no effect on this behavior in non-monogamous voles, which was related to differences in receptor gene expression in these two groups (Young et al., 1997). In birds, AVT increased aggression in colonial zebra finches (Goodson and Adkins-Regan, 1999), but inhibited aggression in the closely related, violet-ear waxbill (Goodson, 1998). In the current study, I have shown differences in AVT's effect on courtship and mating in Desmognathine salamanders compared to newts. Newts and Desmognathine salamanders belong to the Families Salamandridae and Plethodontidae, respectively. The plethodontids are separated from the salamandrids by 200 million years of evolution (Mauro et al., 2005). Newts are aquatic and therefore, males court and mate females in an aquatic habitat, and Desmognathine salamanders reside near streams and court and mate on land (Houck and

Arnold, 2003). AVT's strong effect on mating in newts may be due to their aquatic nature. To test this hypothesis, AVT's role in courtship and mating should be examined in an aquatic plethodontid species, such as *Pseudoeurycea aquatica*.

The lack of an effect of AVT on courtship and mating in *D. ochrophaeus* and *D. ocoee* does not preclude a lack of an effect of AVT in plethodontid salamanders altogether. More studies need to be performed to further examine the behavioral role of AVT in plethodontid salamanders. Future studies should examine pheromone secretion and mate attraction. Other behaviors, such as agonistic interactions and activity, should also be examined. Additionally, more studies need to be performed on other species of salamanders, including aquatic plethodontids to further understand the conserved mechanisms of AVT. Given the divergent behavioral roles of AVP/AVT in mammals and birds, it is possible that AVT's role in courtship and mating evolved independently in newts. AVT has a various effects on social behaviors among all groups of vertebrates, so understanding the conserved effects of this neuropeptide is important. Amphibians are basal tetrapods, so studies in this group can provide information on how AVT has evolved.



67 Figure 3.1. Photomicrograph of *D. ochrophaeus* brain section of the pallium with a) AVT antibody staining and b) an alternate brain section processed without the AVT primary antibody showing an absence of AVT-ir neurons or fibers. Scale bar is 50 μ m. Brains were sectioned in the transverse plane.

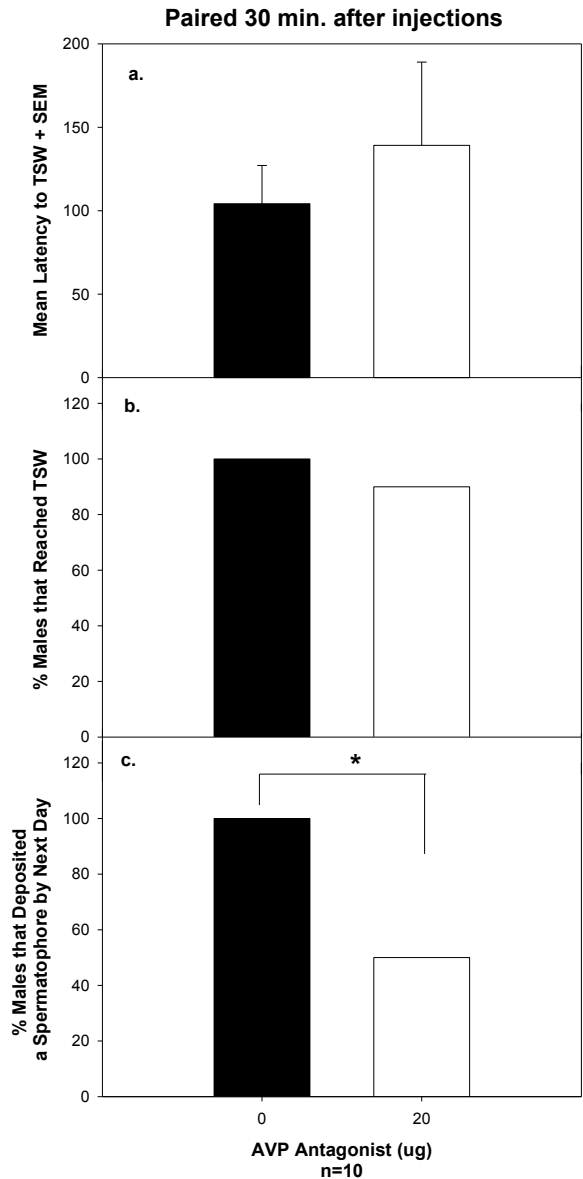


Figure 3.2. Courtship and mating in male *D. ochrophaeus* that were injected with AVP antagonist or vehicle (ringers) and paired with females 30 minutes after injection. Behaviors were recorded for 10 hours by digital camcorder. (a) Mean latency time for male *D. ochrophaeus* to enter tail-straddling walk (TSW). (b) Percentage of male *D. ochrophaeus* that reached TSW. (c) Percentage of males that deposited a spermatophore. * P=0.025, Wilcoxon signed ranks test. Each male was tested once with AVP antagonist and once with vehicle.

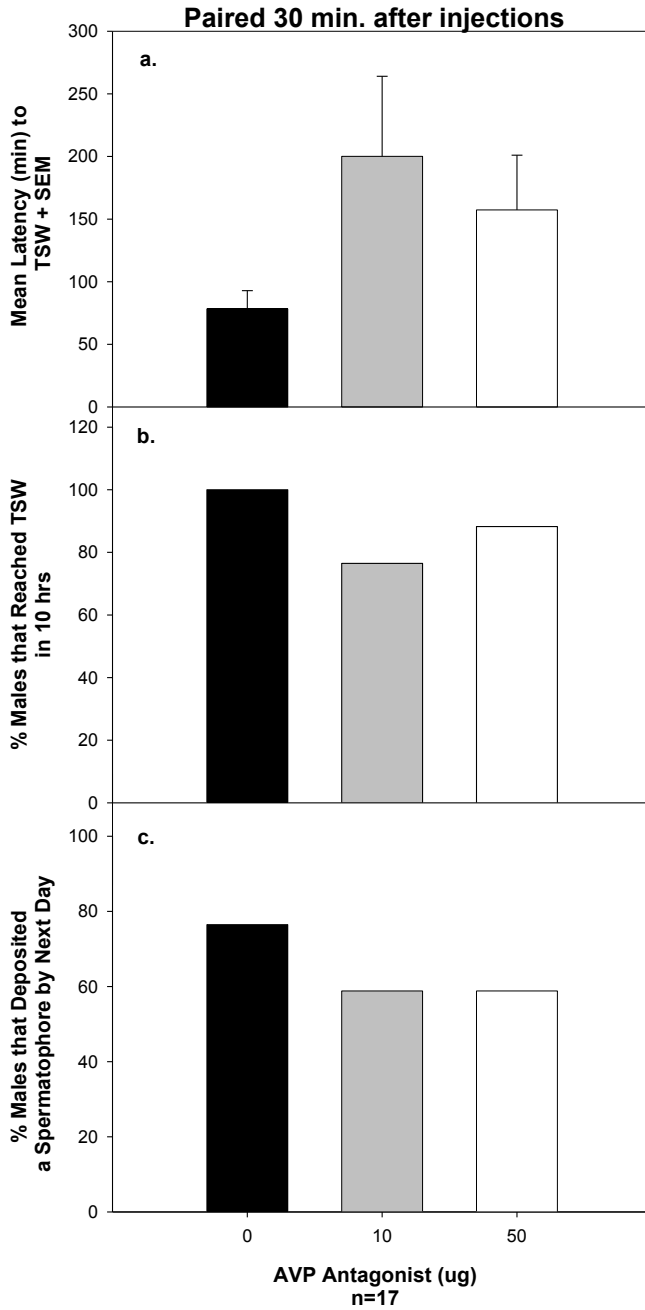


Figure 3.3. Male *D. ocoee* injected with two doses of AVP antagonist or vehicle (ringers) and paired with females 30 minutes after injections. Behaviors were recorded for 10 hours by digital camcorder. (a) Mean latency time for males to enter into tail-straddling walk (TSW). (b) Percentage of males that enter TSW. (c) Percentage of males that deposited a spermatophore.

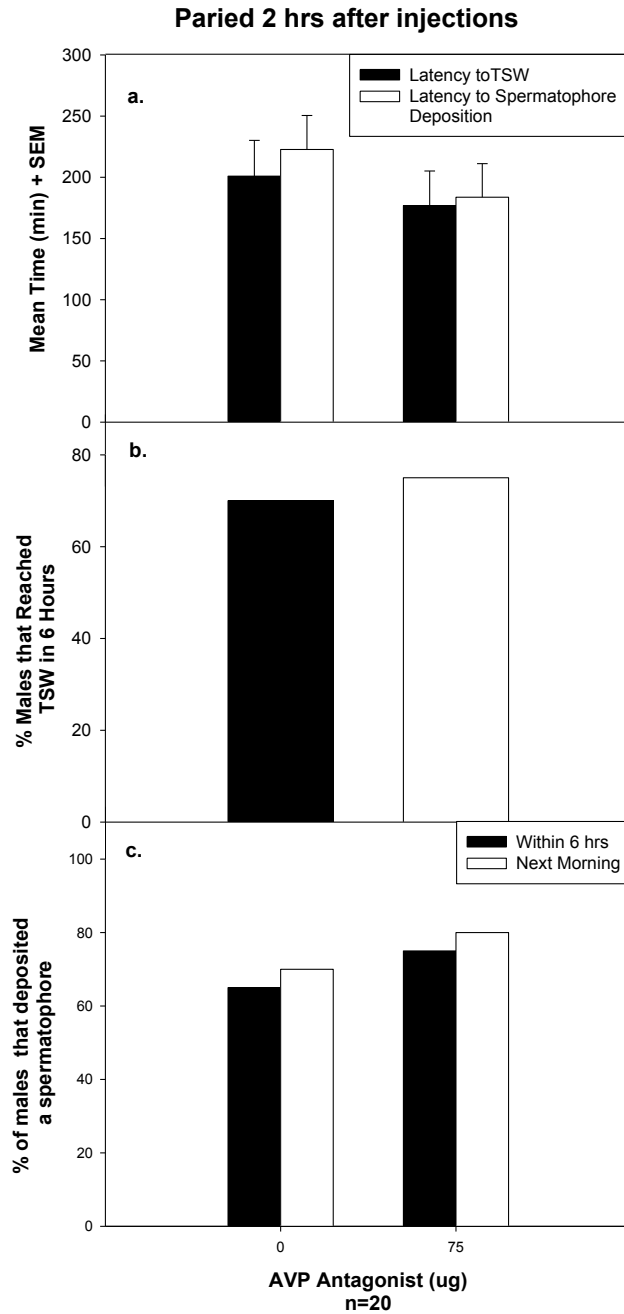


Figure 3.4. Courtship and mating in male *D. ocoee* injected with AVP antagonist or vehicle (ringers) and paired with females 2 hours after injections. Behaviors were recorded by scan sampling for 6 hours. (a) Mean latency times for males to enter into tail-straddling walk (TSW) and deposit a spermatophore. (b) Percentage of males that entered into TSW. (c) Percentage of males that deposited a spermatophore within 6 hours after pairing and by the next morning.

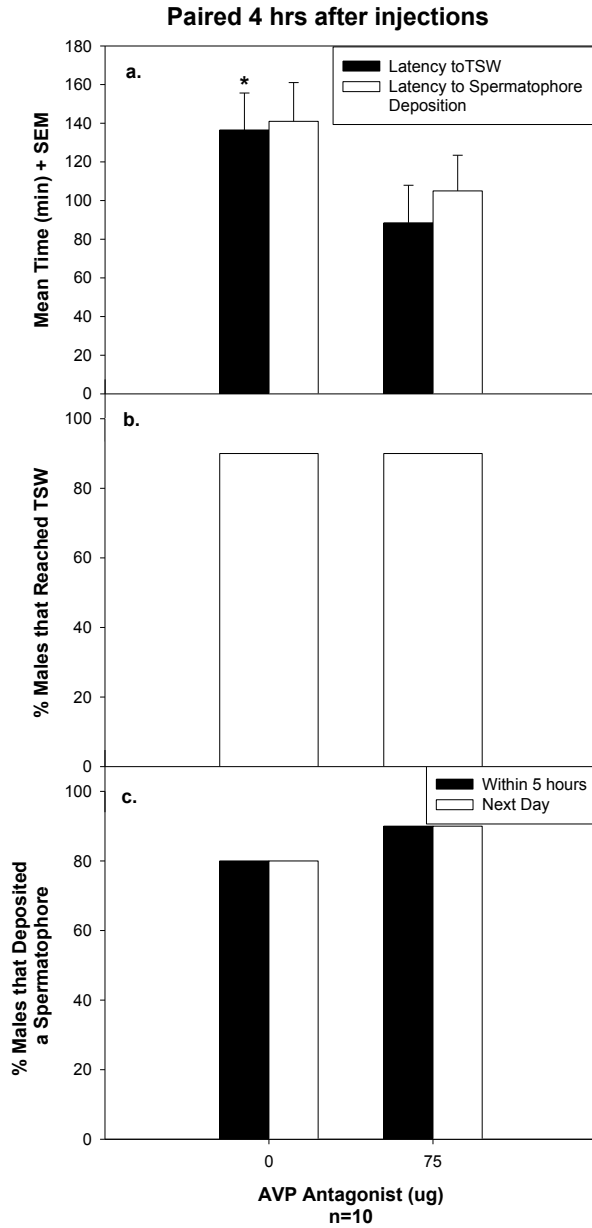


Figure 3.5. Courtship and mating in male *D. ocoee* injected with AVP antagonist or vehicles (ringers) and paired with females 4 hours after injections. Behaviors were recorded by scan sampling for 5 hours. (a) Mean latency time for males to enter tail-straddling walk and to deposit a spermatophore. (b) Percentage of males to enter into TSW. (c) Percentage of males that deposited a spermatophore within the first 5 hours of pairing or by the next morning. Asterisk indicates significant difference within latency time of TSW based on a repeated measures ANOVA.

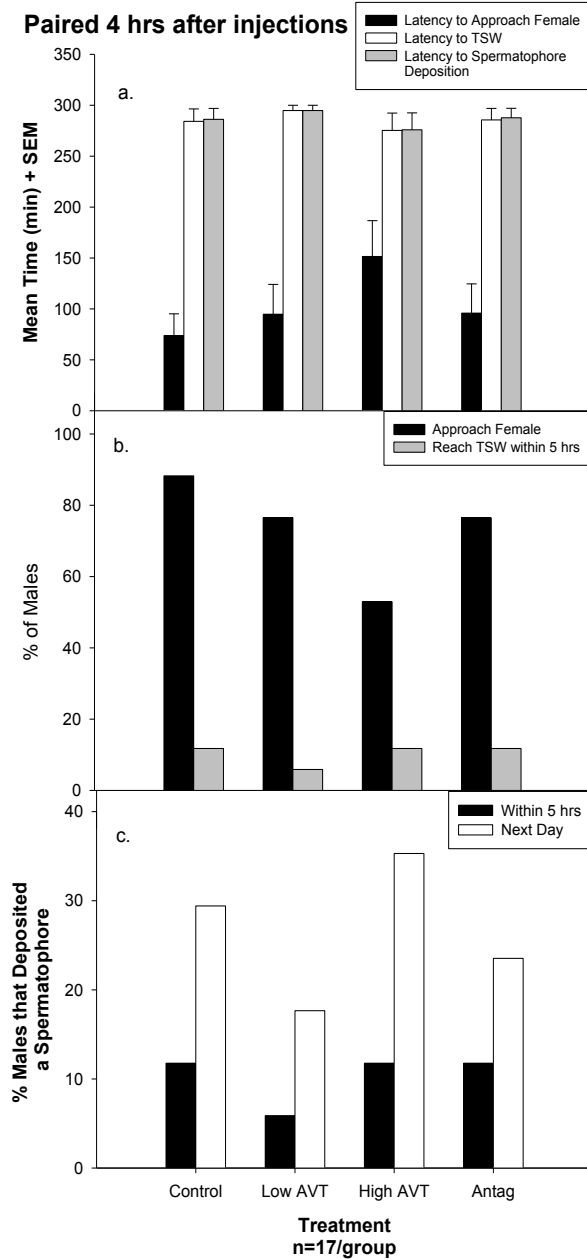
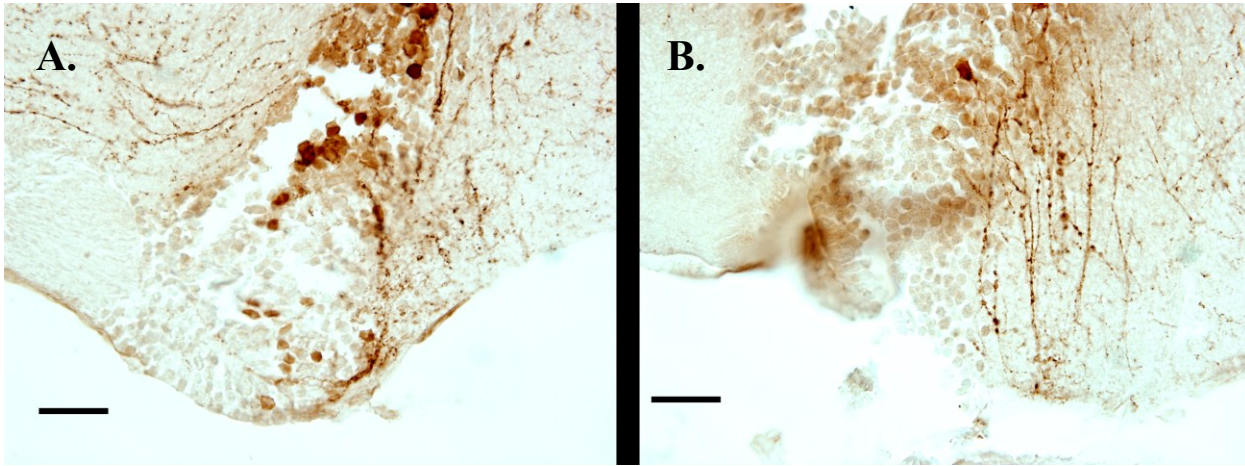


Figure 3.6. Courtship and mating in male *D. ochrophaeus* injected with 10 or 50 ug/g AVT, 20 ug/g AVP antagonist, and vehicle (ringers) and paired with females 4 hours after injections. Behaviors were recorded by scan sampling for 5 hours. (a) Mean latency times for males to enter into particular stages of courtship. (b) Percentage of males that approached a female and entered in TSW. (c) Percentage of males that deposited a spermatophore within the first 5 hours after being paired and the following morning.

Table 3.2. Number of AVT-ir neurons in male and female *D. ochrophaeus*.

Brain Area	Sex	Mean No. AVT Neurons + SEM	P-value
Medial Pallium	Male	78.9 + 10.1	0.29
	Female	64.5 + 8.7	
Dorsal Pallium	Male	6.8 + 2.3	0.89
	Female	7.4 + 3.5	
Septum	Male	1.9 + 0.66	0.46
	Female	3.1 + 1.4	
Anterior POA	Male	25.4 + 4.1	0.74
	Female	28.0 + 6.4	
Magnocellular POA	Male	7.4 + 1.8	0.071
	Female	15.7 + 3.9	
Ventral POA	Male	4.9 + 2.2	*0.011
	Female	16.7 + 3.6	
Posterior POA	Male	24.8 + 6.5	0.92
	Female	23.9 + 6.0	

*Indicates statistical significance as determined by ANOVA.



74 Figure 3.7. Photomicrograph of *D. ochrophaeus* brain sections of the preoptic area in a) female and b) male. Scale bars indicate 50 μm . Brains were sectioned in the transverse plane

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Chapter 4

Transdermal delivery of corticosterone in terrestrial amphibians

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ABSTRACT

Stressors elicit allostatic responses that allow animals to cope with changing and challenging environments and also cause release of glucocorticoid hormones (GCs). Compared to other vertebrate classes, relatively little is known about amphibian behavioral and physiological responses to GCs. To understand the effects of elevated plasma GCs in amphibians, exogenous application of GCs is necessary, but traditional methods to elevate GCs require handling and/or anesthesia which themselves are stressors. A less invasive alternative successfully used in birds and reptiles utilizes transdermal delivery by applying GCs via a dermal patch. We asked whether dermal patches containing corticosterone (CORT, the main GC in amphibians) would elevate plasma CORT in terrestrial salamanders and frogs. We explored the use of the dermal patch to deliver CORT in an acute, sustained, and repeated manner. Patches adhered well to the amphibians' moist skin and were easily removed to regulate the time course of CORT delivery. Application of CORT treated patches elevated plasma CORT concentrations compared to vehicle patches in all species. Patches delivered

physiological levels of plasma CORT in ecologically relevant time frames. Repeated application and removal of CORT patches were used to simulate exposure to repeated stressors. Application of patches did not represent a stressor because plasma CORT concentrations were similar between animals that received vehicle patches and untreated animals. Thus, transdermal delivery of GCs represents a potentially useful tool to better understand amphibian allostatic responses to stressors, and perhaps amphibian population declines.

INTRODUCTION

Stressors are unpredictable aversive stimuli that can be acute or prolonged. Responses to stressful stimuli comprise an allostatic response and help an organism cope with challenging and changing environments (McEwen, 1998; McEwen and Wingfield, 2010; Sapolsky, 2002; Wingfield et al., 1998). For example, when exposed to stressors, energy is diverted to muscles and essential organs, whereas functions nonessential for immediate survival, such as reproduction and growth, are suppressed (Martin, 2009; Wingfield and Sapolsky, 2003). In the short-term, allostatic responses are beneficial, allowing an animal to “endure, avoid, or recover” from a stressor (Martin, 2009). However, severe or repeated allostatic responses are hypothesized to produce “wear and tear”, or allostatic load, which can eventually lead to allostatic overload that can lead to sickness and disease (McEwen and Wingfield, 2010).

Although many physiological mediators are involved in allostatic responses, GCs are believed to be central to mediating allostatic responses (Breuner et al., 2008; Landys et al., 2006; Romero, 2002; Wingfield et al., 1998). Many stressors trigger elevated

plasma GCs, and studies seek to manipulate GCs to better understand the role of GCs in allostatic responses. To elevate GCs acutely, animals are injected with solutions containing GCs. However, injections themselves elicit a physiological stress response (e.g. Burmeister et al., 2002). To elevate GCs chronically, animals are typically implanted with capsules (Silverin, 1986; Tokarz, 1987) that release GCs tonically for weeks to months, depending on the species. However, implants are administered via surgery which is a severe stressor (Wilmore, 2002). Another method of elevating GCs is treatment with gelling implants in which animals are injected with a GC-containing polymer that hardens shortly after injection. The gelling implant provides sustained constant elevation of GCs for several weeks, but the polymer components may have toxic effects in small animals (French et al., 2007; Ricciardella and Woodley, unpublished).

Less invasive methods to treat animals with GCs have been developed to better understand the role of GCs in allostatic responses. In aquatic organisms, GCs added to the water elevated plasma GCs (Glennemeier and Denver, 2002). In birds and fish, GCs added to food elevated plasma hormone concentrations (Breuner et al., 1998; Overli et al., 2002). Another useful method is transdermal delivery of GCs (Knapp and Moore, 1997). This method takes advantage of the ability of GCs, which are lipid-soluble, to pass through the epidermis of the skin to enter the bloodstream. In some cases, GCs are applied directly to the skin (Belluire et al., 2004; Meylan et al., 2003; Robert et al., 2009), but in other cases, GCs are applied via dermal patches. To transdermally apply GCs, animals are briefly restrained and GCs are applied directly or via patches. Transdermal delivery is less invasive than injections and surgery and is being increasingly utilized

(Knapp and Moore, 1997; Ruiz et al., 2010; Thaker et al., 2009; Wada and Breuner, 2008).

The goal of the experiments described in this paper was to explore and develop the dermal patch method to transdermally deliver CORT in terrestrial amphibians. Most of the experiments were conducted in salamanders because background information on physiological concentrations of GCs was available (Ricciardella, 2008; Ricciardella et al., 2010; Schubert et al., 2009), but we also tested whether CORT patches had potential for use in a terrestrial anuran, *Eleutherodactylus coqui*. In the experiments, we transdermally delivered CORT via the patch for various time periods in an attempt to mimic exposure to acute, sustained, or repeated stressors. Experiments used different species and often had slightly different experimental designs. Our design choices for each particular experiment were based on our particular research agendas for each species. For example, we study the effects of acute handling stressors in dusky salamanders and the effects of sustained exposure to social cues in red-legged salamanders. Additionally, we used different species depending on their availability during the various times of the year. Although the use of different experimental designs for experiments involving different species preclude species comparisons, it is our hope that our work will provide a starting point for other investigators using CORT patches in different species of terrestrial amphibians.

METHODS

Animals

Experiments used 3 species of terrestrial salamanders and 1 species of frog, depending on availability and the goal of the experiment. Red-legged (*Plethodon shermani*) and Ocoee (*Desmognathus ocoee*) salamanders of both sexes were hand caught in Macon County, NC in August 2008 (83° 33' 37" N longitude; 35° 11' 13" W latitude and 83° 33' 9" N longitude; 35° 2' 22" W latitude, respectively). Allegheny dusky salamanders (*Desmognathus ochrophaeus*) were hand caught in Westmoreland County, PA (79° 13' 51" N longitude; 40° 9' 54" W latitude) in April – May 2009. Each of these salamanders are members of *Plethodontidae* family. Animals were collected with appropriate permits from Pennsylvania Fish and Boat Commission and North Carolina Wildlife Resources Commission. They were individually housed in 16x16x5 cm plastic home boxes lined with moist paper towels. They were maintained in the laboratory on a 14:10 light:dark cycle at 16°C and were fed wax worms. Coqui frogs, *Eleutherodactylus coqui*, were collected from Hawaii with Injurious Wildlife Export permits provided by the Hawaiian Department of Land and Natural Resources. Frogs were housed in 16x17x13 plastic containers with moist paper towels and a wooden perch. Methods conformed to relevant regulatory standards and were approved by Duquesne University's Institutional Animal Care and Use Committee.

Steroid preparation

Crystalline CORT (Cat. no. Q1550-000, Steraloids Incorp.) was dissolved in sesame oil at a stock concentration of 5 mg/ml. Final doses of CORT were made from the

5 mg/ml stock solution. Sesame oil was used as the vehicle control. CORT doses were selected in an effort to achieve physiological concentrations of plasma CORT, depending on the species. We did not adjust CORT patch doses according to body weight. Subsequent analyses showed no relationship between body weight and plasma CORT concentrations achieved in animals that received CORT patches.

Patch methods

Dermal patches consisted of low protein binding filter paper (Cat No. 1820-070 from Whatman) cut into 1.5 x 3 mm rectangles. Patches were applied with forceps to animals located in their home boxes at room temperature between 09:30 and 10:30, unless noted otherwise. Paper towel substrates were removed from home boxes 30 minutes prior to patch application, so patches would not be removed if animals positioned themselves under the substrates. Patches were placed on the dorsum of the animal between the two front legs, and 2.5 μ l of CORT or sesame oil vehicle was pipetted onto the patch using a 10 μ l eppendorf pipetman. For all experiments, a volume of 2.5 μ l contained the appropriate CORT dose. After a period of time, depending on the experiment, patches were removed gently using forceps.

The patches adhered well to the moist skin of amphibians. In salamanders, patches almost always stayed on, unless an animal rubbed its back against the side of the home box. In these cases, animals were not sampled. In contrast to salamanders, coqui frogs were agile and often removed the patches with their legs. To minimize patch removal in the frogs, we habituated the frogs to the patches by applying dermal patches containing oil ten times over 15 days. Even after the habituation period, some frogs

would still remove patches. Therefore, if a frog removed a patch prematurely during the experiment, the investigator immediately reapplied the patch and continued with the experiment. Reapplication of patches was noted.

Single Patch Application in Salamanders

In Allegheny dusky salamanders (*D. ochrophaeus*), plasma CORT was significantly elevated 30 minutes after capture in the field or handling in the laboratory (Ricciardella et al., 2010). To determine whether dermal patches could elevate plasma CORT to physiological concentrations in a dose-dependent manner, dermal patches containing vehicle, 0.625 μg CORT, or 1.25 μg CORT were administered to male *D. ochrophaeus*. Patches were removed 30 minutes after application and trunk blood was collected.

To determine whether dermal patches themselves elicited an increase in plasma CORT, a second experiment was done using female *D. ochrophaeus* that included a group of animals that did not receive a dermal patch (“no patch”). Patches were removed 30 minutes after application and trunk blood was collected.

To determine the time course of hormone delivery after a 30 minute application of a dermal patch, patches containing vehicle or 0.625 μg CORT were applied to female *D. ochrophaeus* and were removed 30 minutes after application. Trunk blood was collected at 30, 60 or 180 minutes after initial patch application.

In male red-legged salamanders (*P. shermani*), exposure to social cues increased plasma CORT for up to 2 hrs (Schubert et al., 2009). To determine whether dermal patches could elevate CORT in a sustained manner, patches that contained vehicle, 2.5

μg CORT, or 5 μg CORT were applied to male *P. shermani*. Patches were left on animals for 30, 90, or 240 minutes, and trunk blood was collected with immediately after patch removal.

Single Patch Application in Frogs

Capture and handling elevated plasma CORT at 30 minutes after capture in *Rana esculenta* (Mosconi et al., 2006) and at 3 hrs after capture in whistling frogs (Coddington and Cree, 1995). To determine whether patches would elevate plasma CORT in terrestrial frogs, dermal patches containing oil vehicle or 10 μg of CORT were applied to the lower back of 9 males and 11 female coqui frogs. Twenty minutes after patch application, animals were sacrificed by rapid decapitation and trunk blood was collected.

Repeated Patch Application in Salamanders

To test whether the patch could be used to simulate exposure to repeated stressors, Ocoee salamanders (*D. ocoee*) received patches that contained either 1.25 μg of CORT or oil vehicle daily (30 minutes/day) for nine consecutive days. To control for the effects of repeated patch application, one experiment included animals that did not receive any patches and were not disturbed in any way until the day of blood collection (NO PATCH). To determine whether repeated exposure to a patch affected baseline concentrations of CORT, blood was collected either 1 hr after initial patch application, or 8 hrs after initial patch application. We picked 8 hrs as an attempt to capture “baseline” concentrations of plasma CORT, assuming that CORT administered transdermally would have been cleared from the circulation by 8 hrs after removal of the patch. Blood was

collected from all animals at the same time of day to avoid confounding effects of a diel CORT cycle. Thus, patches were applied either at 9:30 and blood was collected at 17:30, or patches were applied at 16:30 and blood samples were collected at 17:30. Differences in plasma CORT concentrations 8 hrs after patch application among oil patch, CORT patch, and NO PATCH animals would indicate that repeated patch application altered baseline plasma CORT.

Hormone measurement

Blood samples were collected to measure plasma CORT. In some experiments, plasma testosterone in males and plasma estradiol (E2) in females were measured to determine whether treatment with CORT altered sex steroid hormone concentrations. To obtain blood samples, unanaesthetized animals were decapitated and trunk blood was collected within 2 minutes of handling via a heparinized capillary tube. Blood was centrifuged and the plasma was placed in a heparinized microcentrifuge tube and stored at -20°C. Six experiments were conducted, and samples from a given experiment were assayed together. Samples from 5 of the experiments were assayed for steroid hormone concentrations by Dr. Matthew Lovern at Oklahoma State University. Samples from the experiment using female *D. ochrophaeus* were measured by the Endocrine Services Laboratory at the Oregon National Primate Research Center. Briefly, in assays in which only CORT was measured, a double ether extraction was performed on the plasma and CORT was measured via radioimmunoassay. In assays in which CORT and T or E2 both were measured, hormones were separated by column chromatography after double ether

extraction and measured via radioimmunoassay (Gruenewald et al., 1992; Lovern et al., 2000; Resko et al., 1980).

Intra-assay coefficients of variation (CVs) for the 6 CORT assays ranged from 2.4% to 14.5%. The intra-assay CV for the E2 assay of female *D. ocoee* was 15.6%. Intra-assay CVs for the T assays of male *D. ochrophaeus*, *P. shermani*, and *D. ocoee* were 4.5%, 14.2%, and 5.2%, respectively.

Statistical Methods

Out of a total of 6 experiments consisting of 182 samples, 2 outliers for plasma CORT were identified using the Grubb's test and were removed from analyses. Data were analyzed with ANOVA. When needed, data were log transformed to meet assumptions of normality (Kolmogorov-Smirnov, $P < 0.05$) and homogeneity of variances (Levene, $P < 0.05$). When the significance level of 0.05 was reached for overall effects, differences among groups were determined via a Student-Newman Keuls (SNK) post hoc test. Additionally, Pearson correlations were performed on CORT and sex steroid hormone (T for males and E2 for females) concentrations. All tests were performed using SPSS version 12.0.

RESULTS

Acute Patch Applications in Salamanders

A 30 minute application of a patch containing CORT elevated plasma CORT relative to vehicle controls in a dose dependent manner in male *D. ochrophaeus* (Fig. 4.1; $F_{2,22}=81.03$, $P < 0.001$). There was no effect of CORT application on plasma T

concentrations (data not shown) in males ($F_{2,22}=0.28$, $P=0.756$), nor was there a correlation between plasma CORT and T concentrations ($r=-0.07$, $P=0.739$). Although patches containing CORT elevated plasma CORT relative to patches containing oil vehicle in female *D. ochrophaeus*, (Fig. 4.2; $F_{2,13}=217.09$, $P<0.001$), SNK pairwise comparison tests indicated that there was no difference between animals that received the patch containing oil and animals that did not receive a patch.

In female *D. ochrophaeus*, treatment with a CORT patch for 30 minutes elevated plasma CORT concentrations for up to 3 hrs following initial patch application (Fig. 4.3; effect of patch: $F_{1,21}=92.87$, $P<0.001$). There was no significant difference in plasma CORT 30, 60 or 180 minutes following initial patch application (effect of time: $F_{2,21}=1.50$, $P=0.245$), but plasma CORT concentrations were lowest 180 minutes after the initial patch application (Fig. 4.3).

In male *P. shermani*, treatment with CORT patches for 30, 90, or 240 minutes elevated plasma CORT concentrations compared to vehicle controls (Fig 4.4; effect of patch: $F_{2,19}=58.41$, $P<0.001$). Plasma CORT concentrations achieved were similar regardless of the duration of patch application (effect of duration of exposure: $F_{2,19}=0.42$, $P=0.663$). In contrast to results in *D. ochrophaeus* (Fig. 4.1), there was no evidence for a dose dependent response to the CORT patches: there was no difference in plasma CORT between animals that received a 2.5 μg dose versus a 5 μg dose (SNK, $P=0.205$). There was no effect of CORT patches on T concentrations (data not shown) ($F_{2,19}=1.32$, $P=0.291$), and plasma CORT and T concentrations were not correlated ($r=-0.21$, $P=0.282$).

Acute Patch Applications in Frogs

There was no difference in plasma CORT between male and female coqui frogs ($F_{1,14}=0.30$, $P=0.591$), so values for males and females were combined. Frogs that received patches containing CORT had elevated plasma CORT concentrations compared to controls (Fig. 4.5; $F_{1,17}=9.79$, $P=0.006$). To determine the effects of re-applying patches in those animals that self-removed the patches, we compared plasma CORT concentrations between control animals that had an oil patch replaced (mean \pm se = 10.3 \pm 3.53; n= 5) and control animals that did not have an oil patch replaced (mean \pm se = 5.5 \pm 1.12; n=5). Although sample sizes were small, replacing the patch had no statistically significant effect on plasma CORT concentrations ($F_{1,6}=1.73$, $P=0.260$).

Repeated Patch Application in Salamanders

One hour after patch application, male *D. ocoee* treated with CORT patches for 30 minutes daily for 9 days had elevated plasma CORT concentrations compared to animals treated similarly with oil patches and the “no patch” control animals (Fig. 4.6a). By 8 hrs after patch application, there was no difference in plasma CORT among groups (Fig. 4.6a; effect of patch: $F_{2,27}=9.55$, $P=0.001$; effect of hr: $F_{1,27}=9.25$, $P=0.005$; patch x hr interaction: $F_{2,27}=8.02$, $P=0.002$). Post-hoc pairwise comparison tests confirmed that there were no differences in plasma CORT between salamanders that received an oil patch for nine days and the “no patch” controls. Application of CORT via dermal patches had no effect on plasma T concentrations (data not shown) in males ($F_{2,27}=1.02$, $P=0.374$), nor was there a correlation between plasma T and CORT concentrations ($r=0.039$, $P=0.830$).

Likewise, a second experiment using female *D. ocoee* found that plasma CORT was elevated 1 hr after treatment with a CORT patch compared to treatment with an oil patch. By 8 hrs after patch treatment, there was no difference in plasma CORT between oil and CORT treated animals (Fig. 4.6b; effect of patch: $F_{1,21}=13.6$, $P=0.001$; patch x hr interaction: $F_{1,21}=6.24$, $P=0.021$). There was no effect of patch application ($F_{1,21}=0.087$, $P=0.771$) or time after patch application ($F_{1,21}=2.88$, $P=0.105$), on plasma E2 concentrations (data not shown). Additionally, there was no correlation between plasma CORT and E2 concentrations ($r=0.135$, $P=0.519$).

DISCUSSION

To understand the role of plasma GCs as physiological mediators of allostasis, allostatic load, and allostatic overload, noninvasive methods for manipulating plasma GCs are necessary. The use of dermal patches to transdermally deliver steroid hormones in wild animals was first described in 1997 (Knapp and Moore). Since then, at least 22 other studies have used transdermal delivery of steroid hormones, indicating that dermal patches and transdermal delivery are increasingly used to elevate steroid hormone concentrations in wild animals. Thus far, transdermal delivery has been used in birds and reptiles (Ruiz et al., 2010; Thaker et al., 2009; Wada and Breuner, 2008). Here, we adapted the method for use in terrestrial amphibians, further characterized the transdermal mode of hormone delivery, and expanded the CORT patch method for simulating exposure to repeated stressors.

Amphibians were well suited for treatment with dermal patches because the dermal patches adhered well to their moist, sticky skin and did not require sedation or

excessive handling to apply. Patches were also easily removed. In salamanders, the patches remained on for up to 4 hrs. In coqui frogs, patches also adhered well to their moist skin, but coqui frogs were able to remove patches with their feet. Habituation to patches helped familiarize the coqui frogs to the patches, but some individuals continued to remove the patches after attempts at habituation. Thus, the patches may be better suited to less dexterous species of anurans.

In both salamanders and frogs, treatment with CORT patches rapidly elevated plasma CORT relative to treatment with oil vehicle patches. It is important to note that animals treated with oil patches did not have higher plasma CORT relative to untreated, undisturbed animals. Even 9 days of patch treatment did not elevate plasma CORT, as evidenced by similar concentrations of plasma CORT in animals treated daily with oil patches for nine days compared to animals undisturbed for 9 days. Thus, neither single nor repeated applications of the oil dermal patch triggered an increase in endogenous plasma CORT, indicating that patch treatment did not itself induce a stress-induced CORT response.

Treatment with CORT patches elevated plasma CORT in a dose dependent manner in the Allegheny dusky salamander. In contrast, CORT patches did not elevate plasma CORT in a dose dependent manner in the red-legged salamander. The species differences may be related to species differences in skin properties. It is possible that, in the red-legged salamander, the amount of CORT applied to the patches saturated the ability of the hormone to pass through the skin of red-legged salamanders. Future experiments with a greater range of doses could address this possibility.

Two studies tested how the profile of plasma CORT was related to the length of time that the patches were in contact with the skin. In one experiment (using red-legged salamanders), continuous treatment with a CORT patch for up to 4 hrs resulted in sustained tonic elevation of plasma CORT. In another experiment (using Allegheny dusky salamanders), CORT patches were applied for 30 minutes and then removed. Relative to controls, plasma CORT remained elevated for up to 3 hrs after patch removal, with a nonsignificant trend for plasma CORT to be lower by 3 hrs compared to 1 hr after CORT patch removal. Sample sizes for that experiment were relatively small, and a power analysis (using G power 2.0 software) indicated that the drop in plasma CORT at 3 hours would be statistically significant if sample sizes had been increased by 2 to 3 subjects in each group (a total experiment-wise increase from 27 to 42 subjects). Thus, we suspect that with a slightly larger sample size we would have been able to show that CORT was being cleared from the circulation by 3 hours after the removal of the CORT patch.

CORT patches elevated plasma CORT to physiologically relevant doses. In male Allegheny dusky salamanders (*D. ochrophaeus*), capture and handling significantly elevated plasma CORT to an average of about 45 ng/ml (Ricciardella et al., 2010). In the current study, application of a patch containing 0.625 µg of CORT increased plasma CORT to an average of 50 ng/ml. In male red-legged salamanders, exposure to mental gland pheromones elevated plasma CORT to approximately 50 ng/ml (Schubert et al., 2009). In the current study, application of a patch containing 2.5 µg of CORT increased plasma CORT to approximately 100 ng/ml, which is reasonably similar to 50 ng/ml. CORT patches also significantly elevated plasma CORT in male and female Ocoee

salamanders, although concentrations were somewhat higher than those reported in response to a handling stressor (Woodley and Lacy, 2010). Endogenous plasma CORT concentrations have not been published for coqui frogs, so we cannot determine whether the CORT patches elevated CORT to physiologically meaningful concentrations in coqui frogs.

In some cases, the same dose of CORT produced different concentrations of plasma CORT in males and females of the same species. For example, in Allegheny dusky salamanders, a patch containing 0.625 μg of CORT produced higher concentrations of plasma CORT in females compared to males. In Ocoee salamanders, a patch containing 1.25 μg of CORT produced higher concentrations of plasma CORT in males compared to females. We did not statistically test for a sex effect, because males and females were used in different experiments conducted at different times of the year with slightly different experimental methods; therefore, it is difficult to interpret the sex differences. However, in our experience, male and female plethodontid salamanders often have different baseline concentrations of plasma CORT (Schubert et al., 2009; Woodley and Lacy, 2010), so there could be sex differences in clearance rates of plasma CORT.

The dermal patch was also used to simulate repeated elevation of plasma CORT in response to repeated exposure to stressors. In this way, patches could potentially be used to mimic the plasma CORT responses that accompanies allostatic load and overload in future studies. Patches were applied for 30 minutes/day for 9 days. On the final day of patch application, blood samples were collected 1 hr or 8 hrs after patch removal. Animals treated with CORT patches had elevated plasma CORT by 1 hr after patch application, indicating transdermal delivery of CORT. By 8 hrs after CORT patch

application, plasma CORT concentrations were indistinguishable from concentrations after oil patch application or concentrations in untreated animals. Thus, repeated acute elevations of plasma CORT over the course of 9 days did not alter baseline concentrations of CORT. This is in contrast to what was found in a similar study performed in lizards (Meylan et al., 2003). In Meylan et al., 2003, CORT dissolved in oil was directly placed on the back of lizards everyday for 20 days. One hour after CORT application, plasma CORT was elevated compared to animals that received oil vehicle. After a few days of treatment with CORT, plasma CORT measured 12 hrs after application was elevated compared to animals receiving oil vehicle. Thus, repeated application of CORT produced long-term elevations in plasma CORT. It is not clear if the increased baseline CORT was due to continual transdermal delivery of the topically applied CORT for 12 hrs after application or if it was due to alterations in clearance rates due to repeated application of CORT. Regardless of the exact mechanism accounting for the differences between our study and Meylan et al., 2003, applying and removing a CORT patch appears to mimic repeated elevations of CORT (our study), whereas topical application of CORT without use of a patch mimics continual elevation of CORT (Meylan et al., 2003).

In most vertebrates, exposure to stressors can alter plasma sex steroid hormones concentrations (Guillette et al., 1995; Wingfield and Sapolsky, 2003). For example, an acute handling stressor in Ocoee salamanders resulted in elevated plasma CORT in both males and females, and decreased testosterone in males but increased estradiol in females (Woodley and Lacy, 2010). In some cases, the change in sex steroid hormones was due to elevated plasma CORT (e.g. Knapp and Moore, 1997). Thus, we were surprised that

elevation of plasma CORT using a dermal patch had no impact on plasma sex steroid hormone concentrations. The results suggest that other factors, such as activation of the sympatho-adrenal medullary system, contributed to stress-induced changes in plasma sex steroid hormone concentrations observed in Woodley and Lacy (2010).

In summary, terrestrial amphibians are well suited for transdermal delivery of CORT via a dermal patch because their moist skin facilitates adhesion of the patch to the skin. There was no need to sedate the animals to apply or remove the patches. In the amphibian species examined, CORT patches successfully simulated acute and repeated elevation of plasma CORT concentrations. Application of patches required minimal handling such that it is unlikely that sympatho-adrenal medullary responses were triggered. This method should be useful in other terrestrial amphibian species, although pilot studies will be need to be conducted to determine doses and time courses of delivery for each species. Future studies will use CORT patches to better understand the behavioral, metabolic, and immunological responses to GCs in *D. ochrophaeus*, *P. shermani*, and *D. ocoee*. With a better understanding of the role of GCs in amphibian stress responses, we hope to better understand potential physiological mechanisms contributing to amphibian population declines.

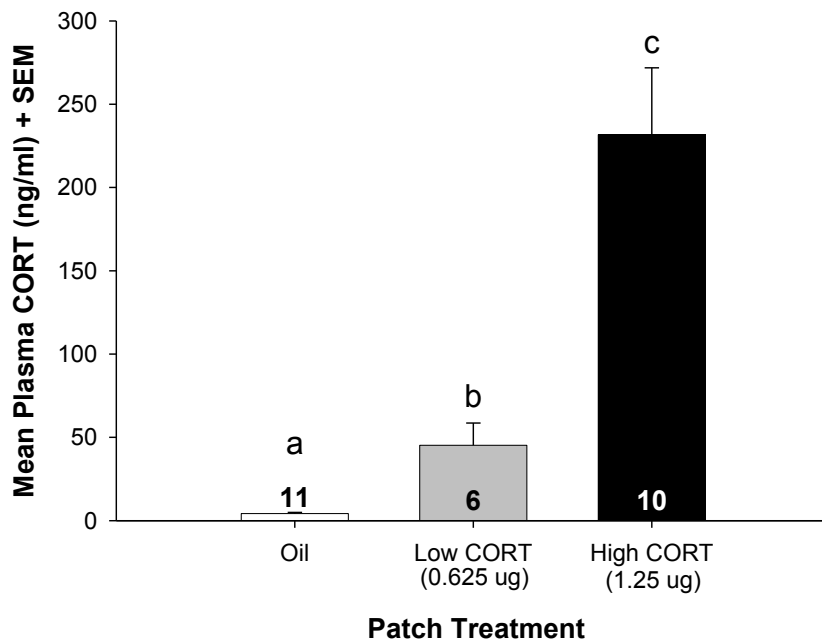


Figure 4.1. Mean plasma CORT concentrations of male *D. ochrophaeus* after application of patches containing oil or CORT. Patches were removed after 30 minutes and blood samples were obtained immediately. Patch treatment significantly affected plasma CORT ($P < 0.001$). Letters denote statistical differences after SNK post hoc test. Sample sizes are listed within or above bars.

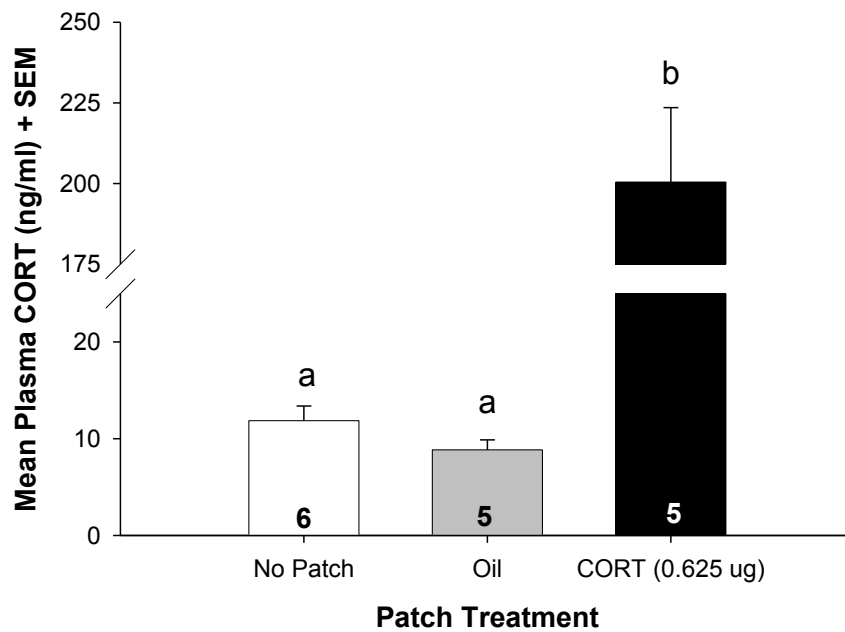


Figure 4.2. Mean plasma CORT concentrations in female *D. ochrophaeus* immediately after a 30 minute application of a patch containing oil or CORT. A “no patch “control group was included. Patch treatment significantly affected plasma CORT ($P < 0.001$). Letters denote statistical differences after SNK post hoc test. Sample sizes are listed within or above bars.

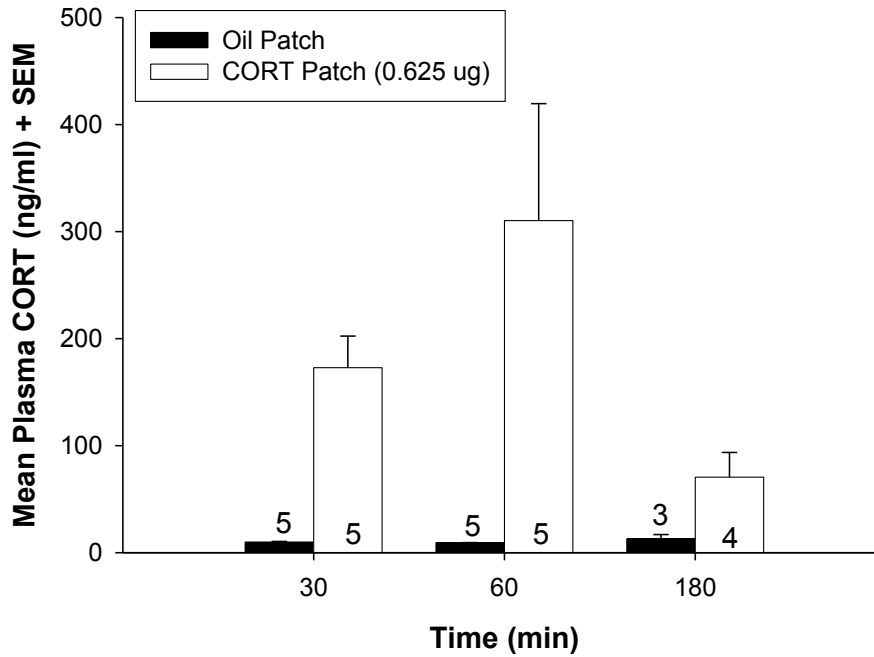


Figure 4.3. Mean plasma CORT concentrations for female *D. ochrophaeus* after a 30 minute application of a patch containing oil or CORT. Blood was collected 30, 60, or 180 minutes after initial patch application. Patch treatment had a significant effect on plasma CORT ($P < 0.001$). Sample sizes are listed within or above bars.

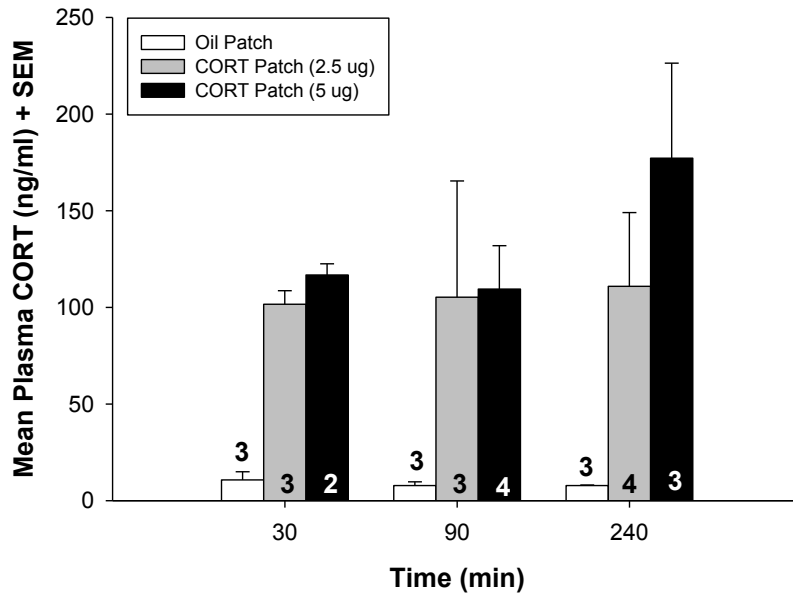


Figure 4.4. Mean plasma CORT concentrations in male *P. shermani* after application of patches containing oil or CORT. Blood was collected after 30, 90, or 240 minutes of continuous patch treatment. Significant main effects are listed in the panel. Sample sizes are listed within or above each bar.

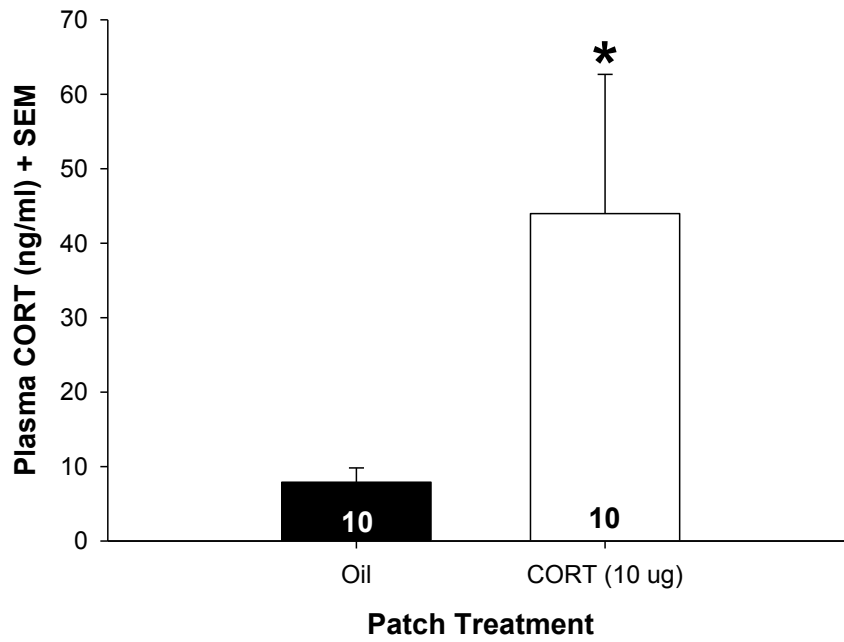


Figure 4.5. Mean plasma CORT concentrations in coqui frogs (*E. coqui*) after a 20 minute application of a dermal patch containing oil or CORT. Animals treated with CORT had significantly higher plasma CORT than oil treated animals ($P = 0.006$). Sample sizes are listed within each bar.

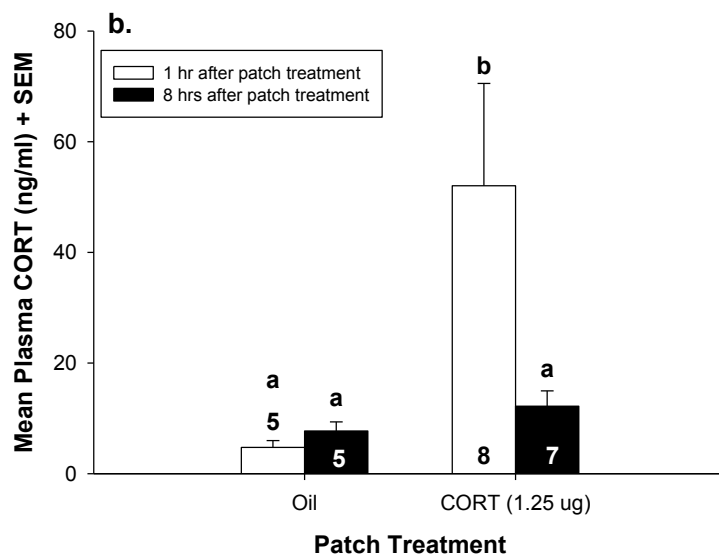
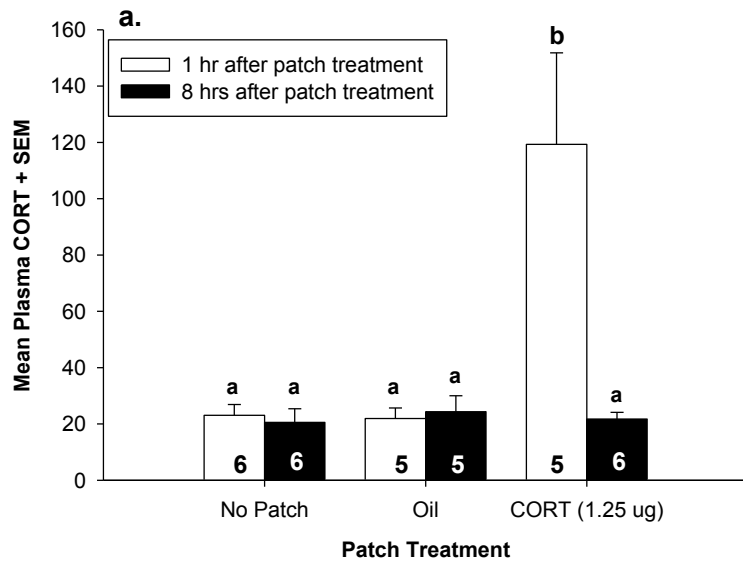


Figure 4.6. Mean plasma CORT concentrations in (a) male and (b) female *D. ocoee* after application of patches containing oil or CORT or no patches. Patches were applied daily for 9 consecutive days for 30 minutes. Blood was collected either 1 hr or 8 hrs after patch application. “No patch” controls were not treated with patches. Significant main effects are listed in the panel. Letters denote statistical differences after SNK post hoc test. Sample sizes are listed within each bar.

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Chapter 5

Effects of corticosterone on behavior in a terrestrial salamander

ABSTRACT

Corticosterone (CORT) is a central physiological mediator of allostatic responses. Plasma CORT increases during unpredictable (stressful) and predictable (e.g. seasonal changes) events and alters behavior in many vertebrates (Romero et al., 2009). In a previous study, exposure to male courtship pheromone elevated plasma CORT concentrations in male red-legged salamanders (*Plethodon shermani*) (Schubert et al., 2009). This elevation in CORT could produce physiological or behavioral changes in males. I investigated the role of CORT in chemoinvestigation and locomotor activity in male *P. shermani* in four experiments. Behavior was measured when males were placed on substrates moistened with either water or biologically relevant cues. For two experiments, I transdermally elevated plasma CORT via a dermal patch. For two additional experiments, dermal patches were used to deliver CORT and also the glucocorticoid receptor blocker, mifepristone. Nosetapping (a measure of chemoinvestigation) and activity were measured for 75 minutes after treatment with patches. Elevation of plasma CORT via dermal patches did not have any consistent effects on chemoinvestigation or activity. In conclusion, I found no evidence that CORT was a primary mechanism mediating rapid behavioral responses to challenging stimuli in male red-legged salamanders.

INTRODUCTION

Animals are often exposed to unpredictable aversive stimuli (i.e., stressors) as well as predictable but challenging life history events or seasonal routines. Together, these changing and challenging events initiate behavioral and physiological responses termed allostatic responses. Stressors include some conspecific social interactions, exposure to predators, and inclement weather (Amaral et al., 2010; Wingfield et al., 1983; Woodley et al., 2000). Typical responses to stressors include an increase in cardiac output, diversion of blood to active tissues, mobilization of glucose from fuels, enhancement of skin immunity, activation of coping behaviors, and suppression of nonessential functions such as reproduction and growth (Martin, 2009; Wingfield and Sapolsky, 2003). Challenging but predictable life history events or seasonal routines include transition to the breeding season, migration, and changes in social status (Greenberg et al., 1984; Holberton et al., 1996; Romero, 2002).

Although many physiological mediators are involved in allostatic responses, glucocorticoid hormones (GCs) such as cortisol and corticosterone (CORT) are believed to be central to mediating allostatic responses (Landys et al., 2006; Romero et al., 2009). Allostatic GC responses may contribute to energy mobilization or behavioral changes, or may function to prepare organisms for future events (Romero, 2002). For example, plasma levels of corticosterone (CORT; the primary GC of most vertebrates) tend to be elevated during the breeding season in amphibians when animals are more active to find potential mates and are therefore utilizing more energy than during the non-breeding season (Romero, 2002).

Understanding the effects of CORT on behavior can give us insight into the mechanisms underlying allostatic responses. Many studies focus on the behavioral effects from chronic exposure to stress or corticosterone, but few studies examine the behavioral effects resulting from acute exposure to CORT (Breuner et al., 2008). Even fewer studies examine the effects of a predictable life history change that induces elevations in CORT. In male red-legged salamanders (*Plethodon shermani*), exposure to a male courtship pheromone increased plasma CORT concentrations, which remained elevated from 45 minutes to two hours (Schubert et al., 2009). Mental gland pheromones are delivered by males to females during courtship, and it acts to increase female receptivity (Rollmann et al., 1999). A role for this mental gland pheromone in male-male interaction has yet to be discovered, but it is possible that males scent mark their territories with their mental gland. The elevation in CORT resulting from exposure to mental gland pheromone is not a stressor, but a predictable event, to which the animal may be exposed daily, primarily throughout the breeding season.

To understand the functional role of elevated CORT from exposure to mental gland pheromones, I tested the hypothesis that CORT alters behavior in male *P. shermani*. I tested the predictions that 1) acute elevation of plasma CORT increases activity and chemoinvestigation (mate searching) and 2) treatment with a CORT receptor blocker, mifepristone would reduce activity and chemoinvestigation. To test these predictions, I delivered CORT or the glucocorticoid receptor blocker, mifepristone, via a dermal patch (Chapter 4). I conducted four experiments examining the effects of CORT on activity and chemoinvestigation. If CORT increased activity and/or

chemoinvestigation, it could increase a male's ability to detect a possible male intruder on its territory or to detect reproductively active females.

METHODS

Animals

Red-legged salamanders (*Plethodon shermani*) were collected in Macon County, North Carolina (83° 33' 37" N longitude; 35° 11' 13" W latitude) in August 2009 with appropriate permits from the North Carolina Wildlife Commission and the US Forest Service. The mental glands of *P. shermani* were removed for biochemical analysis of courtship pheromones (procedures conducted by Dr. Lynne Houck). Animals were individually housed in a 16 x 16 x 5 cm plastic boxes lined with moistened paper towels, kept at 16°C on a 14:10 light:dark cycle, and were fed wax worms. All procedures were approved by Duquesne University's Institutional Animal Care and Use Committee.

Patch application

CORT and the glucocorticoid receptor blocker mifepristone were applied to the subjects via dermal patches as described in chapter 4. Paper towels were removed from home boxes and a 1.5 x 3 mm patch (Cat No. 1820-070 Whatman filter paper) was placed on the dorsum between the front two legs of the salamander. A volume of 2.5 µl of 1 mg/ml CORT (Cat. no. Q1550-000, Steraloids Incorp.), 1 mg/ml mifepristone (Cat. no. M8046, Sigma Aldrich), or vehicle (sesame oil) was pipetted onto the patch. Patches were removed two hours later using forceps. This CORT dose produced physiological concentrations of CORT that were similar to the elevation in plasma CORT produced in

response to treatment with courtship pheromone (Schubert et al., 2009; Chapter 4). Additionally, the exposure time of two hours mimicked the period that CORT was elevated in response to treatment with courtship pheromones (Schubert et al., 2009). The mifepristone dose was based on the dose used in Western spadefoot toads (Crespi and Denver, 2004). Immediately, after patch removal, animals were placed individually in a testing chamber (4.5x4.5x3 cm) and behavioral tests were performed as described below.

In those experiments in which subjects were exposed to both CORT and mifepristone, animals received two patches simultaneously: oil or CORT was applied to the first patch and oil or mifepristone was applied to the second patch.

Activity and nosetapping

Two hours after initial patch application, patches were removed and animals immediately were placed in a testing chamber (4.5x4.5x3 cm) lined with unbleached paper towels moistened with 5 ml of *ddH*₂O or a biologically relevant cue. Scan sampling methods (Martin and Bateson, 1993) were used to quantify locomotor activity and chemoinvestigation. Each testing chamber was visually scanned for 2 seconds every minute or every 2 minutes, and the location of the animal (> 50% of the animal's body) and the presence of nosetaps, a chemoinvestigative behavior, was noted. I scanned for activity and nosetapping for 75 minutes. In some experiments, behavior was observed for 2 seconds every 2 minutes for a total of 75 minutes (37 observations). In other experiments, behavior was observed for 2 seconds every minute for a total of 75 minutes (75 observations). Animals were then returned to their home boxes and placed back in

incubators at 16°C. In all experiments, each male received each treatment on separate nights, with treatments separated by at least 6 days.

All behavioral testing was conducted in the evening, when animals are normally most active, under low incandescent lighting. Behavioral scanning was conducted by a single observer, who was blind to the treatments.

Preparation of testing substrates

In two experiments, subjects were tested in testing chambers lined with paper towel moistened with *ddH*₂O. In another two experiments, testing chambers were lined with paper towels moistened with biologically relevant chemosensory cues derived from conspecifics or prey items. To prepare chemosensory stimuli from conspecifics, two male and two female red-legged salamanders were each placed separately in 100 ml *ddH*₂O for 48 hours to collect body rinses. After 48 hours, rinses from an identical sex were combined. Males used in preparing body rinses had their mental glands removed, thus removing the courtship pheromone that elevated plasma CORT in males. Additionally, I should note that exposure to female-derived chemosensory cues do not elevate plasma CORT in males (Schubert et al., 2009).

The prey cue was made from wax moth larvae (“wax worms”), because this is what the salamanders were fed in the laboratory. Six gm of wax worms were macerated in 20 ml *ddH*₂O. The macerate was centrifuged at 4400 RPM for 45 minutes. The supernatant was removed and centrifuged for another 45 minutes. The supernatant from the second run was diluted 1:5 with *ddH*₂O. Water controls were exposed to the same

containers as the wax worm macerate and were kept in containers for 48 hours as were the conspecific cues.

Analysis

When data did not meet assumptions of normality (Kolmogorov-Smirnov, $P > 0.05$) or homogeneity of variances (Mauchly's test of sphericity, $P > 0.05$), a non-parametric Friedman test was performed. To analyze differences among groups after a significant effect from the Friedman test, Mann-Whitney pairwise comparisons were performed with a sequential Bonferroni adjustment. Data were sometimes square-root or log transformed to meet assumptions of normality ($P < 0.05$) and homogeneity of variances (Mauchly's test of sphericity, $P < 0.05$). Normally distributed data were analyzed with a repeated measures ANOVA.

Non-parametric regression analyses revealed that nosetapping and activity were correlated in each experiment (CORT effects on males exposed to water: $R^2 = 0.55$, $P = < 0.001$; CORT and mifepristone effects on males exposed to water: $R^2 = 0.64$, $P = < 0.001$; CORT effects on males exposed to biological cues: $R^2 = 0.61$, $P = < 0.001$; CORT and mifepristone effects on male exposed to biological cues $R^2 = 0.64$, $P = < 0.001$). Despite the correlation between activity and nosetapping, I was unable to statistically analyze nosetapping with activity as a covariate, because nosetapping data were not normally distributed.

RESULTS

When tested on a neutral substrate moistened only with water, treatment with CORT patches decreased nosetapping (Fig. 1a; $Z=-1.97$, $P=0.048$), but had no effect on activity (Fig. 5.1b; $F_{1,19}=2.33$, $P=0.14$). However, in a subsequent experiment, treatment with CORT or mifepristone did not alter nosetapping (Fig. 5.2a; $\chi^2=6.57$, $P=0.087$) or activity (Fig 5.2b; $\chi^2=1.59$, $P=0.66$).

When tested on substrates moistened with biologically relevant cues, treatment with CORT patches had no effect on nosetapping (Fig 5.3a; $\chi^2=10.46$, $P=0.16$). However, CORT patches had different effects on activity depending on the cue on which the male was tested (Fig 5.3b; patch x cue interaction, $F_{3,14}=3.49$, $P=0.027$). In a second experiment, there was no effect of patch treatment (Fig 5.4a; $F_{3,17}=0.45$, $P=0.72$) or cues (Fig. 5.4a; $F_{1,17}=0.001$, $P=0.98$) on nosetapping. Additionally, there was no effect of patch treatment (Fig 5.4ab; $F_{3,17}=0.54$, $P=0.66$), cue (Fig 5.4b; $F_{1,17}=0.20$, $P=0.66$) on activity.

DISCUSSION

I initially predicted that CORT would increase, and mifepristone would decrease, nosetapping and activity to aid males in finding potential mates or rival males. However, elevation of plasma CORT via dermal patches did not have any consistent effects on chemoinvestigation or activity. Despite an initial finding that CORT altered nosetapping when males were tested on a substrate moistened with water, subsequent experiments failed to find consistent effects of CORT on nose tapping or locomotor activity. In addition, treatment with the glucocorticoid receptor blocker, mifepristone, also failed to

alter locomotor activity or nosetapping. Due to the non-parametric nature of the data, I was unable to use locomotor activity as a covariate for the nosetapping data. However, it is unlikely that differences in activity would contribute to differences in nosetapping given the absence of treatment effects on activity.

Behavioral responses when tested on substrates moistened with water were very low overall. In order to increase levels of chemoinvestigation and activity, I tested animals on biologically relevant cues. Even at increased levels of behavior in response to the biologically relevant cues, treatment of CORT patches had no effect on chemoinvestigation and locomotor activity. However, there was a significant CORT treatment by substrate cue interaction. This interaction is difficult to interpret with no clear trends of CORT's actions on locomotor activity.

The CORT dose and time course used in this study were chosen because they elevate plasma CORT concentrations in the physiological range for a physiologically relevant time frame (Chapter 4). However, it is possible that the plasma concentrations of CORT in this study were not adequate to have an effect on the behaviors examined. A study performed in white crowned sparrows showed that only intermediate concentrations of CORT, not high concentrations, altered activity (Breuner and Wingfield, 2000). Therefore, it is possible that lower levels of CORT may alter behavior in *P. shermani*. Additionally, CORT treatments may have an effect in a different time period. CORT may have delayed actions, which would occur during classical genomic mechanisms via a mineralocorticoid or glucocorticoid receptor. Intracerebroventricular injections of corticotropin-releasing factor (CRF) and mifepristone altered behaviors in tadpoles at six hours after injections but not at one hour (Crespi and Denver, 2004). It is

possible that it takes a longer time period of two hours used in the current study for mifepristone to be effective.

Much of the work examining the effects of exogenous CORT on behavior derived from studies done in the roughskin newt. In male roughskin newts, CORT administration decreased amplexic claspings compared to males injected with vehicle controls (Moore and Miller, 1984). However, CORT administration did not have any effects on males' responses to visual or olfactory stimuli. CORT did not affect the amount of time males spent in proximity to a female in a glass beaker or an empty beaker (Coddington, 2004). CORT injections also did not alter the time males spent in investigating same sex and opposite sex conspecifics. Additionally, males spent similar amounts of time investigating a pad that was scent-marked by reproductive females and a pad with a control (Coddington, 2004). These particular data are similar to the current study showing that CORT does not alter investigation of sensory cues.

Although activity was not altered by CORT administration in male red-legged salamanders, CORT increased activity in lizards (Belluire and Clobert, 2004), fish (Overli et al., 2002), and birds (Breuner et al., 1998). In each of these studies, CORT was delivered non-invasively either via a dermal patch (Belluire and Clobert, 2004), water (Overli et al., 2002), or injected in prey (Breuner et al., 1998). Other physiological mediators are involved in allostatic responses (Romero et al., 2009). These factors may be involved in altering activity in *P. shermani*. Another hormone of the hypothalamo-pituitary-interrenal (HPI) axis, CRF administration increased locomotor activity in roughskin newts, but were blocked by a CRF antagonist (Lowry and Moore, 1991). Similar increases in locomotor activity from CRF administration have been shown in the

Western spadefoot toad (Crespi and Denver, 2004) and rats (Koob and Bloom, 1985). Other factors also may be involved in altering activity, such as cytokines. Interleukin1-beta injections reduced activity in lizards (Dunlap and Church, 1996). These and other factors should be examined in salamanders to understand the mechanism by which activity is altered.

CORT is a metabolic hormone and alters metabolism in vertebrates (reviewed in Guillette and Cree, 1995). Physiological elevation of CORT increased metabolic rate in lizards (DuRant et al., 2008). Elevation in metabolism could aid animals through an energetically costly event, such as an agonistic interaction. Thus, the acute elevation in CORT seen in male *P. shermani* after exposure to male courtship pheromone may elevate metabolism rather than having behavioral effects as originally predicted. In fact, CORT administration does increase metabolic rates in male *P. shermani* (Chapter 6). The effect of CORT on metabolism should be investigated to further understand the functional role of CORT elevation after exposure to male courtship pheromones (Schubert et al., 2009).

In conclusion, CORT had no effect on chemoinvestigation or activity in male red-legged salamanders. Most studies examine the CORT elevations as a result from a stressor, but fewer studies examine predictable elevations in CORT. Given the broad range of allostatic effects on behavior in vertebrates, it is important to understand the mechanism involved in altering behavior. CORT is a primary mediator of allostatic responses in many species, but may not be involved in altering chemoinvestigation and activity in *P. shermani*.

Substrates Moistened with Water

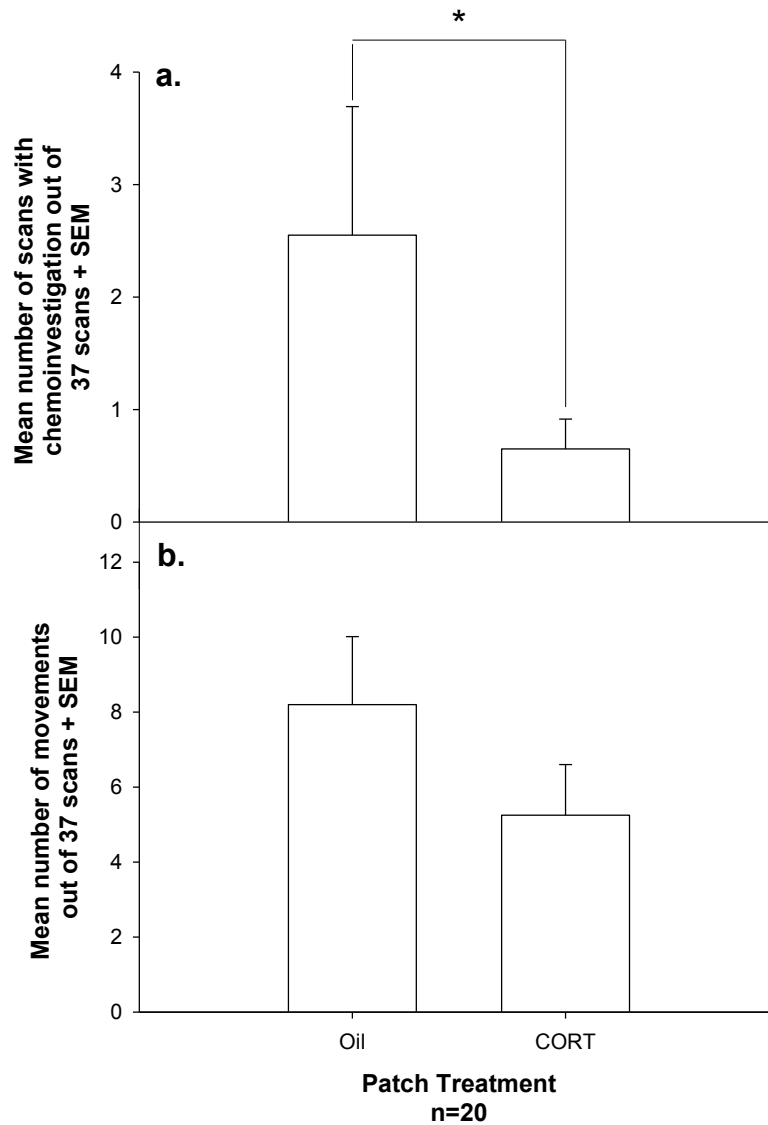


Figure 5.1. Nosetapping and activity in male *Plethodon shermani* exposed to oil or CORT patches for two hours. Behavior observed by visual scan sampling immediately after patch removal. (a) Mean number of nosetaps observed during a 75 minute observation period. (b) Mean number of movements (quadrant changes) observed during a 75 minute observation period.

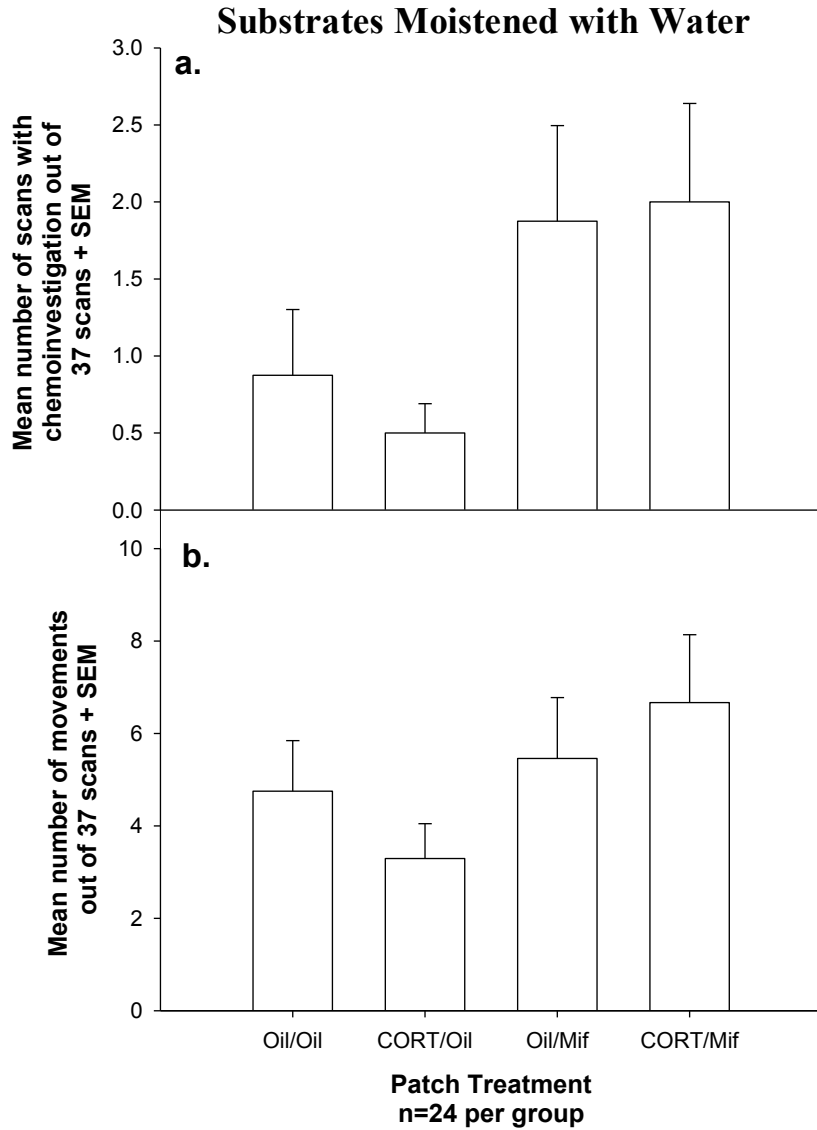


Figure 5.2. Nosetapping and activity in male *P. shermani* exposed to oil, CORT, or mifepristone patches for 2 hours. Behavior observed by visual scan sampling immediately after patch removal. (a) Mean number of nosetaps observed during a 75 minute observation period. (b) Mean number of movements observed during a 75 minute observation period.

Substrates Moistened with Biologically Relevant Cues

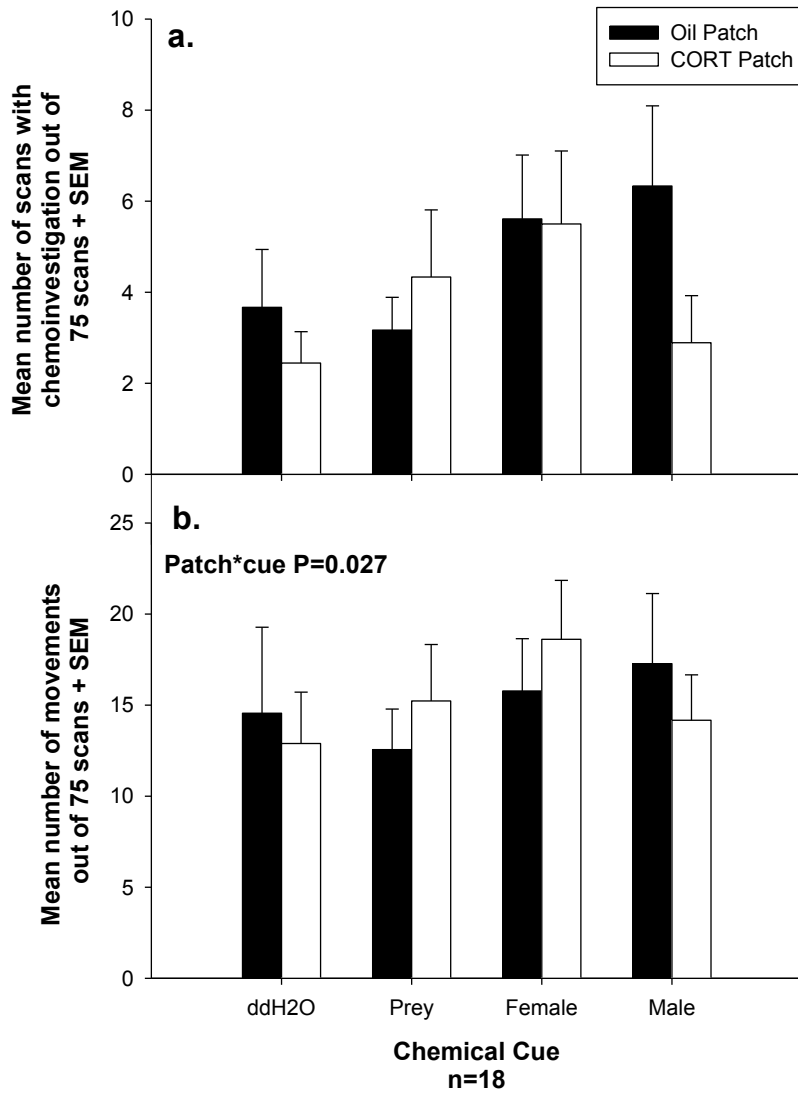


Figure 5.3. Nosetapping and activity in male *P. shermani* exposed to oil or CORT patches for 2 hours and presented with chemosensory cues. Behavior recorded by visual scan sampling immediately after patch removal. (a) Mean number of nosetaps observed during a 75 minute observation period. (b) Mean number of movements observed during a 75 minute observation period.

Biologically Relevant Cues

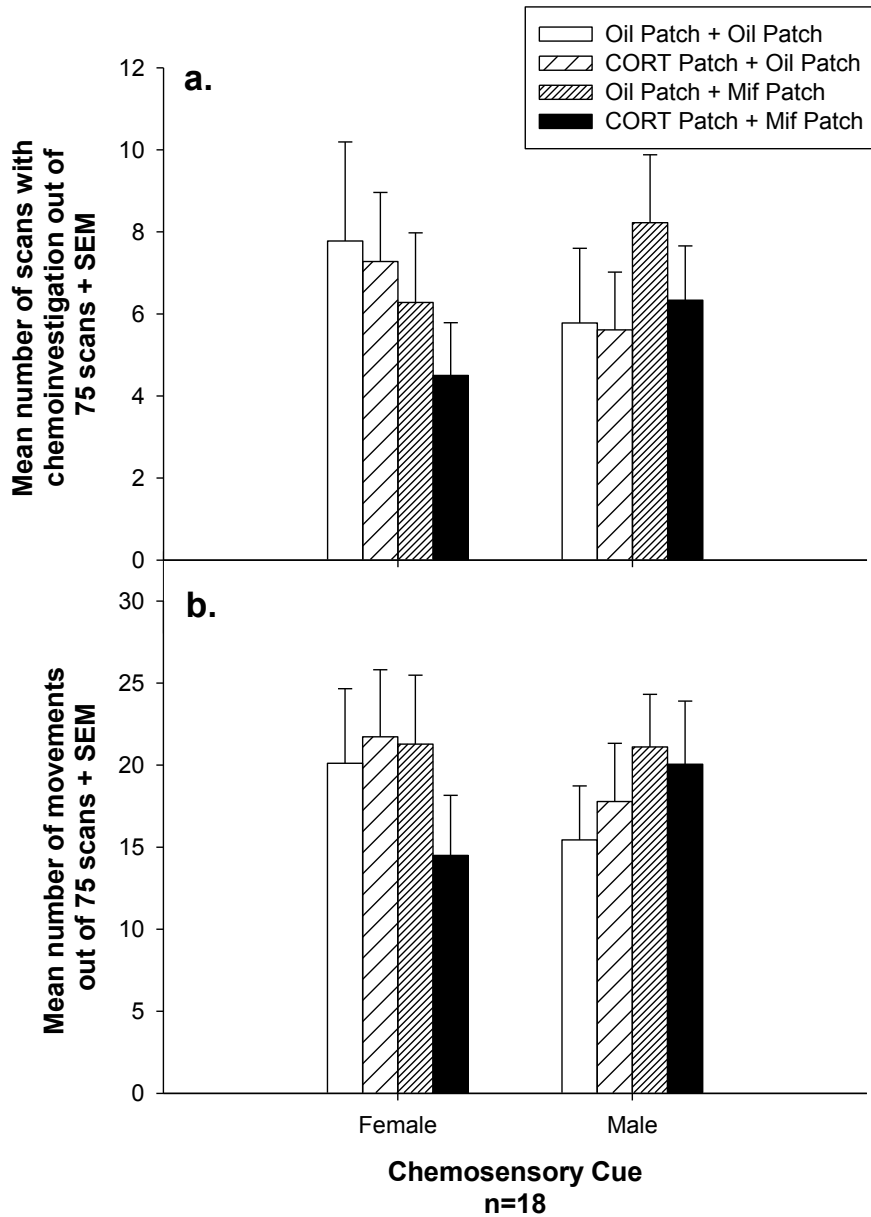


Figure 5.4. Nosetapping and activity in male *P. shermani* exposed to oil, CORT, or mifepristone patches for two hours and presented with chemosensory cues. Behavior observed by visual scan sampling immediately after patch removal. (a) Mean number of nosetaps observed during a 75 minute observation period. (b) Mean number of movements observed during a 75 minute observation period

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Chapter 6

Elevation of plasma corticosterone to physiologically relevant levels increased metabolic rate in a terrestrial salamander

ABSTRACT

Plasma glucocorticoid hormones (GCs) become elevated when individuals are exposed to stressors. GCs alter intermediary metabolism to increase blood glucose concentrations. In the absence of compensatory mechanisms, this increase in intermediary metabolism may be reflected in whole-animal metabolic rate. Studies in fish, birds, and reptiles have shown that GCs may alter whole animal metabolic rates, but results are conflicting and often involve GCs levels that are not physiologically relevant. A previous study in red-legged salamanders found that male courtship pheromone increased plasma corticosterone (CORT; primary GC in amphibians) concentrations in males but not females. To understand the possible metabolic effect of elevated plasma CORT, I measured the effects of male courtship pheromone and exogenous application of CORT on oxygen consumption in male red-legged salamanders. Exogenous application of CORT elevated plasma CORT to physiologically relevant levels. Compared to treatment with male courtship pheromone and vehicle, treatment with CORT resulted in 12% more oxygen consumed over a 2 hr period. Contrary to our previous work, treatment with pheromone did not increase plasma CORT, perhaps because subjects used in this study were not in breeding condition. Our study is one of the few to evaluate the influence of physiologically relevant elevations in CORT on whole animal metabolism in

free-ranging animals, and the first to show that elevated plasma CORT increases metabolism in an amphibian.

INTRODUCTION

Glucocorticoid hormones (GCs) are released by the adrenal cortex upon activation of the hypothalamic-pituitary-adrenal axis (Norris, 2006). Plasma concentrations of GCs may change diurnally, seasonally, and in response to exposure to acute and chronic stressors (e.g. Coe and Levine, 1995; Moore et al., 1991; Romero, 2002; Wingfield et al., 1998). Basal concentrations of GCs are believed to support basic processes, such as maintenance of vascular tone and blood sugar levels (Norris, 2006). GCs also change on a seasonal basis, typically being higher during the breeding season relative to other times of the year (e. g. Licht et al., 1983). The seasonal elevation of GCs may function to support behavioral and/or energetic changes associated with breeding, to prepare an organism for potentially stressful events, or to suppress processes incompatible with a particular life history event (Romero, 2002). Finally, exposure to unpredictable threats or challenges (stressors) typically causes an elevation in plasma GCs. Elevation of plasma GCs may mediate behavioral, immunological, or metabolic responses to stressors (e.g. Amaral et al., 2010; French et al., 2006; Kitaysky et al., 1999; Martin, 2009; Ricciardella et al., 2010).

In temperate-zone amphibians, GCs tend to be elevated during the breeding season relative to the non-breeding season (Homan et al., 1979; Leboulenger et al., 1979; Licht et al., 1983; Pancak and Taylor, 1983; Zerani and Gobbetti, 1993). Furthermore, exposure to stressors typically elevates plasma levels of GCs in amphibians. Confinement

stress, food restriction, handling, and exposure to contaminants increased plasma corticosterone (CORT; the primary GC of most vertebrates) concentrations in amphibians (Crespi and Denver, 2004; Glennemeier and Denver, 2002; Hopkins et al., 1997; Moore and Zoeller, 1985). Exposure to social cues may also cause plasma GCS to increase. For example, plasma CORT concentrations were increased in male toads after mating with females compared to single males (Orchinik et al., 1988).

Stress-induced increases in GCs may have behavioral, immunological, and/or metabolic effects in amphibians. In male roughskin newts, CORT administration inhibited amplexic clasping (Moore and Miller, 1984). CORT caused toads to switch from a calling tactic to a non-calling satellite tactic (Leary et al., 2004). Tadpoles exhibited decreased foraging when administered CORT (Crespi and Denver, 2004). Seasonal and diurnal increases in plasma CORT are associated with mate searching in amphibians (Leboulenger et al., 1979; Zerani and Gobbetti, 1993). Regarding amphibian immunity, blood lymphocyte numbers were decreased after acute exposure to a stressor or treatment with GCs (Davis and Maerz, 2010).

Stress-induced elevations of GCs may also alter whole animal metabolism via changes in intermediary metabolism. GCs are directly or indirectly involved in a number of intermediate metabolic pathways that involve glucose, amino acid and lipid metabolism (Mommsen et al., 1999; Norris, 2006). Through each of these pathways, GCs act to increase glucose concentrations in order to meet energetic demands. In the absence of compensatory mechanisms, changes in intermediary metabolism may be reflected in whole animal metabolism. For example, both acute and chronic increases in GCs resulted

in elevated whole animal metabolic rates in lizards (DuRant et al., 2008; Preest and Cree, 2008).

Plethodontid salamanders are an excellent amphibian model for examining the function of acute increases in plasma GCs. They are abundant, available for study most of the year, and are amenable to both field and laboratory studies. Plasma levels of GCs increase in plethodontid salamanders in response to capture and/or handling (Ricciardella et al., 2010; Schubert et al., 2009; Woodley and Lacy, 2010). Social cues also trigger CORT release in plethodontid salamanders. Schubert et al. (2009) found that exposure to pheromones increased plasma levels of CORT in the red-legged salamander. Courtship in male and female red-legged salamanders is a lengthy process, whereby males and females communicate with pheromonal and tactile cues to coordinate sperm transfer (Houck and Arnold, 2003; Houck and Verrell, 1993). Males possess a submandibular gland (mental gland) that they rub across the nares of females during courtship. Application of mental gland pheromones increases female receptivity to mating (Rollmann et al., 1999). Although mental gland pheromones are involved in male-female interactions, they may also be involved in male-male interactions. I found that exposure of males to mental gland pheromones, but not to female-derived chemosensory cues, resulted in elevated plasma CORT. Plasma CORT was not elevated in females (Schubert et al., 2009). Although a role for mental gland pheromones in male-male interactions has not been definitively established, it is possible that males scent mark territories by tapping their mental glands to the substrate.

In order to better understand the role of elevated plasma CORT in red-legged salamanders, I adapted dermal patches to noninvasively elevate plasma CORT to

physiologically relevant levels (Wack et al., 2010). Other studies using dermal patches indicated that elevated plasma CORT did not alter locomotor behavior or chemo-investigative behavior when tested a few hours after treatment (Ricciardella et al., 2010; Wack, et al., in preparation). Thus, I asked whether elevated plasma CORT might mediate metabolic changes. In the current experiment, I tested the effects of male courtship pheromone and exogenous CORT on metabolic rate measured via oxygen consumption. I used a non-invasive dermal CORT patch to elevate plasma CORT. I tested the hypothesis that courtship pheromones alter metabolic rate via an increase in CORT. I predicted the following: (1) courtship pheromones increase oxygen consumption, (2) courtship pheromones increase plasma CORT concentrations, and (3) elevation of plasma CORT increases oxygen consumption.

METHODS

To test the effects of CORT patches and pheromone on plasma CORT concentrations and oxygen consumption, two experiments were performed. In the first experiment, the effect of male courtship pheromone and exogenous application of CORT on plasma CORT concentrations was measured. In the second experiment, the effects of male courtship pheromone and exogenous application of CORT on oxygen consumption were examined. Oxygen consumption was measured with a closed-circuit respirometer. Methods were approved by Duquesne University's and Virginia Tech's Institutional Animal Care and Use Committees.

Animal collection and husbandry

Adult male red-legged salamanders (*Plethodon shermani*) were caught by hand in Macon County, North Carolina (83° 33' 37" N longitude; 35° 11' 13" W latitude) in August 2009. Animals were collected with appropriate permits from the North Carolina Wildlife Commission and US Forest Service and transported to Duquesne University. To measure metabolic rate, animals were transported from Duquesne University to Virginia Tech two weeks before the start of the experiment. One day after measurement of metabolic rate was completed, animals were returned to Duquesne University. Two weeks after completing metabolic measurements, the effects of male pheromone and exogenous application of CORT on plasma CORT concentrations were measured. Throughout the experiments, animals were individually housed in 16 x 16 x 5 cm plastic boxes lined with moist paper towels, maintained on a 14:10 light:dark cycle at 16°C, and fed wax moth larvae (“wax worms”).

Experimental treatments

Animals were randomly placed into one of three treatment groups: vehicle control (n=16), pheromone (n=16), or CORT (n=15). Depending on the treatment group, pheromone or PBS vehicle was delivered to the nares of salamanders, and CORT or oil vehicle was delivered transdermally via a patch that was placed on the dorsum of the salamanders. Animals in all treatments were handled similarly, such that animals in the control group had an oil patch and had vehicle (PBS) delivered to the nares, animals in the pheromone group received an oil patch and had pheromone delivered to the nares,

and animals in the CORT group received a CORT patch and had vehicle (PBS) delivered to the nares.

Preparation and application of pheromone

Pheromone consisted of extract from male mental glands (Schubert et al., 2006; Wirsig-Wiechmann et al., 2002). To obtain mental gland extract, an additional group of males were captured and mental glands were removed. For surgical details, see Wirsig-Wiechmann et al. (2002) (procedure approved by Oregon State University ACUP to Dr. Lynne Houck). Mental glands from multiple males were pooled and pheromone was extracted with acetylcholine-chloride. Pheromone concentration was standardized at 1 μg of total protein from mental gland extract per μl . This concentration elevated plasma CORT concentrations (Schubert et al., 2009) and activated vomeronasal sensory neurons in male *P. shermani* (Schubert et al., 2006; Wirsig-Wiechmann et al., 2002). A volume of 5 μl of pheromone or vehicle (PBS) was pipetted onto the nares every 5 minutes for 45 minutes for a total of 10 applications, as described in Schubert et al. (2009).

Dermal Patches

I used dermal patches containing CORT to non-invasively elevate plasma CORT. Patches consisted of a 1.5 x 3 mm rectangle cut from filter paper (Cat No. 1820-070 from Whatman) that was placed onto the dorsum between the front two legs of the salamander with forceps. Using a pipette, 0.625 μg of CORT was applied to the patch in a volume of 1.25 μl (Cat. no. Q1550-000, Steraloids Incorp.; 0.5 mg/ml). This amount of CORT was half of a dose previously shown to elevate plasma CORT to high physiological

concentrations in red-legged salamanders (Wack et al., 2010). Controls received vehicle (sesame oil) pipetted onto the patch. Patches were removed with forceps 45 minutes after initial application.

Plasma CORT concentrations

To determine the effects of treatments on plasma CORT, animals were treated with pheromone or PBS vehicle to the nares over a period of 45 minutes. At the same time, oil or CORT patches were applied to the back for 45 minutes. After the last pheromone or PBS vehicle treatment, patches were removed. Treatments were delivered between 1515 and 1545. Trunk blood was collected from animals at 2, 4, or 10 hours after patch removal. Blood was centrifuged and the plasma portion was frozen at -20 °C until assayed. CORT concentrations were measured by the Endocrine Services Laboratory at the Oregon National Primate Research Center. Briefly, a double ether extraction was performed on approximately 3 µl of plasma, and CORT concentrations were then determined by standard radioimmunoassay procedures (Gruenewald et al., 1992; Resko et al., 1980). Intra-assay coefficient of variation was 8.5%.

Oxygen consumption

To determine the effect of treatments on oxygen consumption, I used a computer-controlled, closed circuit respirometer to measure the rate of oxygen consumption (V_{O_2}) as well as total oxygen consumed, calculated by taking the integral (i.e., area under the curve) of V_{O_2} . Oxygen consumption was measured in salamanders that were individually placed in glass respiratory chambers (150 ml) lined with a kimwipe moistened with

ddH₂O. Measurements were made while animals were maintained at 16 °C in constant darkness. Testing was done at least 2 weeks after the last feeding to ensure that animals were post-absorptive, in this way, digestion did not contribute to V_{O₂} (Roe et al., 2005).

Oxygen consumption was measured both before and after treatments. Incurrent air passed through columns of Drierite® to absorb water before passing into individual respirometry chambers. Air leaving respirometry chambers was dried again using magnesium perchlorate before passing into the oxygen sensor. Further details of the respirometry system are discussed elsewhere (Hopkins et al., 2004; Hopkins et al., 1999).

Trials were initiated between 0900 and 0930. During the pre-treatment trial, V_{O₂} was measured every 42 minutes over 6.3 hours. After 6.3 hours (i.e., between 1515 and 1545), salamanders were removed from their chambers and exposed to one of three treatments: control, pheromone, or CORT. Either pheromone or PBS vehicle was applied to the nares over a period of 45 minutes. At the same time, either an Oil patch or a CORT patch was applied to the back for 45 minutes. Treatments took approximately 50 minutes to complete. After treatment, animals were immediately returned to their chambers for post-treatment respirometry measurements. The post-treatment trial measured V_{O₂} resulting from the experimental treatments. V_{O₂} was measured every 42 minutes for 14.73 hours. After post-treatment respirometry measurements, each salamander was weighed. Total oxygen consumed was calculated by taking the integral (i.e., area under the curve) of V_{O₂}.

Analysis

All plasma CORT, V_{O_2} , total oxygen consumption, and mass data were log-transformed to meet assumptions of normality (Kolmogorov-Smirnov, $P < 0.05$) and homogeneity of variance (Levene's test, $P < 0.05$). For all analyses of V_{O_2} and total oxygen consumed, mass was used as a covariate to control for effects of mass on metabolic rate (Andrews and Pough, 1985). All V_{O_2} and total oxygen consumption data are adjusted for mass by displaying the least squares means (aka estimated marginal means). Tukey's post hoc analyses were performed after significant main and interaction effects were found from ANCOVA and ANOVA analyses.

Plasma corticosterone data: Effects of treatments and time of blood collection on plasma CORT concentrations were analyzed with an ANOVA with treatment and time as factors.

Pretreatment respirometry data: The effect of mass on V_{O_2} was determined with a regression analysis (proc reg, SAS 9.2, SAS Institute, Carey, NC). To determine if groups differed initially in V_{O_2} and total oxygen consumption, V_{O_2} was analyzed with a repeated measures ANCOVA with time of V_{O_2} measurement as the repeated variable and treatment group as the between subjects factor. Total oxygen consumed during the 6.3 hour pre-treatment period was analyzed using an ANCOVA with mass as the covariate and treatment group as the between subjects factor

Post-treatment respirometry data: To analyze post-treatment respiration, data were divided into three intervals. The first interval included all V_{O_2} measurements taken in the first four hours post-treatment. At this time, animals that have received CORT patches or pheromone typically have elevated plasma CORT concentrations. The second

interval included all V_{O_2} measurements taken during hours 4 to 10 post-treatment. At this time, plasma CORT concentrations in animals treated with CORT patches are declining. The final interval included measurements from 10 to 14.73 hours. By this time after application of CORT patches, plasma CORT concentrations have returned to control levels. The length of intervals was based on previous observations of how plasma CORT changed after exposure to CORT patches and pheromones (Schubert et al., 2009; Wack et al., 2010) and was confirmed by plasma CORT results of the current study.

The effect of treatment on V_{O_2} was analyzed with a repeated measures ANCOVA for each time interval separately, with time as the repeated measures variable. In addition, total oxygen consumption for each interval was calculated for each time interval: 2-4, 4-10, and 10-15 hours. The effect of treatments on total oxygen consumption were analyzed with a repeated measures ANCOVA with time interval as the repeated variable and mass as the covariate.

RESULTS

Plasma CORT concentrations

Overall, there was a significant effect of treatment on plasma CORT concentrations (Fig. 1; $F_{2,64}=3.43$, $P=0.038$). Treatment with CORT, but not pheromone, elevated plasma CORT relative to treatment with vehicle control (CORT versus control, $P=0.05$; pheromone versus control, $P=0.87$). There was a marginally significant interaction between time of sampling and treatment on plasma CORT concentrations (Fig. 1; $F_{4,64}=2.08$, $P=0.094$). The effects of CORT treatment were most evident at 4 hours after treatments ($F_{2,23}=7.65$, $P=0.0032$) and males with CORT patches had elevated

plasma CORT concentrations compared to males treated with pheromone and vehicle controls (CORT versus pheromone, $P=0.0033$; CORT versus control, Tukey's $P=0.025$). In contrast, plasma CORT concentrations were similar among treatment groups at two ($F_{2,22}=2.41$, $P=0.11$) and 10 hours ($F_{2,21}=0.03$, $P=0.98$) after treatment application. Finally, plasma CORT concentrations were not affected by body mass ($P=0.60$).

Pre-treatment oxygen consumption

Masses of salamanders ranged from 2.19 to 4.09 g ($\bar{x}=3.06$ g \pm 0.07) and there was no significant difference in masses of individuals allocated to the three treatment groups ($F_{2,44}=0.69$, $P=0.51$). There was a significant positive relationship between mass and V_{O_2} (Fig. 6.2; $r^2=0.21$, $P<0.001$). There was no difference in respiration among animals allocated to the different groups during the pre-treatment trial (V_{O_2} data not shown; $\bar{x}=0.079$ ml/hr \pm 0.0023; $F_{2,43}=0.32$, $P=0.73$). Nor was there a difference in total oxygen consumed among animals allocated to different treatment groups (Fig. 6.3; $F_{2,43}=0.64$, $P=0.53$).

Post-treatment oxygen consumption

V_{O_2} in the first four hours was highest in animals treated with CORT patches relative to treatment with pheromone or vehicle (Fig. 6.4; $F_{2,43}=7.12$, $P=0.0021$). There was no effect of treatment on the V_{O_2} 4-10 hours (Fig. 6.4; $F_{2,43}=0.49$, $P=0.62$) or 10-14 hours (Fig. 6.4; $F_{2,43}=0.97$, $P=0.97$) after treatment .

Additionally, the total amount of oxygen consumed in the first four hours was highest in animals treated with CORT related to animals treated with pheromone or

vehicle (Fig. 6.5; $F_{2,43}=7.07$, $P=0.0022$). There was no effect of treatment on the total oxygen consumed 4-10 hours (Fig. 6.5; $F_{2,43}=0.29$, $P=0.75$) or 10-14 hours after treatment (Fig. 6.5; $F_{2,43}=0.04$, $P=0.96$).

DISCUSSION

Elevation of plasma CORT resulted in an elevation in oxygen consumption in male red-legged salamanders. The plasma CORT concentrations achieved by the CORT patches were physiologically relevant, being of similar magnitude to levels measured previously after handling or treatment with pheromones (Schubert et al., 2009). In contrast, oxygen consumption was not altered by pheromone or vehicle administration. Our study is one of the few to evaluate the influence of physiologically relevant elevations in CORT on whole animal metabolism in free-ranging animals, and the first to show that elevated plasma CORT increases metabolism in an amphibian.

Compared to treatment with oil patches or pheromone, treatment with CORT patches elevated plasma CORT concentrations, which was most evident at four hours after CORT patch removal. By 10 hours after CORT patch removal, plasma CORT levels were no longer elevated compared to treatment with oil patches or pheromone. CORT delivery by the patches was similar to previous studies in plethodontid salamanders, where plasma CORT was elevated for several hours following treatment with CORT patches, but had returned to baseline by eight hours after removal of CORT patches (Wack et al., 2010).

Treatment with CORT patches increased whole animal metabolic rate as measured by changes in oxygen consumption. Due to the positive relationship between

mass and oxygen consumption, demonstrated here and in other studies (Gatten et al., 1992; Homyack et al., 2010), mass was accounted for in analyses. Before treatments, oxygen consumption did not differ among groups. From two to four hours after removal of a CORT patch, oxygen consumption rates and total oxygen consumption were elevated compared to treatment with oil patches or pheromone. After four hours following patch removal, there were no differences among treatment groups in oxygen consumption rate or the total amount of oxygen consumed.

Studies in other vertebrate classes have examined the effects of CORT on oxygen consumption, but findings are inconsistent. Exogenous CORT increased metabolic rates in titmice and gulls (Hissa and Palokangas, 1970; Palokangas and Hissa, 1971), decreased nocturnal metabolic expenditures in pine siskins and white-crowned sparrows (Astheimer et al., 1992; Buttemer et al., 1991), and had no effect in pigeons (Hissa et al., 1980). CORT increased oxygen consumption in geckos and fence lizards (DuRant et al., 2008; Preest and Cree, 2008), but decreased metabolic rates in side-blotched lizards (Miles et al., 2007). Additionally, exogenous CORT increased oxygen consumption in salmon and trout (Chan and Woo, 1978; DeBoeck et al., 2001; Morgan and Iwama, 1996), but decreased oxygen consumption in coho salmon (Davis and Schreck, 1977). These contradictory results may be due to differences in plasma CORT concentrations. Plasma CORT concentrations were in the pharmacological range in some studies (Hissa and Palokangas, 1970; Palokangas and Hissa, 1971), but were at physiological concentrations in others (Buttemer et al., 1991; Davis and Schreck, 1977; DuRant et al., 2008; Hissa et al., 1980; Morgan and Iwama, 1996). Differences in methodology of CORT delivery (i.e., acute versus chronic administration) and measurement of oxygen

consumption (i.e., measurement over a period of time versus a single discrete measurement) may also explain the difference in results among these studies. Our study is an important contribution to the field because it is one of the few studies that measured the effects of physiologically relevant elevations of CORT on oxygen consumption.

There are many potential mechanisms whereby treatment with CORT increased whole animal metabolic rate in red-legged salamanders. First, metabolic rate may increase as a direct outcome of the effects of CORT on metabolic pathways involved in intermediary metabolism and glucose mobilization. Another possibility is that CORT may potentiate the metabolic effects of epinephrine (Sapolsky et al., 2000; Staten et al., 1987). CORT potentiates the actions of epinephrine in the cardiovascular system by modulating enzymes involved in the synthesis and breakdown of epinephrine (Križanová et al., 2001; Wurtman and Axelrod, 1966). GCs might also potentiate some of epinephrine's metabolic effects (Sapolsky et al., 2000). The time course of changes in CORT and oxygen consumption is consistent with a mechanism that involves an interaction between CORT and epinephrine. The effects of CORT treatment on oxygen consumption were waning by four hours after treatment even though plasma CORT was still elevated. This mismatch between plasma CORT and oxygen consumption could be explained if CORT potentiated the effects of epinephrine that was presumably released when subjects were handled during applications of the treatment. The decline in oxygen consumption from two to four hours may have reflected the decline in epinephrine-induced metabolism.

CORT binds to several different types of receptors. When CORT binds to intracellular receptors, changes in gene expression require at least one to two hours to be

evident (Wehling, 1995). However, CORT also can have rapid behavioral effects that suggest the CORT can act non-genomically. For example, CORT administration altered reproductive behavior (i.e. amplexic clasping) in male roughskin newts within minutes (Moore and Miller, 1984). The mechanism whereby CORT has effects is still being described, but may involve a membrane bound receptor (Orchinik et al., 1992). The time course of CORT effects on oxygen consumption shown in this study is consistent with either a genomic or a non-genomic mechanism.

A number of important life history events are associated with elevated metabolic rates in amphibians, including being gravid (carrying oocytes/eggs) or nest-building (Finkler, 2006). A classic example of an energetically demanding behavior is male anuran advertisement calling (Taigen and Wells, 1985). Male advertisement calling is also associated with elevated plasma CORT. Calling male anurans have increased plasma CORT concentrations compared to non-calling or satellite males (Emerson and Hess, 2001; Leary et al., 2008; Leary et al., 2004; Mendonca et al., 1985). The energetic-hormone-vocalization hypothesis posits that the energetic demands of male vocalization drives the increase in CORT which consequently modifies endocrine and behavioral endpoints (Emerson, 2001). In contrast, our study in red-legged salamanders showed that increased CORT can drive an increase in metabolic rate. A CORT-induced increase in metabolic rate may act to prepare an organism for a future event (Romero, 2002; Sapolsky et al., 2000).

CORT-induced increases in oxygen consumption likely have important implications for red-legged salamanders. Plethodontid salamanders have among the lowest metabolic rates of any terrestrial vertebrate. Adult plethodontids only need to

ingest 12 mealworms per year to sustain their energetic demands (Feder, 1983). Predatory, agonistic, and breeding interactions represent less than one percent of the daily dietary intake of plethodontid salamanders (Bennett and Houck, 1983; Feder and Arnold, 1982). In the current study, oxygen consumption from 2-4 hours after patch removal was 12% greater than oxygen consumption in oil patch controls. For comparison, courtship increased oxygen consumption rate by 38% in the plethodontid salamander, *Desmognathus ochrophaeus*, compared to non-courting pairs (Bennett and Houck, 1983). Additionally, male-male agonistic interactions increased oxygen consumption rate by 27% compared to individual males (Bennett and Houck, 1983).

In contrast to an earlier study (Schubert et al., 2010), application of male courtship pheromone did not elevate plasma CORT concentrations. The lack of a CORT response to pheromone treatment in the current study may be due to the use of males that were no longer in breeding condition, whereas males from the Schubert et al. (2009) study were recently collected from the field and tested during the breeding season. Unfortunately, unforeseen logistical constraints precluded using males in breeding condition in the current study. Consistent with the lack of increase in CORT in response to pheromone treatment, pheromone treatment had no effect on oxygen consumption. Additional studies using reproductively active males should be conducted to better evaluate the hypothesis that pheromones alter metabolic rate.

In conclusion, elevation of plasma CORT to physiological levels increased metabolic rates in red-legged salamanders. The increase in oxygen consumption observed in salamanders treated with CORT was comparable to oxygen consumption associated

with social interactions like mating. This is the first study to demonstrate that physiological elevation of plasma CORT alters metabolic rate in an amphibian.

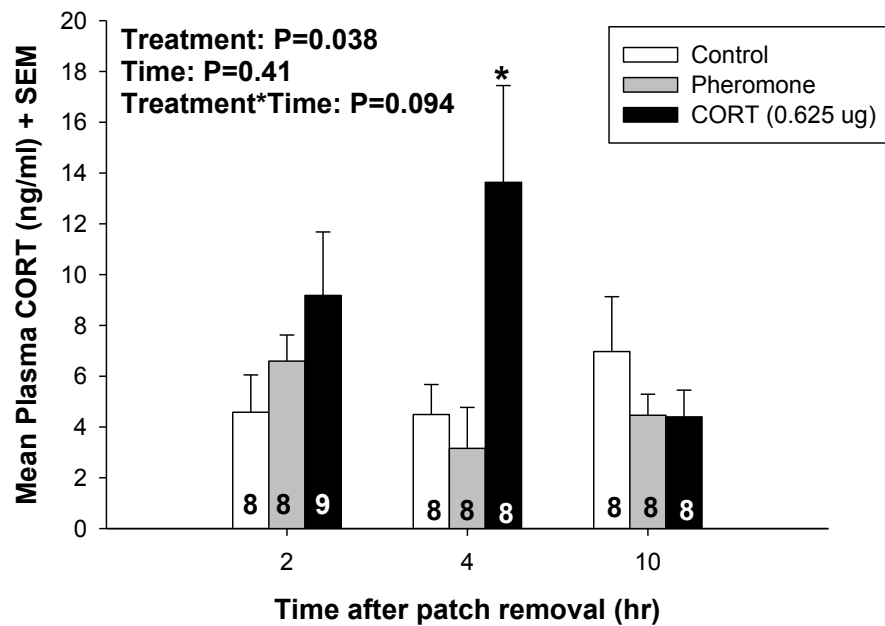


Figure 6.1. Mean plasma CORT concentrations in male *P. shermani* exposed to male courtship pheromone, CORT patches, or vehicle controls for 45 minutes. Blood was collected at 2, 4, and 10 hours after last treatment application. Sample sizes are listed within each bar. P-values of main and interaction effects from ANOVA are listed in the panel. Asterisk denotes difference from other treatment groups at 4 hours.

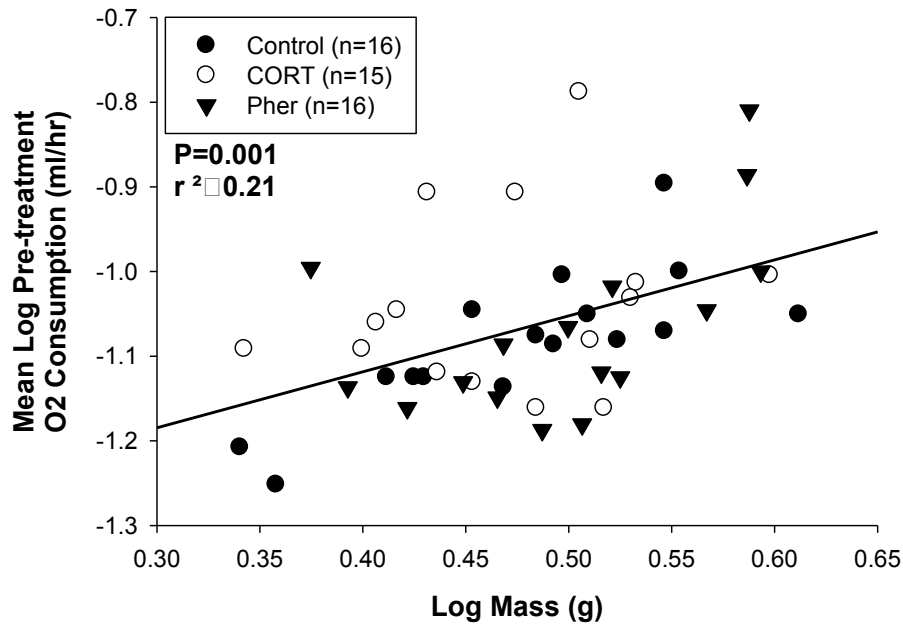


Figure 6.2. Relationship between body mass and rate of oxygen consumption (VO₂) measured in male *P. shermani* before experimental treatments. Corresponding P and r-squared values are listed in the box. Sample sizes are listed within legend.

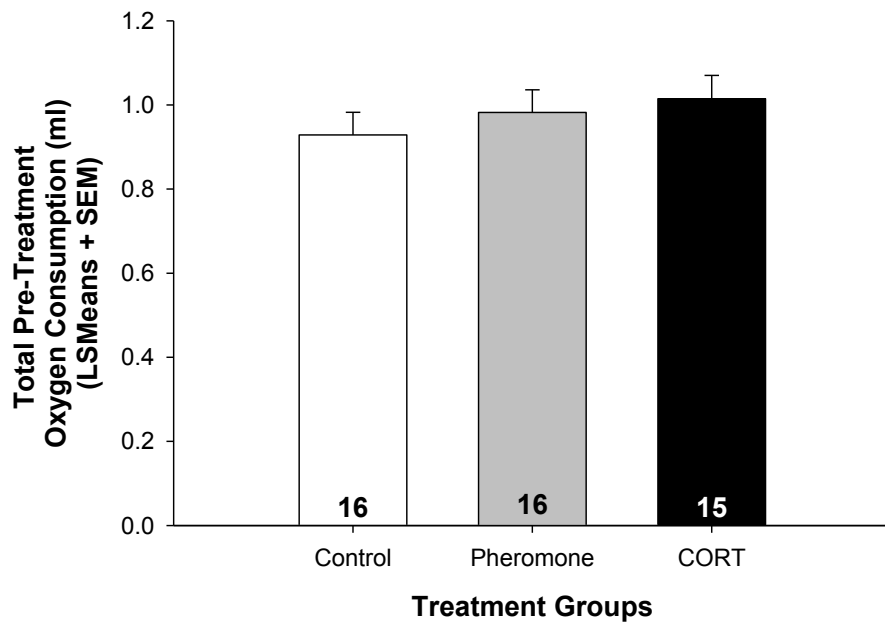


Figure 6.3. Total oxygen consumption of male *P. shermani* before treatment. Data are presented as least squares means of total oxygen consumption over a 6.3 hour period corrected for mass. Sample sizes are listed within the bars above.

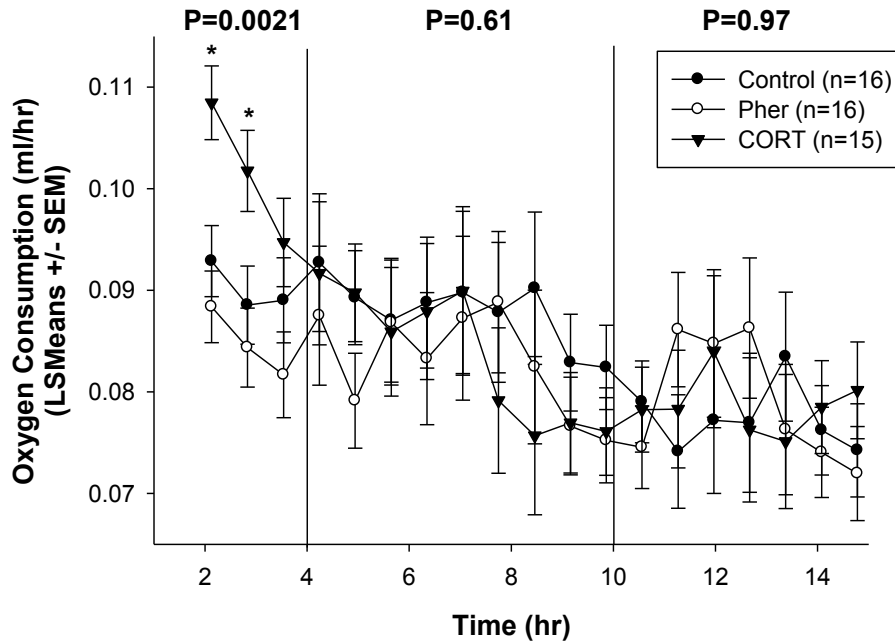


Figure 6.4. Rate of oxygen consumption (V_{O_2}) in male *P. shermani* after exposure to CORT patches, male courtship pheromone, or vehicle controls. Treatments were applied for 45 minutes and oxygen consumption was measured from 0 to 15 hours after the end of the treatments. Results were analyzed according to time interval, indicated by the vertical lines P-values from analyses for each interval are listed. Data are presented as least squares means of V_{O_2} corrected for mass. Asterisk indicates significant difference from other treatment groups based on Tukey post-hoc analyses. Data are plotted as LSMMeans of oxygen consumption corrected for mass. Sample sizes are listed in the box.

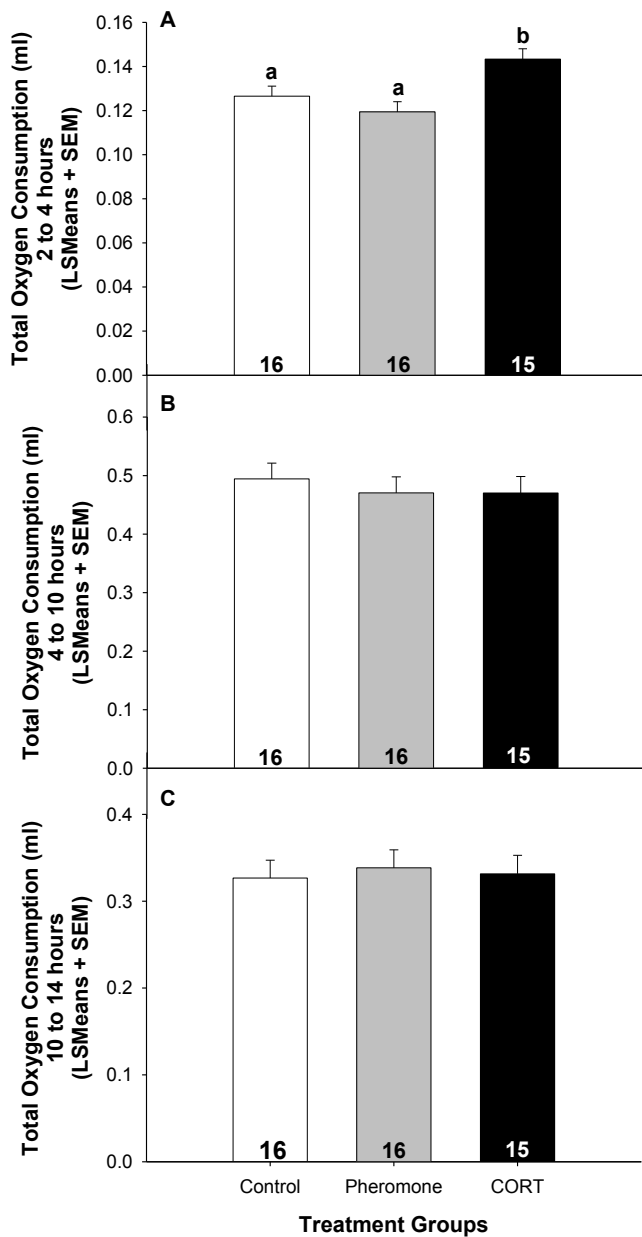


Figure 6.5. Total oxygen consumption of male *P. shermani* from (a) 2 to 4 hours, (b) 4 to 10 hours, and (c) 10 to 14 hours. Data are presented as least squares means of total oxygen consumption corrected for mass. Letters denote significant differences after Tukey post hoc tests. Sample sizes are listed within each bar

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Chapter 7

Conclusions

Pheromones can alter behavior and physiological functions in animals. Many of the mechanisms involved in these processes are still unknown. Furthermore, much work needs to be done to understand the evolution of pheromonal mechanisms in vertebrates. I have investigated the interactions of pheromones and neuroendocrine mechanisms in a basal tetrapod to add to our understanding of chemical communication. I have shown that mental gland pheromones altered levels of the neuromodulator, gonadotropin-releasing hormone (GnRH), which may act to alter expressions of behavior, particularly those related to reproductive behavior. However, mental gland pheromones did not interact with the arginine vasotocin (AVT) system nor were there large sex differences in the AVT system. AVT also did not alter reproductive behavior in plethodontid salamanders as it does in newts. Thus, I provided evidence that mental gland pheromones may have a releasing effect by altering behavior via GnRH, but not AVT.

Mental gland pheromones also were shown to have a priming effect in plethodontid salamanders. Data collected and published from an earlier master's student, Nikki Schubert, and I showed that mental gland pheromones increased plasma corticosterone (CORT) concentrations in males. Mental gland pheromones were previously shown to increase receptivity in females, thus were thought to only play a role in male-female interactions. I investigated this new role of mental gland pheromones by examining the functional role of increased plasma CORT in males. I determined that CORT had no consistent effects on locomotor activity and chemoinvestigation. However,

increases in plasma CORT concentrations within the physiological range increased metabolic rates in male *P. shermani*. This was an important contribution to the field of bioenergetics and stress physiology. Studies in vertebrates examining the role of CORT on metabolism present conflicting results. These conflicting results are likely due to the method that was used to elevate plasma CORT and the levels of plasma CORT achieved. Many of these studies elevate plasma CORT concentrations at pharmacological or high physiological levels. The plasma CORT concentrations achieved in my study were well within the physiological range. My study also is the first to show the connection between CORT and metabolism in an amphibian.

Below I describe the contributions and significance of my work in more detail. First, I discuss my work describing the role of GnRH and AVT in pheromonal communication. Next, I discuss my work describing the role of CORT in pheromonal communication. Table 7.1 shows how results from my research fit into the general mechanism of how pheromones alter behavior through a priming or releasing effect.

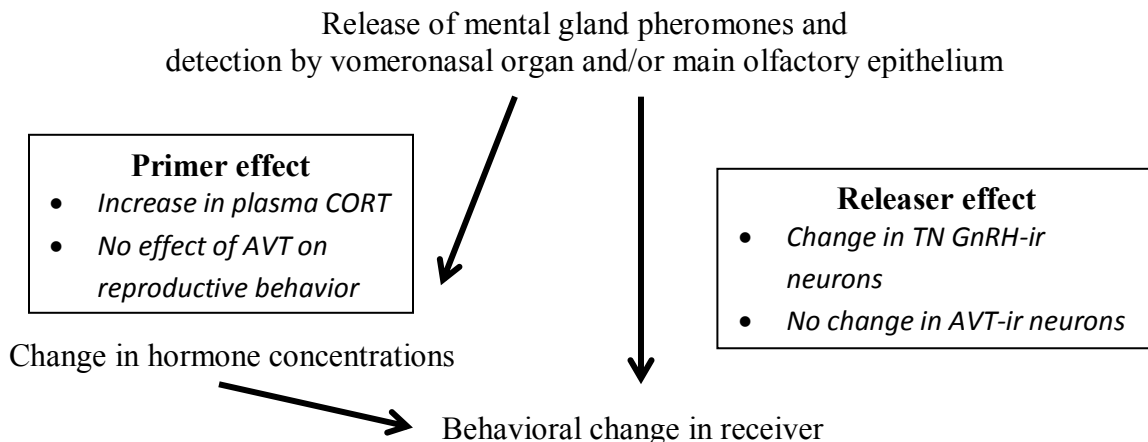


Figure 7.1. Diagram of how pheromones alter behaviors in vertebrates and how results from my research fit into how mental gland pheromones alter behavior in plethodontid salamanders.

Interactions of pheromones, neuromodulators, and behavior

Pheromones may act to alter behavior in the receiver via the GnRH system. In my study, application of mental gland pheromones tended to increase the number of terminal nerve GnRH-ir neurons in females compared to males. Other studies show that the terminal nerve GnRH-I system is intimately associated with the olfactory system. GnRH altered the vomeronasal system by modulating responses of vomeronasal sensory neurons (Eisthen et al., 2000; Park and Eisthen, 2003; Zhang and Delay, 2007). Social signals increased the number of GnRH-ir neurons and concentrations of GnRH in amphibians (Burmeister and Wilczynski, 2005; Propper and Moore, 1991). Male plethodontid salamanders deliver mental gland extract to females during courtship, which increases receptivity of the female (Rollmann et al., 1999). GnRH increased receptivity in other vertebrates (Kelley, 1982; Moore et al., 1987; Pfaff et al., 1987). Terminal nerve GnRH might modulate reproductive behavior by modulating vomeronasal function. Therefore, pheromones might increase sensitivity of the GnRH system to subsequent exposure to pheromones, which ultimately would increase female receptivity.

Sex differences were also found in the hypophysiotropic GnRH system, where females had more GnRH neurons of the caudal, hypophysiotropic GnRH system. It is unknown what the functional role of this sex difference is, but it is possible that it could also be related to female sexual behavior. Since these animals were collected during the breeding season and in reproductive condition, it is possible the GnRH is being primed to activate the HPG axis during this reproductive period. In vertebrates, GnRH acts to increase estrogen concentrations in females, and estrogen levels are typically increased during breeding seasons (Norris, 2006).

Pheromones had no effect on the number of AVT-ir neurons in *P. shermani* nor were there large sex differences in the anatomical distribution of AVT-ir neurons in *P. shermani* or *D. ochrophaeus* as there has been seen in the roughskin newt (Lowry et al., 1997). In addition, treatment with AVT or AVP antagonists had no observable effects on courtship behavior in male *D. ochrophaeus* and *D. ocoee*. In five separate studies examining courtship behavior in *Desmognathus spp.*, there was no effect of AVT or AVP antagonist at various concentrations or time courses on behavior. It is possible that I used the incorrect dose or time course to demonstrate behavioral responses to AVT or AVP antagonists, but it is also possible that AVT is not a major mediator of reproductive behavior in these terrestrial salamanders.

Much of the work understanding the effects of AVT on reproductive behavior was performed in the roughskin newt by Frank Moore and his colleagues. They determined that AVT was involved in the sensorimotor processing of clasping behavior in male newts. When the somatosensory neurons of the abdominal area of males are activated, AVT is released and alters the motor output to initiate clasping behavior (Moore and Miller, 1983; Moore and Rose, 2002). In addition to these behavioral effects, large male-biased sex differences in the distribution of AVT-ir are found throughout brain regions controlling sexual behavior in roughskin newts (Hollis et al., 2005; Lowry et al., 1997; Moore et al., 2000). There also are seasonal and steroidal influences on the AVT system in roughskin newts, which suggest that AVT has strong behavioral effects in the roughskin newt (Moore, 1992; Moore et al., 2000). Female chemosensory signals also increased firing of neurons in the medulla, which were blocked by an AVP antagonist. These data suggest that pheromones can mediate the effects of AVT (Thompson et al.,

2008). However, using similar experimental designs and doses, I have not found similar results in plethodontid salamanders. I propose that there are species differences in responses to AVT. At the very least, there are differences between salamandrid and plethodontid salamanders, which could be related to the different lifestyles of roughskin newts (aquatic) and plethodontid salamanders (semi-terrestrial). Generalizations made from studies performed in roughskin newts to other amphibians and vertebrates should be done with caution.

Future Directions

Because pheromones altered the number of GnRH-ir neurons in females, future studies should investigate the behavioral effects of GnRH on vomeronasal function and behavior in females. Additional experiments might include examining the effects of female-derived pheromones on GnRH-ir in males, and the use of a chicken GnRH antibody to examine the effects of pheromones on the GnRH-II system.

Future studies on the role of AVT in plethodontid salamander should focus on female behavior, because I found that the sex differences in the AVT system in plethodontid salamanders was female biased. Female *P. shermani* and *D. ochrophaeus* had more AVT-ir neurons in the preoptic area, which controls reproductive behavior in vertebrates. Given these results, I would further investigate how AVT alters courtship and mating in female *D. ochrophaeus*. Furthermore, relatively little work has been done examining the effects of AVT in females, so these studies would yield novel data. Future studies should also examine AVT effects in aquatic plethodontids, to test the role of

lifestyle (aquatic versus terrestrial) on whether AVT is a neuromodulator of reproductive behavior.

Interactions of corticosterone, behavior, and metabolism

The second half of my thesis was based on an observation that application of mental gland pheromones increased CORT concentrations in males, but not females (Schubert et al., 2009). This was the first time that a pheromone was shown to have an effect on the endocrine system in an amphibian and suggested that CORT may play an important role in pheromonally-mediated behaviors. Mental gland extract was originally implicated in male-female courtship interactions, because it increased female receptivity during courtship (Rollmann et al., 1999). However, after finding that application of mental gland pheromones increased plasma CORT concentrations in males, it seemed reasonable to study the role of CORT in male behavior and physiology.

In order to understand the role of CORT in males, I developed a non-invasive method to deliver exogenous CORT in salamanders. Traditional methods of CORT delivery require handling and/or anesthesia, which in and of themselves would increase plasma CORT concentrations. A non-invasive patch method had already been developed and used successfully in lizards (Knapp and Moore, 1997) with continued success in birds (Wada and Breuner, 2008). However, transdermal delivery of steroid hormones had not been utilized in amphibians. Amphibians are an excellent group for the transdermal delivery of steroid hormones because of their moist and sticky skin. I validated the CORT dose and time course of delivery in order to produce physiological plasma concentrations of CORT over a physiologically relevant time course. Once this was accomplished, I

used the dermal patch method to measure behavioral and metabolic responses to elevated CORT.

CORT alters a number of behavioral and physiological functions in vertebrates. As a metabolic hormone, CORT is typically elevated during stressful or energetically expensive events. Courtship in plethodontid salamanders is fairly lengthy (Arnold, 1976; Houck and Arnold, 2003). Males deliver mental gland extract to females over several hours of courtship. It is plausible that mental gland pheromones are feeding back onto the male to cause an increase in plasma CORT. Increased plasma CORT might help a courting male to meet the energetic needs of a lengthy courtship by increasing glucose concentrations.

CORT may also act to aid males during agonistic interactions. Male plethodontid salamanders are territorial and engage in agonistic interactions, which can escalate to biting (Verrell and Donovan, 1991). Confrontations with an intruding male can be considered a stressful situation, thus increasing CORT concentrations to aid in the possible contest that may ensue. Plethodontid salamanders do scent mark, primarily with glands from their tails (Simons et al., 1999; Wise et al., 2004). It is possible that males may also scent mark with their mental gland. If so, males could detect an intruding male through detection of mental gland pheromones. Detection of a conspecific male's pheromone could increase plasma CORT concentrations in the receiver.

I found that treatment with CORT had no effect on activity or chemoinvestigation in male *P. shermani*. My results are consistent with other studies that have found that elevation of CORT via dermal patches both acutely and repeatedly over several weeks had no effects on activity, feeding, or courtship in *Desmognathus spp.* (Ricciardella et al.,

2010; Woodley and Bliley, in preparation). However, it is possible that different doses of CORT may have behavioral consequences, or that the behavioral effects induced by CORT may be delayed. Although acute elevations in CORT had no immediate behavioral effects, CORT did increase metabolic rates in male *P. shermani*. Our study is one of the few to evaluate the influence of physiologically relevant elevations in CORT on whole animal metabolism in wildlife, and the first to show that elevated plasma CORT increases metabolism in an amphibian. In my study, oxygen consumption rates increased by 12% over controls. For comparison, metabolic rates increased by 38% and 27% during courtship and agonistic interactions, respectively in a related species of salamander, *D. ochrophaeus* (Bennett and Houck, 1983). Plethodontids have among the lowest metabolic rates of any vertebrate and can sustain their low energy lifestyle with only 12 worms per year (Feder, 1983). Therefore, any increase in plasma CORT concentrations, even a minor increase, could come at a large metabolic cost and reduce aerobic scope. The uncoupling of behavioral changes from CORT changes may be an adaptation to minimize metabolic costs of elevated plasma CORT.

Future Directions

More work needs to be done to better understand the role of CORT in vertebrate behavior and physiology. Plethodontid salamanders are a particularly useful group for this work, due to their low energy lifestyles. Anything that alters metabolic rate in plethodontid salamanders could come at a large metabolic cost, because these animals have the lowest metabolic rates of any terrestrial vertebrate (Feder, 1983). Increases in plasma CORT come at a metabolic cost for these animals, which could result in fitness

consequences. These fitness consequences could be measured in plethodontid salamanders by examining survival and reproductive output. These studies would be best performed in the field.

Metabolic rates were increased in plethodontid salamanders engaged in courtship and agonistic interactions. I would also like to see if CORT is involved in these metabolic increases. CORT concentrations should be measured in males after courting a female or being exposed to female chemosensory cues. Additionally, CORT concentrations can also be measured in males exposed to male conspecific chemosensory cues to simulate male-male interactions. I would then suggest measuring oxygen consumption in males exposed to these cues. If exposure to chemosensory cues result in increased CORT concentrations and rates of oxygen consumption, then antagonists should be used to further verify if CORT is directly involved in increasing metabolic rates. Metyrapone blocks endogenous CORT concentrations by interfering with CORT synthesis, and mifepristone blocks the action of CORT by binding to glucocorticoid receptors. Animals exposed to these antagonists should have similar rates of oxygen consumption to controls if CORT is involved in the mechanism to increase metabolic rates. I would also like to repeat my original metabolic experiment with males that are collected directly from the field, so that a connection between pheromone delivery, increased CORT concentrations, and increased rates of oxygen consumption can be made.

Conclusion

My dissertation research has made several contributions to the field of chemical communication, comparative endocrinology, animal behavior, and stress physiology. I

hope that these data will add to our understanding of the mechanisms used by pheromones to alter behavior. The increase in metabolism from exogenous CORT is also an exciting result. This result should stimulate more research understanding the connection between CORT and metabolism, particularly in amphibians. Additionally, I hope that the non-invasive dermal patch method I validated will be utilized to further understand the role of CORT in amphibians. Amphibians are experiencing a global decline, the largest vertebrate decline since the dinosaurs. Given these declines, information on how amphibians respond to stressors are imperative. Unfortunately, we know very little about the amphibian stress response. These patches can be used as a valuable tool to manipulate CORT concentrations in terrestrial amphibians to gather data on how amphibians respond behaviorally and physiologically to elevated plasma CORT.

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