THE ROLES OF THE MAIN OLFACTORY AND VOMERONASAL SYSTEMS IN
PREY DETECTION BY TWO TERRESTRIAL SALAMANDERS

A Thesis
Presented to
The Faculty of Graduate Studies
of
The University of Guelph

By
ANGELA C. TELFER

In partial fulfilment of requirements
for the degree of
Master of Science
September, 2011

© Angela C. Telfer, 2011
ABSTRACT

The Roles of the Main Olfactory and Vomeronasal Systems in Prey Detection by two Terrestrial Salamanders

Angela Telfer
University of Guelph, 2011

Adviser: Dr. F. Laberge

Terrestrial salamanders of the genus *Plethodon* are among many vertebrates possessing both main olfactory and vomeronasal systems, which the Volatility Theory posits are for detection of volatile and soluble olfactory cues, respectively. Further recent work showing a high amount of convergence between the two olfactory subsystems at the level of the central nervous system suggests complementary or overlapping roles for them. This study examined the use of the olfactory subsystems in prey detection from the perspectives of behaviour and neurobiology. Red-backed salamanders, *Plethodon cinereus*, were observed in standardized behavioural assays with both volatile and soluble prey olfactory cues. Naïve salamanders showed an increase in nosetapping as well as a side preference in the presence of soluble and volatile prey cues when tested in a 22°C day/20°C night room. In a 15°C day /12°C night room, salamanders increased nosetapping in the presence of soluble prey cues. Salamanders showed a pattern of responses that differed based on their previous experience with the assay, as well as the temperature of the testing room. Attempts to study the neurobiology of olfactory function in *Plethodon shermani* were inconclusive up to this point, but future directions are discussed. This study shows the importance of olfaction in prey detection by salamanders and that prey searching behaviour is exhibited in the exclusive presence of olfactory cues.
ACKNOWLEDGEMENTS

Completing my MSc. research at the University of Guelph was challenging and rewarding, and I owe a lot to the people who have supported me throughout. First of all, I greatly appreciate the support, encouragement, and guidance offered to me by my advisor, Dr. Fred Laberge. The door was always open for quick questions or in-depth discussions, and I appreciate the time and effort you invested in me and this project. Thanks also go to Dr. Rob McLaughlin and Dr. Elena Choleris for being part of my advisory committee and providing constructive criticisms and advice.

Thank you to Robert Frank and Matt Cornish for help, materials, advice and friendly faces to turn to in the Hagen Aqualab. Much appreciation for the CGS-M scholarship I received goes to the Natural Sciences and Engineering Research Council (NSERC). Thanks also go to my lab mates Zach Ramsay, Patrick Kelly and Arielle Duhaime-Ross, as well as the numerous volunteers who helped shoulder the workload associated with care of research animals. I also thank my office-mates for providing great company and advice.

Finally, I would like to thank my parents, Scott and Penny, and other family and friends who have encouraged me throughout my studies. You helped me persevere through the bad times and celebrated with me in the good times.
# TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS....................................................................................................................... iii

TABLE OF CONTENTS............................................................................................................................ iv

LIST OF TABLES ..................................................................................................................................... v

LIST OF FIGURES ................................................................................................................................... vi

CHAPTER 1 – General Introduction...................................................................................................... 1
  Olfaction........................................................................................................................................ 1
  Theories of Olfactory Subsystem Function...................................................................................... 5
  Prey Foraging ................................................................................................................................. 8
  Salamanders as a Model Organism .............................................................................................. 9
  Thesis Objectives ........................................................................................................................... 11

CHAPTER 2 – Behavioural Responses of Red-backed Salamanders to Soluble and Volatile Prey Cues......................................................................................................................... 13
  Abstract ...................................................................................................................................... 13
  Introduction ................................................................................................................................. 14
  Methodology .............................................................................................................................. 19
  Results ....................................................................................................................................... 32
  Discussion ................................................................................................................................. 40

CHAPTER 3 – The Roles of the Olfactory Subsystems of Red-legged Salamanders in Prey Detection.............................................................................................................................................. 50
  Abstract ...................................................................................................................................... 50
  Introduction ............................................................................................................................... 51
  Methodology ............................................................................................................................. 55
  Results ....................................................................................................................................... 61
  Discussion ................................................................................................................................. 67

CHAPTER 4 – General Discussion...................................................................................................... 75

REFERENCES ....................................................................................................................................... 80
LIST OF TABLES

CHAPTER 3

Table 1. Number of c-Fos labelled cells in salamanders receiving different soluble and volatile control or prey cues. .......................................................... 66
LIST OF FIGURES

CHAPTER 2

Figure 1. Schematic drawing of the testing boxes used for all behavioural assays .................. 23

Figure 2. Responses of naïve red-backed salamanders in a preference/avoidance assay to control and prey cues in a 20-22°C room ........................................................................................................... 35

Figure 3. Responses of experienced red-backed salamanders in a preference/avoidance assay to control and prey cues in a 20-22°C room .................................................................................................................. 36

Figure 4. Responses of naïve red-backed salamanders in a preference/avoidance assay to control and prey cues in a 12-15°C room .......................................................................................................................... 37

Figure 5. Responses of naïve red-backed salamanders in an volatile attraction assay to control and prey cues in a 12-15°C room .......................................................................................................................... 38

Figure 6. Responses of red-backed salamanders to control and prey cues depending on olfactory block received in a 20-22°C room .......................................................................................................................... 39

CHAPTER 3

Figure 1. Responses of naïve red-legged salamanders in a preference/avoidance assay to control and prey cues in a 12-15°C room ...................................................................................................................... 63

Figure 2. Micrographs of transverse brain sections from P. shermani after c-Fos immunocytochemistry (20x magnification), taken from a control animal ...................... 64

Figure 3. Comparison of c-Fos labelling in four brain areas in salamanders receiving different treatments (100x magnification). .................................................................................................. 65
CHAPTER 1

GENERAL INTRODUCTION

Olfaction

Successful detection and recognition of chemical cues in the environment is an invaluable ability for many organisms. A wide variety of chemicals exist in the environment and their identity cannot be predicted before they are encountered. The sense of olfaction, responsible for detection of chemical cues along with the sense of gustation, displays remarkable sensitivity, range, and discriminatory ability (Buck and Axel 1991; Firestein 2001; Bargmann 2006). Even something as minute as a change in chirality or concentration can change the smell of a molecule, yet very dissimilar molecules can smell similar (Malnic et al. 1999; Kauer 2002). This ability to discriminate between odourants provides a functional advantage to organisms as they can change their behaviours in response to specific cues they perceive in their environment. Many organisms depend wholly or in part on their olfactory subsystems for finding prey (e.g. Nevitt 2000; Placyk & Graves 2002; Catania et al. 2008), avoiding predators or dangerous situations (e.g. Chivers & Smith 1995; Madison et al. 1999a, b; Apfelbach et al. 2005), attracting or finding mates (Simon & Sternberg 2002; Schubert et al. 2008), and territoriality (e.g. Roberts & Dunbar 2000; Zub et al. 2003; Martin et al. 2005).

Chemical cues play a large role in predator/prey interactions, both for the predator and the prey. Predators that can successfully interpret and respond to prey chemical cues are likely to benefit from increased foraging success. Chemical cues have been shown to indicate to predators if their prey has been in the vicinity recently and can provide a scent trail to follow and find the
prey (e.g. Smith et al. 2005; Cunningham et al. 2008; Thomas et al. 2008). They can alert a predator to the presence of prey earlier than visual cues, possibly giving the predator the advantage of a surprise attack. Conversely, prey animals that can detect and interpret chemical cues from their predators may benefit from reduced predation risk by displaying an avoidance response. This relationship has been widely documented in aquatic systems (reviewed in Wisenden 2000), though the literature on the role of olfaction in terrestrial predator/prey systems is scant. Many tetrapods possess two olfactory subsystems for detection of chemical cues, the main olfactory and vomeronasal (or accessory olfactory) systems. A few aquatic organisms possess both subsystems (i.e. salamanders from the amphiumid and sirenid families: Eisthen 2000), but no fish have been shown to have a vomeronasal system. However, it should be noted that Hashiguchi & Nishida (2006) detected main olfactory and vomeronasal type receptors in teleost fishes.

The general model of olfactory sensing in the main olfactory system begins with the detection of an odourant by G-protein-coupled receptors (GPCRs), known as odourant receptors (ORs), which belong to a large multigene family; each receptor will detect a certain odourant or suite of odourants (Buck & Axel 1991; Touhara et al. 1999; Bargmann 2006). Odourant receptors are borne by the olfactory receptor neurons (ORNs) interspersed throughout the main olfactory epithelium of the nasal cavity. The olfactory epithelium also contains trace amine-associated receptors (TAARS), another type of G-protein receptor from a different family thought to function in volatile amine detection (Liberles & Buck 2006). The odourant itself is viewed as a ligand for the receptor and the pattern of receptor binding across the various ORs is believed to be responsible for identification of individual ‘odours’ (Buck & Axel 1991; Malnic et al. 1999; Kajiya et al. 2001; Nara et al. 2011). Binding of the ligand causes increases in the
second messenger cyclic AMP (cAMP), activating a cation-selective cyclic nucleotide-gated channel and causing external Ca$^{2+}$ influx into the receptor neuron(s). If a sufficient number of channels are open for long enough, an action potential is generated (described in Buck & Axel 1991; Touhara et al. 1999; Firestein 2001; Laberge & Hara 2001). The axons of the ORNs join to form the olfactory nerve, which projects to the olfactory bulb. At the olfactory bulb, these axons make synaptic contact with the dendrites of second-order olfactory neurons, the mitral cells. The ORN nerve terminals and the apical dendrites of mitral cells form glomeruli, which are centers for integration of the olfactory information detected by specific ORs (Sharp et al. 1975; Stewart et al. 1979; Hamdani & Døving 2007). This convergence to glomeruli may increase the sensitivity of the main olfactory system to low concentrations of an odour (Buck 2000). The axons of the mitral cells form the olfactory tract, which then projects to various higher brain regions, including the piriform cortex (in mammals), hippocampus, amygdala, and hypothalamus (Scalia & Winans 1975; ten Donkelaar 1998; Firestein 2001). The projection of the second-order olfactory neurons is subdivided into medial (MOT) and lateral (LOT) olfactory tracts. The MOT is formed mainly from projection fibers from the medial olfactory bulb, while the LOT is formed from fibers in the lateral olfactory bulb (Sheldon 1912; Laberge & Hara 2001). These subdivisions, along with the ORNs leading to them, appear to have functional differences (Hamdani & Døving 2007), where the LOT is associated with feeding (Hamdani et al. 2001) and the MOT with pheromonal responses (Sorensen et al. 1991).

The vomeronasal system (VNS) is believed to follow a similar model of olfactory sensing and processing as the main olfactory system (MOS), though some key differences exist. While GPCRs are still believed to be responsible for odourant detection, the GPCRs of the VNS belong to a family unrelated to those of the ORs in the MOS and are known as the V1R and V2R.
families of receptors (Dulac and Axel 1995; Matsunami & Buck 1997; Ryba & Tirindelli 1997; Mombaerts 2004). Recently, another GPCR family referred to as the formyl peptide receptors (FPR-rs) was found in the vomeronasal epithelium of mammals; they are believed to function in pathogen identification (Rivière et al. 2009). A member of the transient receptor potential (TRP) superfamily of ion channels, TRP2, is expressed exclusively in the vomeronasal organ (VNO) sensory microvilli (Stowers et al. 2002). The signal transduction mechanism in the VNS is believed to be mediated by the TRP2 ion channel and phospholipase C activation; it may further include Inositol(1,4,5)-triphosphate (IP3), diacyl glycerol, and Ca^{2+} release (Luo et al. 1994; Liman et al. 1999; Lucas et al. 2003). Notably, it is not believed to include cyclic nucleotides as second messengers, as in the cAMP pathway of the MOS (Firestein 2001; Stowers et al. 2002). The vomeronasal receptor neurons project to the accessory olfactory bulb, a distinct structure located caudal to the olfactory bulb. They also do not converge onto single glomeruli, as is the case in the MOS (Rodriguez et al. 1999; Belluscio et al. 1999), though a more convergent pattern appears at the level of the mitral cells (Del Punta et al. 2002). Lévai et al. (2006) found a subset of main olfactory ORs in the vomeronasal organ of mice whose axons projected to the accessory olfactory bulb, and use the TRP2 channel; their role is however unclear.

There are other differences between the subsystems. The MOS epithelium has receptor cells possessing cilia, and the lamina propria of the epithelium possesses Bowman’s glands. The VNO epithelium has receptor cells possessing microvilli, and the presence of Bowman’s glands has not been shown (Dawley & Bass 1988). Mode of delivery to the epithelia differs slightly; odour delivery is easily made between the MOS and external environment via inhalation, but requires more effort on the part of the organism for the VNS (Hamlin 1929). In mammals, the Flehmen response is a documented means of delivering molecules to the VNO (Lindsay &
Burton 1983; Hart & Leedy 1987); other examples are tapping of the nasolabial grooves to the substrate in salamanders (Brown 1968; Dawley & Bass 1988, 1989), tongue flicking in garter snakes (Kubie & Halpern 1975), and head bobbing in guinea pigs (Beauchamp et al. 1982, 1985). These methods all involve a voluntary response from the organism to deliver molecules to the VNO, as opposed to passive inhalation delivering molecules to the MOS.

**Theories of Olfactory Subsystem Function**

In terrestrial systems, two types of chemical cues are generally identified. The first, soluble cues, are dissolved in water. They are generally thought to be of higher molecular weight (Wysocki et al. 1980; Halpern and Kubie 1980; Kikuyama & Toyoda 1999) and may be amino acids, proteins, or other large, low volatility molecules (Li et al. 2005; Hemilä & Reuter 2008). The second type, volatile cues, are airborne and thought to be of low molecular weight. Much research has been performed on the perception of these two types of odourants, especially in rodents. The functional significance of these classifications is still being debated in the literature, as it has been shown that both subsystems can detect both types of cues (Xu et al. 2005), but the physiological relevance of these findings is not yet clear.

Although early literature suggests the VNS is meant only for pheromone detection (the ‘Equivalency Theory’), this theory has fallen into disfavour. First described in 1959, a pheromone is a cue emitted by one member of a species and detected by another of the same species, resulting in a specific reaction like a stereotyped behavioural or endocrine response (Karlson & Lüscher 1959). Yet the VNS has been implicated as playing a role in many behaviours, including courtship (Wirsig-Wiechmann et al. 2002, 2006; Laberge et al. 2008; Schubert et al. 2008), prey detection (Placyk & Graves 2002), and home recognition (Graves
Interspecific cues like those emitted by prey would not fit the definition of a pheromone, negating the possibility the VNS detects pheromones only. Furthermore, other studies have shown that the MOS is responsible for the detection of some pheromones (Dorries et al. 1997a; Swann et al. 2001; Xu et al. 2005).

Another theory that gained popularity suggests that each subsystem is specialized for detection of chemical cues differing in volatility (referred to hereafter as the ‘Volatility Theory’; Wysocki et al. 1980; Wysocki et al. 1985; Li et al. 2005; Baxi et al. 2006; Spehr et al. 2006b). The VNS is thought to be responsible for detection of soluble cues, and the MOS of volatile cues. Mode of delivery is important, as Li et al. (2005) found differential activation of MOB and AOB neurons depending on whether the odour was delivered in vapour or liquid form in garter snakes. Volatiles have been shown to stimulate the VNS (O’Connell & Meredith 1984; Meredith 1991), non-volatiles the MOS (Spehr et al. 2006a), and some molecules may be able to be detected by both systems (Xu et al. 2005). Recent literature suggests there may be a greater interdependence of the subsystems than was previously thought (Xu et al. 2005; Kelliher 2007; Martinez-Marcos 2009; Martinez-García et al. 2009), yet these ideas have existed in the literature for over 25 years (Powers et al. 1979; Beauchamp et al. 1982; Meredith 1986). Male guinea pigs were found to maintain discriminatory abilities between male and female urine following vomeronasal organ removal, initially spending more time investigating female urine; this response became extinct after approximately 6 months post-vomeronasal organ removal (Beauchamp et al. 1982). The authors suggested that the main and accessory olfactory systems may play interacting or redundant roles, and that vomeronasal input from urine may have intrinsically rewarding properties. Meredith (1986) showed that male hamsters who had sexual experience before vomeronasal organ removal showed no deficits, but those with no experience
lacked sexual behaviours following removal; Meredith suggested the vomeronasal input from previous sexual experience could be associated with main olfactory input to be recalled when needed. Similarly, Powers et al. (1979) found both subsystems participated in sexual responses of male hamsters to females. Previously, the VNS was believed to exclusively mediate the response to courtship and reproductive pheromones, but the MOS may also carry these signals to the vomeronasal amygdala in mice (Kang et al. 2009), suggesting not exclusivity of subsystem function but convergence. This was supported by Roth & Laberge (2011), which found abundant convergence between the two subsystems at the level of the CNS in salamanders. This theory of convergent or complementary functions is now referred to as the ‘Learning Theory’ of olfaction.

The Learning Theory explains the convergence of the subsystems in terms of function, where the VNS would detect a limited number of biologically relevant cues that trigger non-learned responses. Cues detected by the MOS concomitantly with VNS stimulation could acquire the ability to trigger the behavioural responses elicited originally only by the VNS. If these cues were not ‘learned’ after vomeronasal stimulation, then detection by the MOS alone would not trigger that same behavioural response. This is not to say that the organism would lack discriminatory ability, but that the cue’s biological importance would not have been learned (Martínez-García et al. 2009). The MOS would also function in general olfaction of novel stimuli. In hamsters, both volatile and non-volatile components of female vaginal discharge are responsible for changes in mating behaviour, and both the main olfactory and vomeronasal systems are implicated in attraction and mating behaviours (O’Connell & Meredith 1984). Garter snakes will tongue-flick in response to earthworm wash odours as a function of both the main olfactory and vomeronasal systems, though maintenance of tongue-flicking appears to be more dependent on a functional vomeronasal system (Halpern et al. 1997). Martínez-García et al.
(2009) support this theory with work in mice, where sexually naïve females showed no preference for male urine volatiles, while females raised with male conspecifics did. Furthermore, when chemically naïve females were given accessory olfactory bulb lesions, they were found to lack the typical behavioural response to male urine but did not lack discriminatory abilities between urine and other odours. These studies suggest that the learning of biologically relevant stimuli by the vomeronasal system, followed by subsequent detection of these stimuli by either the vomeronasal or main olfactory system, may be sufficient to explain olfactory subsystem function.

**Prey Foraging**

This study focused on the importance of olfaction in prey detection by two terrestrial salamanders, *Plethodon cinereus* and *P. shermani*, the red-backed and red-legged salamanders. The red-backed salamander is abundant in forests across Northeastern America, often taking shelter beneath rocks or logs (Jaeger 1980; Sullivan et al. 2005). Red-legged salamanders are currently listed as vulnerable to extinction in the IUCN Red List due to the fact only five locations are known to hold these salamanders (Hammerson & Beamer 2004). However, they are abundant in areas where they are found, and their populations are believed to be stable. Their habitat is similar to that of red-backed salamanders. The literature on prey detection in these salamanders is limited. Visual cues were long thought to be the main sensory modality used to detect prey (ten Donkelaar 1998; Wake & Deban 2000), but this description does not fit with the nocturnal lifestyle of these salamanders. They are broad generalist predators of forest invertebrates (Burton 1976; Maerz et al. 2005) and actively forage, primarily using chemical cues, during dusk and evening or directly after a rainfall (Jaeger 1978; David & Jaeger 1981; Placyk & Graves 2001, 2002). Visual cues do appear to be the primary mode of detection during
daytime ambush foraging, but they display increased success when both visual and chemical cues are present (Placyk & Graves 2001). Chemical cue-mediated active foraging appears to be more successful than visual foraging in nocturnal conditions, as red-backed salamanders were more successful catching *Drosophila melanogaster* (fruit fly) prey with this strategy in the dark versus those tested in dim light (Placyk & Graves 2001). To this date, all studies investigating salamander foraging behaviours have included prey items, whether live or dead, in the testing procedure. In the natural environment, salamanders may need to detect cues from their prey without their immediate presence to begin their search for the prey item. Thus, this study was undertaken without the presence of prey items in any task, but only the presence of chemical cues from the prey.

*Salamanders as a Model Organism*

Plethodontid salamanders are a good model for both behavioural and brain research. Red-backed salamanders are abundant and easily accessible in the forest, and are easy to maintain in the laboratory with low levels of mortality. They are hardy in the face of extended periods of food deprivation, due to their low metabolic rate and the large lipid energy stores in their tail (Fitzpatrick 1976; Feder 1983; Yurewicz & Wilbur 2004). Other studies with *P. cinereus* have used food deprivation periods ranging between 4 to 27 days with no reported mortalities (Maerz et al. 2001; Placyk & Graves 2001, 2002). California slender salamanders were held unfed for 4 months and the only reported mortalities were not related to food stress (*Batrachoseps attenuatus*; Maiorana 1977). This hardiness allows for long food deprivation periods to be successfully undertaken to ensure sufficient motivation to explore prey cues, and also to ensure similar levels of hunger among salamanders. Due to their small size many salamanders can be subjected simultaneously to behavioural assays with only one observer. Their prey items are
readily available, as they consist mostly of small insects. Other species of salamanders can prove difficult to observe in behavioural assays, but plethodontids have shown to be reliable subjects in a variety of assays (Madison 1999a,b; Placyk & Graves 2001, 2002; Dantzer & Jaeger 2007a,b). Plethodontid salamanders are particularly well suited for olfactory research as their characteristic nosetapping, where they tap their nasolabial grooves to the substrate, is a clear indicator of chemoinvestigatory behaviour involving the VNS (Dawley & Bass 1989). The nasolabial grooves are clearly visible, and can be altered for manipulation of olfactory abilities.

The relatively simple brain of *P. shermani* is well-suited for research in neuroscience. Generally believed to be very primitive, urodele brains may have undergone a secondary simplification (Roth et al. 1988). Though they lack the piriform cortex of mammals which is associated with olfaction, a number of brain regions including the striato-pallial transition area (SPTA), amygdala, pallium, striatum and hypothalamus have been shown to receive input from either the main olfactory or vomeronasal systems, or both in *P. shermani* (Laberge & Roth 2005; Laberge et al. 2006, 2008; Roth & Laberge 2011). As well, the size of their neurons is relatively large (Roth et al. 1988, 1992), aiding in visualization of neurons when investigating for neuronal activation. In this study, neuronal activation is being visualized using c-Fos immunocytochemistry. Induction of transcription of the immediate-early gene *c-fos* is stimulated externally by many factors, including receptor stimulation. Transcription is highly regulated and elevated for short periods of time after stimulation (Morgan & Curran 1991; Herdegen & Leah 1998). A polyclonal anti-c-Fos antibody was used to detect which neurons were expressing c-Fos following stimulation of the two olfactory subsystems with prey chemical cues. As an indirect measure of neuronal activation, immunocytochemistry against c-Fos was shown to be effective.
in various amphibians, including *P. shermani* (Ubink et al. 1997; Cobellis et al. 1999; Calle et al. 2006; Laberge et al. 2008).

**Thesis Objectives**

This thesis investigated how the two olfactory subsystems mediate detection of prey chemical cues. This was approached using both behavioural and neurobiological analyses that were meant to complement each other. Establishing a behavioural response to prey cues was a prerequisite to investigation of neurobiological responses. Different means of cue delivery were attempted using both soluble and volatile cues to assess olfactory function. This was designed to allow analysis of behaviour in response to both types of cues and to test whether the Volatility Theory was plausible in salamander olfaction. Chapter 2 addresses how the presence of chemical cues from prey affected behaviour of red-backed salamanders. Temperature of testing rooms was varied, as salamander reactions to chemical cues as well as physical properties of the cues themselves may differ according to temperature. Presentation of soluble and volatile cues was attempted to determine their respective roles. If prey chemical cues alone were enough to attract salamanders, then I expected to see spatial preferences toward sites scented with prey cues and increased chemoinvestigatory behaviours (nose-tapping or increased movement) in the presence of the cues. I also predicted that salamanders whose olfactory subsystems were impaired would show a decline in chemoinvestigatory behaviours.

Chapter 3 focuses on brain responses to prey cues in red-legged salamanders. Previous work in the lab (unpublished, P. Kelly and F. Laberge) found little to no c-Fos labelling in *P. cinereus*, so *P. shermani* was chosen due to previous success with this species (Laberge et al. 2008). If, as according to the Volatility Theory, the two olfactory subsystems are activated by
prey cues differing in volatility, then I would expect to see greater amounts of labelled neurons in areas associated with the main olfactory system upon delivery of volatile cues, and more labelled neurons in vomeronasal areas after soluble cue application. When both cues were applied simultaneously, areas where both subsystems converge in the CNS could display higher or lower amounts of labelling than controls and single cue applications due to interactions between the two olfactory subsystems. These interactions could be interpreted as such that when a higher amount of labelled neurons occurs in a brain region compared to single cue deliveries synergy is observed, or when levels of labelling are lowered inhibition is observed. These findings could help support the Learning Theory, which requires associations between the subsystems. If both types of cues indiscriminately activate olfactory brain regions, this would provide evidence against the Volatility Theory.

Chapter 4 is a general discussion of the findings from this research. Contributions to research on chemically-mediated prey detection are highlighted and a discussion of future research possibilities is included.
CHAPTER 2

Behavioural Responses of Red-backed Salamanders to Soluble and Volatile Prey Cues

ABSTRACT

The role of the olfactory subsystems in prey detection by red-backed salamanders (*Plethodon cinereus*) was investigated using both soluble and volatile prey cues of the common house cricket. Salamanders were exposed to these cues in conjunction with controls in assays designed to highlight the role of either the main olfactory or vomeronasal system according to the Volatility Theory of olfaction, which posits that in terrestrial vertebrates volatile cues are detected by the main olfactory system while soluble cues are detected by the vomeronasal system. Salamanders also received olfactory blocks to their vomeronasal system, nares, or a sham procedure before a behavioural assay to gauge the involvement of each subsystem. Temperature and experience of the salamanders with the behavioural assay was varied across experiments. Salamanders naïve to the assay increased chemoinvestigation via nosetapping in the presence of either soluble cues alone, or both soluble and volatile cues. In the warmer temperature, salamanders showed a spatial preference for areas of prey soluble cue deposition. Salamanders receiving the nares and vomeronasal combined block were removed from analysis, and no effect of treatment existed for both sham and vomeronasal blocked salamanders. Alternative methods to conduct that experiment are discussed. This study indicates that prey cues alone are sufficient to change the behaviour of the salamander, and that the roles of temperature and previous experience should be considered when conducting olfactory experiments with salamanders.
INTRODUCTION

Chemical cues in the natural environment provide information to organisms. There has been much research in aquatic environments, where the roles of chemical cues include conspecific signals in courtship and sex identification and interspecific signals mediating predator recognition, alarm reaction and prey detection (i.e. Sorensen et al. 1988; Hara 1994; Wisenden 2000; Webster et al. 2007). Although diffusion of a cue is faster in air than water, odour plumes are more reliable in the aquatic environment because water current is usually more predictable than wind currents (Hemilä & Reuter 2008) and chemical cues will be present for a greater amount of time before they can no longer be detected in water (Wilson 1970; Eisthen & Schwenk 2008). While vision is generally considered more important than olfaction in terrestrial systems, there is mounting evidence that olfaction plays an important role on land as well. With impaired chemosensation, many terrestrial organisms show deficits in their natural behaviours, such as prey trailing in garter snakes (Kubie & Halpern 1975), prey detection in plethodontid salamanders (Placyk & Graves 2002), and diverse deficits in social behaviours in rodents (Powers and Winans 1975; Beauchamp et al. 1982, 1985; O’Connell & Meredith 1984).

Most terrestrial vertebrates possess two olfactory subsystems, the main olfactory and vomeronasal systems (Eisthen 2000 and Reiss & Eisthen 2008 discuss aquatic amphibians possessing both subsystems). Cue detection is governed by different receptors in each subsystem. Both subsystems possess G-protein coupled receptors (GPCRs), but the main olfactory system relies upon a large family of GPCRs referred to as olfactory receptors (ORs) as well as a smaller family of trace amine-associated receptors (TAARS) (Buck & Axel 1991; Liberles & Buck 2006). The receptors of the vomeronasal system belong to separate families than those of the main olfactory system and are referred to as the V1R and V2R families of vomeronasal receptors.
(Dulac and Axel 1995; Matsunami & Buck 1997; Ryba & Tirindelli 1997; Mombaerts 2004). There also exists a smaller unrelated family of receptors, the formyl peptide receptors (FPR-rs: Rivière et al. 2009). The two subsystems also follow different pathways to the brain.

Though the function of each olfactory subsystem is still being debated (Baxi et al. 2006), it is clear that both have roles in detecting a variety of biologically important cues. The Volatility Theory suggests that the subsystems are used for detection of different types of cues, water soluble, higher molecular weight molecules, like proteins, by the vomeronasal system and lower molecular weight air-borne molecules by the main olfactory system (Wysocki et al. 1980; Johnston 1985; Dawley & Bass 1988). I will refer to these cues as soluble and volatile, respectively. Though supporting evidence exists for the Volatility Theory, there is also contradictory evidence where high molecular weight odourants were detected by the main olfactory system (Wysocki et al. 1980; Wysocki et al. 1985; Li et al. 2005; Baxi et al. 2006).

The red-backed salamander, *Plethdon cinereus*, is common across Northeastern America in temperate forest systems, and is found beneath leaf litter, logs and rocks (Jaeger 1980). It is a lungless salamander, and forages mainly at night or after a rainfall, when there is a lower risk of desiccation (Jaeger 1978; David & Jaeger 1981; Placyk & Graves 2001, 2002). It possesses two nasolabial grooves, which arch down from below the external nares to the tip of the mouth. Plethodontid salamanders are able to capture molecules in their nasolabial grooves by tapping the substrate with their snout in a behaviour known as nosetapping. The molecules are then transferred up the grooves to the vomeronasal organ by capillarity (Brown 1968; Dawley & Bass 1988, 1989). As a means of delivering molecules to the vomeronasal organ, this behaviour is comparable to tongue flicking in garter snakes (Kubie & Halpern 1975), head bobbing in guinea
pigs (Beauchamp et al. 1982, 1985), and the Flehmen response in horses and cats (Lindsay & Burton 1983; Hart & Leedy 1987).

In salamanders, chemoreception plays important roles in courtship, home recognition, sex identification, predator avoidance, and prey recognition (Graves 1994; Madison et al. 1999a,b; Rollman et al. 1999; Placyk & Graves 2001, 2002; Martin et al. 2005; Wirsig-Wiechmann et al. 2006; Dantzer & Jaeger 2007a,b; Houck et al. 2007; Laberge et al. 2008). However, there are only a few studies looking at the response of plethodontid salamanders to prey cues. David and Jaeger (1981) embedded fruit flies in agar so the flies were unable to emit chemical cues, and found this manipulation to have a greater effect on salamander foraging success than when salamanders were denied either prey visual cues alone or when neither visual nor chemical cues were available. Placyk & Graves (2001) later showed that the red-backed salamander has greater success in capturing fruit fly prey when using chemical cues in the dark than in dim light. They suggested that the vomeronasal system mediated prey detection as salamanders with cauterized nasolabial grooves were less efficient foragers than normal salamanders, taking longer to attack prey, making less attacks, and catching less prey than their sham-cauterized counterparts (Placyk & Graves 2002). Salamanders will increase their nosetapping to gain information about the location or presence of their prey items, especially in the dark (Placyk & Graves 2001, 2002). Thus far, prey cues have only been presented as emanating from either live or dead prey in an experimental arena. In their natural environment, salamanders would need to be able to sense and follow trails or areas in which prey have been located, with or without the prey’s immediate presence. By providing salamanders only with the chemical cues from these prey items, inferences can be made as to how they might respond in the wild. As well, providing prey in the testing arena does not provide a means of assessing what type of cue the prey was giving off, i.e.
soluble or volatile. Salamanders will forage on a variety of insects, from insect larvae to moths (Burton 1976; Placyk & Graves 2002; Maerz et al. 2005) so it is possible that different types of cues play important roles depending on prey type. Nymphs of the house cricket (*Acheta domesticus*) were chosen as prey items for this study as they are the appropriate size for these salamanders and would plausibly provide both soluble and volatile cues in the environment. Salamanders eat these prey items readily in laboratory settings. Further, salamanders have been shown to react to both soluble and volatile cues of conspecifics (Martin et al. 2005; Dantzer & Jaeger 2007a,b) in the laboratory. For the latter, cues were prepared identically but presented differently (i.e., could make contact with the source of odour or not) to assess salamander responses.

The effect of temperature on the detection of chemical cues is another interesting factor to consider. Throughout the literature, room temperatures where chemical-cue mediated behaviours by salamanders were analyzed varied from 15°C in some studies up to 25°C in others (David & Jaeger 1981; Cupp 1994; Sullivan et al. 2000; Maerz et al. 2001; Placyk & Graves 2001, 2002; Madison et al. 2002; Martin et al. 2005; Schubert et al. 2006; Dantzer & Jaeger 2007a,b). The preferred temperature of *Plethodon cinereus* lies between 16°C and 21°C; humidity also plays a large role in the selection of a microhabitat (Feder & Pough 1975). While most temperatures found in published studies fit within that range, it is possible that behavioural reactions to cues differed slightly based on temperature. The effect temperature may have on chemical cues is two-fold. First, it may affect the metabolic rate of the organism. Ectotherms like salamanders are very sensitive to temperature changes in their environment; in plethodontid salamanders metabolism increases as temperature rises up from 5°C, reaching an optimum between 15°C and 20°C (Whitford & Hutchinson 1967). An increased metabolic rate would
likely result in increased hunger, possibly increasing salamander motivation to explore prey cues at higher temperatures. Second, it may affect the physical properties of the cue itself. Both high humidity and high temperature can cause increases in diffusion of molecules so that a cue would fade much sooner in a warmer environment (Regnier & Goodwin 1977; Alberts 1992). Salamanders were tested at different temperatures throughout the study to determine whether temperature had any effect on their reaction to prey cues.

This series of experiments was designed to test a) whether salamanders would respond to chemical extracts of prey and b) whether responses differed to the presentation of soluble, volatile, or both prey extracts at the same time. The experiments differed in cue delivery as well as olfactory ability of the salamanders. The Series I experiments were preference/avoidance assays, where salamanders were tested on their ability to choose between substrates scented with controls and prey soluble cues. A choice for one side would indicate a preference for that side, and will be referred to as ‘side preference’ for the remainder of this Thesis. I hypothesized that if prey soluble cues were attractive to red-backed salamanders, then I would see an increase in nosetapping and a preference for the side containing the soluble prey cue. Temperature was varied across three different experiments, with the prediction that salamanders would show a greater prey side preference or amount of chemoinvestigation when held and tested at a higher temperature. Series II was conducted in a 15°C/12°C room where attraction to volatiles emitted by point sources was measured. The substrate remained consistent across the entire testing arena, where soluble prey cues were present across the entire substrate or a control substance was. In this experiment, salamanders were presented with a choice between two areas where sources of either control or prey volatile cues were present. If prey volatile cues were attractive to red-backed salamanders, I expected to observe a higher amount of either nosetapping, movement,
and/or time on the side of the volatile cue. Series III involved a temporary block of the olfactory abilities of salamanders. This experiment was conducted in a 22°C/20°C room with soluble prey cues only, and tested the ability of salamanders to respond when they had use of only the main olfactory system or the use of neither of the olfactory subsystems. I hypothesized that salamanders receiving impairments would be less successful at detecting prey soluble cues than their sham-treated counterparts. I predicted that they would display lower amounts of nosetapping and neglect to show a side preference when compared to conspecifics with intact olfactory systems.

**METHODOLOGY**

*Experimental Animals*

Salamanders were collected from Rockwood Conservation Area in Rockwood, Ontario in June 2009 and 2010, and October 2010. Appropriate permits for animal collection were obtained from Ontario's Ministry of Natural Resources (Permits 1056815 and 1051564) and the Grand River Conservation Authority (no permit number provided). Salamanders were transported from the conservation area within a chilled cooler in plastic bags containing moistened paper towels. Upon arrival at the holding room in the Hagen Aqualab, University of Guelph, salamanders were individually transferred into pre-prepared ~ 1 L, 16.5 x 11.5 x 5.8 cm rectangular plastic boxes. The boxes were lined with a moistened paper towel on the bottom and contained a crumpled moistened paper towel for environmental enrichment. Salamanders were fed two crickets or to satiation (usually no more than four crickets) once weekly. Their bedding was changed once a week. They were generally maintained at a 12L:12D photoperiod where light turned on at 7 AM
and the temperature was maintained at 15ºC in the day and 12ºC at night. Dusk and dawn simulations were achieved by an automated, gradual change in the sets of lights turning on or off over a period of one hour. In specified experiments, salamanders were moved to a warmer room at 22ºC in the day and 20ºC at night. All methods were approved under University of Guelph animal utilization protocol 08R128.

Cue Preparation

The soluble prey cue was prepared by placing 20 small crickets in a 250 mL glass beaker which contained cricket food (ground Meow Mix cat food) and a small wet sponge surrounded by a plastic membrane to provide drinking water for crickets. The beaker was covered by a secured paper tissue and crickets remained in the beaker for 5 days, after which the crickets and any remaining food were taken out. A 30 mL distilled water rinse of the beaker was taken at this time. The control for soluble cues consisted of a distilled water rinse of an empty beaker that stood covered by a tissue paper in a room devoid of possible chemical cues from crickets for 5 days. Soluble cue was prepared fresh before each evening of testing.

The volatile prey cue was prepared in a similar manner to the soluble cue, except that there were two 9 x 4.5 cm pieces of crumpled tissue paper placed with the crickets in the beaker for the 5-day duration. The tissue was removed from the beaker and placed into a 20 mL syringe prior to commencement of the assay. The control consisted of an equivalent amount of tissue paper left in a clean beaker for 5 days. Volatile cues were delivered by hand using 20-ml syringes in the first series of experiments and 10-ml cryogenic tubes equipped with porous caps in the second series.
Experimental Series

Three slightly different bioassays, based on those described in Madison et al. (1999a,b), Sullivan et al. (2002), and Dantzer & Jaeger (2007a,b) were used to investigate how salamanders reacted to different cues. All followed a similar pattern in that a choice between a scented and a non-scented side was presented in the treatment trials. Testing was conducted in the dark phase under red light illumination between 18:00 h and 23:00 h in the Hagen Aqualab, University of Guelph. Salamanders were deprived of food for four weeks prior to testing to ensure sufficient motivation to explore chemical cues (Maerz et al. 2001; Placyk & Graves 2001, 2002). Air temperature was also varied as a means to produce differences in feeding motivation between experiments. Room temperature during the food deprivation period was the same as the temperature used during testing. Observations were made using a scan methodology modified from Schubert et al. (2008); four salamanders were tested concurrently, and one salamander of the four was watched for 15 seconds of each minute for a total of 60 minutes. At the end of each 15-second period, the location of the salamander within a quartered grid was recorded (Figure 1), as well as the presence or absence of nosetapping behaviour (0 = no nosetapping, 1 = nosetapping). These two observations allowed three variables to be quantified: the sum of all movements from one quadrant to another (number of location changes), the sum of observations recorded on the side of scent deposition or a randomly selected control side (side preference), and the sum of scans where nosetapping was recorded (nosetapping index).

Sixty animals naïve to the testing procedure were used in each experiment, except for the Series III experiment where 90 salamanders were used. Salamanders not exhibiting normal foraging behaviours were re-tested, and if they failed a trial more than twice they were removed from the experiment. Failure of the test was defined as salamanders that did not change sides at
all, made less than two movements across quadrant boundaries throughout the 60 minute trial, or
spent 15 or more consecutive minutes hiding under the substrate. Only salamanders that
performed the test successfully for each treatment were included in the statistical analysis. This
applied to all testing procedures.
**Figure 1.** Schematic drawing of the testing boxes for all behavioural assays. Testing arenas measured 16.5 x 11.5 x 5.8 cm. Filter paper was cut to 11 x 8 cm dimensions. The first piece of filter paper was placed over the side containing quadrants 1 and 3 and the second over quadrants 2 and 4. Prey soluble cues were then randomly applied to either side ‘1,3’ or side ‘2,4’ for SC and SV treatments. The controls were applied to the other side or both sides for CC and CV treatments. A small area in the middle of the arena was left uncovered by filter paper, so that soluble cues would not run onto the other side. Numbers indicate identity of quadrants used for tracking of location but were not present in the arena during testing.
Series I – Salamander Preference for Soluble Prey Cues

This series consisted of three experiments that differed in the temperature of the testing room and the experience salamanders had with the assay (i.e., whether they had been undergone the assay previously or not). The assay was designed to examine the preference behaviours exhibited by red-backed salamanders in response to prey chemical cues by presenting a choice in substrate conditions. Treatments were assigned in a 2x2 factorial design, with soluble and volatile cues being introduced along with control treatments. For the remainder of this thesis, the treatments will be referred to as follows: CC= control soluble, control volatile; CV= control soluble, prey volatile; SC= prey soluble, control volatile; and SV= prey soluble, prey volatile. Either a control of distilled water was applied on both sides of the testing box, or was applied on one side and the soluble prey cues on the other. An increase in chemoinvestigatory behaviours or a side preference would indicate a preference of the salamanders for the prey cues. Volatile cues were also introduced into the testing box but were not side specific. Thus, the focus of the preference/avoidance assay in Series I was on the soluble cues present on the substrate. If results were to agree with the Volatility Theory of olfaction, salamanders responding to soluble cues would rely on their vomeronasal system and nosetapping responses would be of particular interest.

Filter papers loaded with control or prey cues were placed on top of the paper towel that lined the bottom of the home box so that the salamanders would not experience a novel environment. This was done to minimize escape or marking behaviour (Tristam 1977; Placyk & Graves 2001). Filter paper was cut to 11 x 8 cm dimensions, so that two pieces could fit on the bottom of a testing arena without touching in the middle. One piece of filter paper covered quadrants 1 and 3, and the other piece covered quadrants 2 and 4 (Figure 1). This was adopted
from the methodology of Madison et al. (1999a,b) so that soluble cues would not run between filter papers.

**Experiment One**

Testing took place from mid-February to mid-March of 2010 and the testing room temperature ranged daily from 20-22°C. Testing began at 18:00 h after lighting had faded, mimicking the natural decline. Red-light illumination was used to allow the observer to view the salamanders. Vinyl gloves were worn during all handling of substances containing both control and prey soluble and volatiles cues. One mL of prey soluble cues or a control was applied via pipette to each filter paper according to treatment prior to insertion into the home box. Salamanders were lifted from their box, and the filter paper was pressed down to the bottom of the testing arena. Once the filter paper was in place the salamander was placed in the middle of the testing arena, along the dividing line between filter papers to avoid a biased placement. After the salamander was placed, either control or prey volatile cues were introduced via a 20 mL syringe. In this experiment, paper towel was used (tissue paper was used for the following experiments, see below) in the making of prey volatile cues. This may be a slightly less porous material than tissue paper. The syringe was stuffed near the tip with cut-up pieces of paper towel. Ten mL of air was forced through the syringe into the testing arena with its lid slightly ajar. Following volatile cue introduction into each arena, the box lid was closed and observations commenced according to the scan methodology previously described.

A repeated measures design was used in which each salamander was presented with each treatment in the 2x2 factorial design (CC, CV, SC and SV) in a counterbalanced order. Order of presentation of the first treatment was randomized between salamanders, with the constraint that
each treatment had the same number of salamanders on the first trial. Each salamander was tested once per night until each treatment was tested over a period of one week. The beginning of the food deprivation period was staggered to allow all salamanders to be at the same level of food deprivation when their week of testing began.

Experiment Two

Previous work in the laboratory on the congenere salamander *Plethodon shermani* (P. Kelly, A. Telfer and F. Laberge, Chapter 3 of this Thesis) had tested volatile cues using tissue paper rather than paper towel. To remain consistent with these testing procedures, a switch was made to tissue paper for the remainder of experiments. Experiment one was repeated using the tissue paper in May 2010. During April 2010, when salamanders were in their food deprivation period, the temperature was consistent at 20-22°C; however during testing temperatures ranged from 21-26°C because there was no temperature regulation in the room and temperature in the whole building rose greatly in May. No other changes were made to the experimental procedures. Sixty animals were used in this experiment, but all had previously been used in a predator cue detection assay, which was structured in the same way as the prey detection assays.

Experiment Three

For this experiment, the temperature of this room was regulated at 15°C during the day and 12°C at night, reminiscent of early spring temperatures. The 12L:12D photoperiod was maintained, only light was now ended abruptly at 19:00h. All testing commenced at this point. Methodology did not differ from experiment two except for the inclusion of additional observations during the testing period. Observations of whether an animal was under the substrate (defined by head and at least half of SVL under) or exploring the sides (defined by two
or more limbs touching plastic walls of arena) were recorded upon each viewing to produce a more accurate record of these behaviours. Sixty salamanders naïve to the assay were used for this experiment.

**Series II – Salamander Attraction to Volatile Prey Cues**

This experiment presented a choice between a point source of volatile prey cue and control. The methodology for cue presentation is based on Dantzer & Jaeger (2007a,b), who developed a tube method for presenting conspecific salamander odours without allowing contact via nosetapping. To accomplish this, 10 mL cryotubes had the center portion of the cap cut and replaced with plastic window screen mesh. This provided the ability to change the volatiles emerging from the tube without allowing salamander contact. Enough tubes were prepared for 4 hours of testing (16 salamanders per evening) and tubes were used for either control or prey volatiles only, to avoid confounding effects of possibly lingering cues. As soluble cues were not the focus of this experiment, but were still used as a factor, they were present across the entire substrate or not at all. Filter paper substrates measured 22 x 8 cm, and 2 mL of control or prey soluble cue was distributed over the substrate before a trial.

Treatments remained the same as in experiment one (CC, CV, SC, SV), however the choice in sides was now dependant on differential presentation of prey volatile cues within a tube. Filter paper was placed first into the testing arena, followed by placement of a tube on each side of the arena, and lastly the salamander was placed in the middle of the arena. Observations were recorded as in experiment one, but the observation ‘touch’ was added (similar to Dantzer & Jaeger 2007a,b), which was defined as a salamander touching a tube with any part of its body with one exception. If the salamander had moved away from the tube but part of its tail was still
touching, this was not recorded as a touch. A binary observation method was again employed, where 0 = no touch to the tube and 1 = touch to the tube. Testing took place in July 2010, between 19:00 and 23:00h in a room at 15°C during the day and 12°C at night. Sixty salamanders were used for this experiment; all were naïve to the testing procedures.

**Series III – Manipulation of Olfactory Ability**

In this experiment, the olfactory abilities of red-backed salamanders were manipulated in an attempt to tease apart the contribution of the vomeronasal and main olfactory systems in prey detection. Vetbond tissue adhesive (3M, London, Ontario, Canada) was applied according to one of three treatments: sham block (SHAM), vomeronasal block (VNX), or complete olfactory cavities block (EN+VNX). Salamanders were anaesthetized prior to block application in 1% MS-222 bath. Salamanders were judged to be fully anaesthetized when their turning reflex was gone. The sham block consisted of a drop of glue applied via a needle directly above, but not contacting, the nares. For the VNX, glue was applied to both nasolabial grooves, with caution to avoid plugging the nares as well. The EN+VNX block was applied to the nares directly to block both main olfactory and vomeronasal input. A main olfactory-only block was not feasible with the current method as solutions travelling in the nasolabial grooves need to enter the lateral part of the nares to reach the vomeronasal epithelium. To ensure the treatment was effective, the application to the nares in the EN+VNX block covered both the nares and nasolabial grooves. Salamanders were given one night to recover from glue application and then were exposed to a similar preference/avoidance assay as in Series I. However, they were only exposed to the control/control and control/soluble treatments for this assay (CC and SC treatments, respectively). If these soluble cues were only detected by the vomeronasal system, then both the VNX and EN+VNX blocks should experience similar deficits in behaviour. If both subsystems
are used to detect prey cues, the EN+VNX blocked group should experience a greater deficit than the VNX only blocked group.

The scan method of observations was again used for this assay. The same failure standards were applied, as salamanders not under duress would be expected to maintain a minimum level of movement when introduced to a novel area. Salamanders hiding under the substrate more than 15 minutes or moving less than twice over a 60 minute period would not meet these minimum requirements of normal behaviour. Salamanders were inspected daily to ensure the block was attached and, if it had fallen off, it was re-applied and the salamander was tested the following day. A total of 90 animals were used for this assay, divided randomly into three groups differing in blocks. Twenty-four of the total 90 animals were naïve to the behavioural assay and these animals were divided evenly into the three block groups. The remainder had previously been used in the Series II behavioural assay. The temperature was 22°C during the day and 20°C at night.

Statistical Analysis

GraphPad Prism software (version 5.01) was used to conduct D’Agostino and Pearson omnibus normality tests on all data. Most data sets failed to meet the assumptions of normality, and square root or logarithmic transformations could not normalize the data. Thus, statistical analysis was performed using a Monte Carlo randomization test, which is a more powerful alternative to non-parametric tests using ranks, especially when data distribution is skewed (Manly 1991; Adams & Anthony 1996; Mewhort 2005). The randomization test uses the original data set to create shuffled data sets according to parameters set by the user, described below for my analyses (Crowley 1992). After numerous repetitions it creates a distribution of values based
on the original data set; the significance can be determined by how many test statistics from that
distribution are more extreme than the test statistic of the original data set. The p value is
calculated by dividing the number of more extreme values by the number of repetitions
performed.

A repeated measures ANOVA was calculated in Microsoft Excel for each variable in
each data set based on the design of the Series I and II experiments. The Microsoft Excel add-on
PopTools (Version 3.2.3, available online) was used to perform randomization test procedures.
The resample option was chosen, and data was shuffled without replacement and within rows.
The ‘within rows’ option was chosen as this shuffled the data according to individual
salamanders, so the repeated measures design was maintained. The repeated measures ANOVA
calculations were replicated so F statistics of shuffled data could be obtained. After this, the
Simulation Tools→ Monte Carlo analysis option was chosen and the parameters were set to run
the analysis. The F statistic calculated for the shuffled data was selected as the dependant range
and the F statistic for the original data set was selected as the test value. Number of replicates
was chosen as 10,000 in this design, which is more than ample to test hypotheses at a 5% level of
significance (Manly 1991). The output was produced in an area of blank cells and the test
criterion was set to report values that were greater than or equal to the test value. After the output
was delivered, the number of values greater than the original F statistic was divided by the
number of replicates. If a p-value showed that statistically significant differences existed overall,
multiple comparisons were conducted using t-tests. The randomized t-tests were calculated
between each of the treatments. While the number of comparisons usually performed is less than
or equal to the degrees of freedom of the sample, I chose to perform comparisons between all
groups due to the nature of our factorial treatment design. All treatments could differ from each
other, and even a small difference between treatments could speak to the relative roles of the olfactory subsystems. The randomization procedure using the t-test statistic was an exact randomization test. The number of replicates was tailored to be the maximum number of permutations possible given the number of individual salamanders for that experiment; this was calculated by squaring the number of individuals. A Bonferroni correction for multiple comparisons was not employed, as suggested by Cabin & Mitchell (2000) and Nakagawa (2004).

Statistical analysis for Series III changed slightly because the introduction of different blocks with repeated measures only existing within blocks changed the parameters of analysis. D’Agostino and Pearson omnibus normality tests were first conducted on all data using GraphPad Prism software (version 5.01). An overall one-way ANOVA of side changes was performed on data from the first time a salamander was tested with each treatment because the trial failure rate of salamanders receiving a EN+VNX block was extremely high (only 2/30 met requirements for a successful trial). Reshuffling and a Monte Carlo randomization test were performed on the data and compared to the F statistic from the one-way ANOVA. Further statistical analysis was performed on data from salamanders in the SHAM and VNX groups, and only salamanders that successfully completed the assay were included in this analysis. As there were only two groups, paired t-tests within groups were used to determine if differences between CC and SC treatments existed within in block group. Exact randomization analyses were completed with the t statistic as the test statistic. Comparisons between the VNX and SHAM block were not performed.
RESULTS

Series I – Salamander Preference for Soluble Prey Cues

Experiment One

A total of 48 salamanders were included in the analysis for this experiment. Salamanders exhibited a side preference when soluble prey cues were present ($F_{(3,47)} = 3.11, p = 0.030$; Figure 2a). More time was spent on the side of the soluble prey cue (SC and SV) than was exhibited by salamanders choosing a randomly selected side in the CC, but not CV, treatments ($CC \times SC \quad t_{(47)} = 3.10, p = 0.0003; \quad CC \times SV \quad t_{(47)} = 2.80, p = 0.007; \quad CV \times SC \quad t_{(47)} = 1.02, p = 0.275; \quad CV \times SV \quad t_{(47)} = 0.912, p = 0.369$). There was no difference in side preference between the soluble prey cue treatments ($SC \times SV \quad t_{(47)} = 0.145, p = 0.880$). Salamanders also increased their nosetapping in the presence of both soluble and volatile prey cues ($F_{(3,47)} = 3.43, p = 0.019$; Figure 2c), where they increased nosetapping in the SV, but not SC, treatments as compared to the CC and CV treatments ($CC \times SV \quad t_{(47)} = 2.26, p = 0.038; \quad CV \times SV \quad t_{(47)} = 2.85, p = 0.007; \quad CC \times SC \quad t_{(47)} = 1.76, p = 0.091; \quad CV \times SC \quad t_{(47)} = 1.69, p = 0.100$). There was no difference in nosetapping between the soluble prey cue treatments ($SC \times SV \quad t_{(47)} = 0.965, p = 0.353$). Salamanders showed similar levels of movement across all treatments ($F_{(3,47)} = 0.838, p = 0.478$; Figure 2b). The Monte Carlo simulation for multiple comparison t-tests was computed based on 2304 replications, as this is the maximum number of comparisons possible between the two groups.

Experiment Two

A total of 42 salamanders were included in the data analysis for this experiment. Salamanders did not show a response to the cues presented, as no differences between treatments
were found for movement, side preference, or nosetapping (Side changes $F_{(3,41)} = 2.47, p = 0.063$; Side preference $F_{(3,41)} = 0.758, p = 0.520$; Nosetaps $F_{(3,41)} = 0.332, p = 0.817$; Figure 3).

Experiment Three

A total of 51 salamanders were included in the data analysis for this experiment. Salamanders increased their nosetapping in the presence of prey cues ($F_{(3,50)} = 4.06, p = 0.006$; Figure 4c). Salamanders receiving treatments with a soluble cue nosetapped more than those receiving a CC treatment ($CC \times SC \ t_{(50)} = 2.96, p = 0.002$; $CC \times SV \ t_{(50)} = 2.93, p = 0.005$), but there were no differences between the control and volatile only treatment nor between the two soluble cue treatments ($CC \times CV \ t_{(50)} = 1.87, p = 0.067$; $SC \times SV \ t_{(50)} = 1.06, p = 0.336$). Amount of nosetapping by salamanders exposed to the CV treatment did not differ from when they received either soluble treatment ($CV \times SC \ t_{(50)} = 1.57, p = 0.138$; $CV \times SV \ t_{(50)} = 1.13, p = 0.310$). Salamanders did not show a side preference for the scented side nor did they increase movement across treatments (Side changes $F_{(3,50)} = 0.787, p = 0.508$; Side preference $F_{(3,50)} = 0.156, p = 0.929$; Figures 4a and b). The Monte Carlo simulation for multiple comparison t-tests was computed based on 2601 replications.

Series II – Salamander Attraction to Volatile Prey Cues

A total of 51 salamanders were included in the data analysis for this experiment. Salamanders did not show a response to the cues presented as measured by their movement, side preference, amount of nosetapping, or amount of times touching the tubes containing volatile cues (Side changes $F_{(3,50)} = 0.605, p = 0.615$; Side preference $F_{(3,50)} = 1.29, p = 0.282$; Nosetaps $F_{(3,50)} = 0.712, p = 0.548$; Touch to tube $F_{(3,50)} = 1.11, p = 0.348$; Figure 5).
Series III – Manipulation of Olfactory Ability

Ninety salamanders were used to compare movement between the types of olfactory blocks and an overall difference was found ($F_{(2,89)} = 26.64, p < 0.0001$). Comparison of movement levels between block type showed very significant differences, especially when comparing the EN+VNX block to the VNX and SHAM (SHAM x VNX $t_{(58)} = 2.88, p = 0.006$; VNX x EN+VNX $t_{(57)} = 5.11, p < 0.0001$; SHAM x EN+VNX $t_{(58)} = 6.38, p < 0.0001$). These extreme values, coupled with the fact that only 2 out of 30 salamanders passed the requirements for normal foraging called for the exclusion of the EN+VNX block from further analysis.

Twenty-four sham animals and 20 VNX animals passed the requirements for inclusion in statistical analysis, giving maximum replicate values of 576 and 400, respectively. Salamanders did not behave differently when presented with CC or SC treatments regardless of block applied (SHAM: Side changes $t_{(23)} = 1.34, p = 0.196$; Side preference $t_{(23)} = 1.16, p = 0.259$; Nosetaps $t_{(23)} = 0.271, p = 0.833$; VNX: Side changes $t_{(19)} = 1.48, p = 0.168$; Side preference $t_{(19)} = 0.130, p = 0.925$; Nosetaps $t_{(19)} = 0.246, p = 0.850$; Figure 6).
Figure 2. Responses of naïve red-backed salamanders to control and prey cues in a preference/avoidance assay in a 20-22°C room for A) number of scans where salamanders were on the side of prey cues or a randomly chosen side for soluble control treatments, B) number of movements between quadrants and C) number of scans where salamanders nosetapped the substratum. All values reported are mean ± SEM (n = 48). The p value of the randomization test is shown above each panel and different letters denote significant differences detected by multiple comparison t-tests that followed an overall significant effect. CC: control soluble, control volatile; CV: control soluble, prey volatile; SC: prey soluble, control volatile; SV: prey soluble, prey volatile.
Figure 3. Responses of experienced red-backed salamanders to control and prey cues in a preference/avoidance assay in a 20-22°C room for A) number of scans where salamanders were on the side of prey cues or a randomly chosen side for soluble control treatments, B) number of movements between quadrants and C) number of scans where salamanders nosetapped the substratum. All values reported are mean ± SEM (n = 42). The p value of the randomization test is shown above each panel; no significant differences were detected in this experiment. CC: control soluble, control volatile; CV: control soluble, prey volatile; SC: prey soluble, control volatile; SV: prey soluble, prey volatile.
**Figure 4.** Responses of naïve red-backed salamanders to control and prey cues in a preference/avoidance assay in a 12-15°C room for A) number of scans where salamanders were on the side of prey cues or a randomly chosen side for soluble control treatments, B) number of movements between quadrants and C) number of scans where salamanders nosetapped the substratum. All values reported are mean ± SEM (n = 51). The p value of the randomization test is shown above each panel and different letters denote significant differences detected by multiple comparison t-tests that followed an overall significant effect. CC: control soluble, control volatile; CV: control soluble, prey volatile; SC: prey soluble, control volatile; SV: prey soluble, prey volatile.
Figure 5. Responses of naïve red-backed salamanders to control and prey cues in a volatile attraction assay in a 12-15°C room for A) number of scans where salamanders were on the side of prey cues or a randomly chosen side for soluble control treatments, B) number of movements between quadrants, C) number of scans where salamanders nosetapped the substratum and D) the number of scans where salamanders were observed touching the tube with their bodies or limbs. For D, number of scans observed is separated into touching of the control tube (white column) or the tube containing volatile cues (grey column). All values reported are mean ± SEM (n = 51). The p value of the randomization test is shown above each panel; no significant differences were detected in this experiment. CC: control soluble, control volatile; CV: control soluble, prey volatile; SC: prey soluble, control volatile; SV: prey soluble, prey volatile.
Figure 6. Responses of red-backed salamanders receiving a sham or vomeronasal olfactory block to control and soluble prey cues in a 20-22°C room for A) number of scans where salamanders were on the side of prey cues or a randomly chosen side for soluble control treatments, B) number of movements between quadrants and C) number of scans where salamanders nosetapped to the substratum. All values reported are mean ± SEM (SHAM n = 24; VNX n = 20; naïve n = 14; experienced n = 30). The p value of the randomization test is shown above each panel; no significant differences were detected. SHAM: sham glue block applied to snout; VNX: glue block applied to nasolabial grooves; CC: control soluble, control volatile; CV: control soluble, prey volatile; SC: prey soluble, control volatile; SV: prey soluble, prey volatile.
DISCUSSION

This study is the first to show an increase of chemoinvestigatory behaviours in red-backed salamanders to the presence of prey chemical cues only (i.e., without the immediate presence of prey). It provides further evidence that salamanders will nosetap to gain information about prey location, and shows for the first time that these animals will exhibit a side preference for soluble prey cues. Differences in testing procedures that may have had an effect on the results are discussed, especially in respect to experience and temperature.

The intent of the behavioural assays performed in this chapter was to observe how salamanders responded to soluble and volatile prey cues. If their responses reflected differences in usage of the olfactory subsystems, i.e. greater nosetapping when soluble cues were present versus a lesser emphasis on nosetapping but greater movement or side preference for side of volatile cues, then inferences could have been made regarding whether these results aligned more closely with the Volatility or Learning theories of olfaction. The fact that no differences were detected in the Series II assay with point sources of volatile prey cues, as well as the lack of differences across treatments in the olfactory block experiment, hindered my ability to make any meaningful conclusions on that topic. Without data to show differences in how salamanders approached volatile cues or how olfactory deficits impair their prey-seeking behaviour, I cannot attribute the salamander response to main olfactory or vomeronasal function. While it appears soluble cues increase the nosetapping response of salamanders, this information alone is not enough to confidently attribute it solely to vomeronasal function.
Soluble Cues as an Indicator of Prey Presence

Increased nosetapping in preference/avoidance assays appears mainly due to the presence of soluble prey cues in the testing arena. The role of volatile cues cannot be ruled out, as the presence of both types of cues was the only treatment different from the control in experiment one. In experiment three, however, the control was different from both treatments involving soluble cues regardless of the presence of a volatile cue. In the warmer of the two testing rooms (20-22°C vs. 12-15°C), salamanders also exhibited a side preference for the soluble cue when compared to a randomly chosen side in the CC treatment. This response accounts for any salamanders that would show a biased side preference, because all animals were tested with each treatment and side of scent was randomized.

These findings suggest that prey detection involves the vomeronasal system in the red-backed salamander. Garter snakes also search out prey using tongue flicking to deliver cues to their vomeronasal organ; the vomeronasal system is believed to mediate the continuation of tongue flicking along a prey trail (Kubie & Halpern 1975; Halpern et al. 1997). However, without a functional main olfactory system, garter snakes will not initiate a tongue-flick response when presented with prey cues (Zuri & Halpern 2003). Additionally, virgin female rats reduce their inhibition to foster pups using cues detected by both the main olfactory and vomeronasal systems (Fleming et al. 1979). However, the higher response to the SV treatment in Series I, Experiment One may be indicative of initial volatile detection motivating salamanders to increase nosetapping, as is the case in garter snake tongue flicking (Zuri & Halpern 2003). Though Placyk & Graves (2002) showed that vomeronasal-impaired salamanders have a greater latency to prey capture and a lower number of mobile prey eaten, salamanders were still able to
attack and consume mobile prey. This suggests that the vomeronasal system alone is not essential for prey detection.

_Volatile Cues and the Effect of Temperature_

The response of salamanders to volatile cues is not entirely clear from the results of this study. Naïve salamanders in warmer temperatures only showed a difference between the two control soluble treatments (CC, CV) and the SV treatment; in this warmer temperature it appears that the addition of both soluble and volatile cues worked synergistically to increase nosetapping. Naïve salamanders tested in the warmer conditions also showed a side preference for the soluble cue, but this was not observed in the other experiments. In colder temperatures, the nosetapping response was different, where both treatments with soluble prey cues were different from the CC treatment. The differences in effect of volatility versus solubility may have to do with the temperature in which salamanders were held and tested in the laboratory. There are a few reasons why this could have occurred, relating to the possible effects of temperature on metabolism, on odour sensation and perception, or on odourant properties.

Metabolic rates of all organisms are governed in part by external temperature and the body mass of the organism (Gillooly et al. 2001; Clarke 2004). The effect of varying temperature can cause large increases in metabolic rate in ectotherms, even for small organisms like the red-backed salamander. In _Ambystoma maculatum_ and _Desmognathus quadramaculatus_ (a plethodontid), metabolism increased with temperature, though at a greater rate in the lunged ambystomid than the lungless plethodontid. The ceiling was believed to exist between 15°C and 20°C, as further increases in temperature did not appear to cause increases in metabolic rate (Whitford & Hutchinson 1967). Though the different temperatures red-backed salamanders were
exposed to in this study both fall within the optimal range, the length of time the salamanders were held at these temperatures during their food deprivation could have produced different hunger levels. Early tests with the Eastern newt, *Triturus viridescens*, showed that those receiving a longer period of food deprivation put greater effort into food-searching after being exposed to a solution containing soluble odours from their prey (Goldstein 1960). Perhaps this is why a side preference was seen only when salamanders were held at 20-22°C versus 12-15°C.

Another possibility is that temperature affected responsiveness to the odours themselves. Male moths are less responsive to female sex pheromones when raised in lower temperatures (25°C versus 10°C: Dumont and McNeil 1992); when different species of moth were exposed to odours at different temperatures (20°C versus 26°C) both showed increased responsiveness to odours, but less specificity at higher temperatures (Linn et al. 1988). Fruit flies also show less sensitivity to odourants at higher temperatures (Riveron et al. 2009). Discrimination between similar odours decreased when turtle olfactory epithelium was perfused with Ringer’s solution and odours at different temperatures, but not until temperatures reached 40°C (Hanada et al. 1994); as well, turtles decreased their ability to cluster similar odours when temperature was increased from 25°C to 37°C (Kashiwayanagi & Nagasawa 1995). This may be due to increased membrane fluidity of lipid layers in the cells of the olfactory system (Kashiwayanagi et al. 1997). These temperatures were higher than the maximum temperature red-backed salamanders were exposed to in this study, and salamanders were held within the temperature range of 5°C to 25°C where Hanada et al. (1994) and Kashiwayanagi & Kagasawa (1995) still saw great discriminatory ability. Though an organism may be less able to discriminate between odourants at a higher temperature, they appear to be more likely to respond to an odour. This may be
responsible for the increased responsiveness of salamanders to volatile cues at a higher
temperature.

Lastly, the difference in temperature may have caused a physical difference in the
properties of the chemical cues introduced. The dispersion of chemical cues in the terrestrial
environment is affected by many abiotic factors, such as wind, humidity, and temperature. Early
insect sex pheromone work used a rubber septum to deliver odourants to test organisms. To test
the longevity of the cues their half-lives were determined after the pheromone was released in
chambers of different temperatures. Those emitted at the lowest temperature (15°C) had a
substantially longer half-life than those at higher temperatures (35°C). Even between 15°C and
20°C, the half-lives of the pheromones declined by a factor of 1.6 to 2 times (McDonough et al.
1989). These temperatures match those used in the current experiment and could explain the
effect of volatile cues present in warmer temperatures. This increased diffusion with higher
temperatures would be responsible for shorter half-lives and shorter longevity of the cue.
Conversely, it would result in higher concentrations and possibly easier detection of cues
present. It is possible that more of the volatiles present in the warmer room would have diffused
throughout the testing arena than in the colder room over the hour the experiment was conducted.
In colder temperatures, the volatile cue may not have spread throughout the arena enough for it
to be recognized and deemed important for the salamander to respond to.

The Series II experiment should be repeated in a warmer setting. The interpretation of
which olfactory system is being used (according to Volatility Theory) requires data which speaks
to the effect of volatile cue presentation. The increases in nosetapping found in response to a
combination of soluble and volatile cues in Series I, Experiment One (Figure 2) suggest that both
systems may be used. Perhaps an initial detection of prey cues by the main olfactory system
motivates a salamander to nosetap so more information on the source of the cue can be
determined, though this is just speculation at this point. Further testing with point sources of
volatiles will hopefully lead to clarification of the roles of the olfactory subsystems.

Effects of Experience

Salamanders differed between experiments in their experience with the testing protocols. One theme recurred between all series and experiments, where naïve salamanders showed
differences between soluble treatments but experienced salamanders never did. Many studies of
olfaction using red-backed salamanders use subjects for one experiment only (i.e. Madison et al.
1999b), though repeated measures tests are common (David & Jaeger 1981; Graves 1994; Placyk &
Graves 2001; Dantzer & Jaeger 2007a,b). Placyk & Graves (2002) re-used salamanders that
had their nasolabial grooves cauterized, but the testing procedures differed greatly between
experiments.

Learning via chemical cues is common in aquatic systems. Chivers et al. (1995) showed
that sticklebacks will avoid odours of pike if first paired with alarm cue. This has also been
shown with the newt when smallmouth bass odour was paired with their alarm cue (Woody &
Mathis 1998). In terrestrial systems, amphibians are also capable of sophisticated learning. Marbled frogs were able to learn to avoid preying upon cane toads, an unpalatable introduced
prey item (Greenlees et al. 2010). Tiger salamanders will avoid a conditioned stimulus, be it a
shock (Ray 1970) or an odour (Dorries et al. 1997b) and the Ozark zigzag salamander, P.
angusticlavius, displays spatial recognition of landmarks and their relation to food sources
(Crane & Mathis 2011). P. cinereus exposed to novel prey as neonates showed increasing
foraging efficiency upon further exposures to the same prey as yearlings (Gibbons et al. 2005),
and those with experience preying upon a novel food type in the laboratory consumed more prey during observations than those without experience (David & Jaeger 1981). Thus, it is possible that the salamanders being exposed to consecutive rounds of experiments were no longer responding to the testing protocol. If the salamanders tested by Crane and Mathis (2011) were able to re-locate areas known to previously hold prey in comparison to those that did not, it follows that salamanders tested in this assay could recognize that prey items would not be present when again subjected to the experimental conditions. Though the prey cues used in the testing were attractive, none offered a reward in the form of prey after testing completion. As well, only two out of four exposures offered a soluble prey cue; three out of four offered either a soluble or volatile cue. The experiments where salamanders showed no response to prey cues may be affected by previous experience more than a lack of attraction to the cues presented. The consistency in tests lacking a response, as well as the literature describing learning in amphibians, suggest this may be so. Experiments where experienced salamanders were used should be repeated with naïve salamanders to test if this explanation for lack of response to prey cues is plausible.

Manipulation of Olfactory Ability

Previous studies have had success in manipulating the olfactory ability of salamanders using a cauterization method (Dawley & Bass 1989; Graves 1994; Placyk & Graves 2002). Graves (1994) utilized cold anaesthesia to prepare salamanders for surgery, and Placyk & Graves (2002) used 30% ethyl alcohol. Our use of 1% MS-222 (similar to Dawley & Bass 1989) allowed salamanders to be anaesthetized fairly rapidly, with most salamanders fully anaesthetized between 6 and 10 minutes post-immersion. Salamanders usually regained consciousness and recommenced movement after approximately 5 minutes post-glue application.
(personal observation). The glue block usually fell off naturally after two to three days during a pilot study. This method was chosen over cauterization as these non-invasive methods would allow salamanders to be returned to the wild unharmed. Unfortunately, they may not have been as effective as a side effect of the blocking agent.

Adding even a small amount of adhesive may have contributed a large amount of weight on the snout of the salamander. With cauterization, there would be no additional weight added. This increase in weight may have caused salamanders to perform differently than a normal salamander would have under the same conditions. Those salamanders receiving the EN+VNX block showed a noticeable difference in behaviour, with only 2 of 30 passing the standards for a trial as well as displaying increased buccal oscillations (personal observation). Buccal oscillations are the rhythmic dilation and contraction of the buccopharyngeal cavity in amphibians; they are thought to be primarily related to olfaction, but may also play a small role in respiration (reviewed in Barker Jørgensen 2000). This response was not noted in either the SHAM or VNX blocks. It is possible that salamanders increased their buccal oscillations in response to the complete olfactory deprivation achieved by the EN+VNX block, and that this level of deprivation was responsible for the extremely low movement levels of the salamanders. Salamanders receiving the EN+VNX block changed quadrants an average of 1.8 times in 60 minutes, plus or minus a standard error of the mean of 0.37. In the SHAM and VNX blocks, movement was slightly lower than that exhibited in other experiments, at 13.1 ± 1.7 and 9.0 ± 1.3 respectively. Once salamanders who did not meet the standards for inclusion in the data set were removed, the levels of movement displayed by the SHAM and VNX blocked salamanders were similar to those exhibited in the Series I and II experiments.
Further testing of salamanders with manipulated olfactory ability is required. A different method of olfactory blocking should be considered that allows salamanders to maintain normal activity levels. Cauterization could be attempted, or chemical cauterization such as that with zinc sulphate may be a possibility as well. Unfortunately, this chemical cauterization is selective to the main olfactory system, so an alternative for the vomeronasal system would be needed. Another option is to cut the olfactory or vomeronasal nerves to impair chemosensation. This surgery would be difficult in an animal as small as the red-backed salamander, but feasible. A permanent block would allow salamanders a longer time period to recover from any possible lingering effects of anaesthesia.

**Concluding Remarks**

Overall, this study provides evidence that salamanders can detect and respond to chemical cues left by prey items. Previous experience with an assay may deter salamanders from displaying preferences and chemoinvestigation of prey cues, but this requires further investigation. This study supports previous findings which suggest that salamanders use chemical cues during night-time foraging and that the vomeronasal system plays a primary role in prey detection. Further studies should focus on the roles of the two olfactory subsystems in mediating prey cue detection depending on prey type as well as cue type. Soluble cues may be more important for prey such as insect larvae or crickets, but volatile may be more helpful when searching for fruit flies or other winged prey items. Specifically, the attraction/repulsion assay focusing on volatile cues should be attempted with naïve salamanders or an alternate method of testing volatile cues should be developed. Without results pertaining to salamander reaction to a point source of volatile cues or a successful olfactory block experiment, I cannot make any inferences as to the role of the main olfactory system. Furthermore, without evidence suggesting
which olfactory system is being used or how they interact, no comments on the credibility of 
either the Volatility or Learning theories of olfaction can be made. With future work, as 
suggested above, more insight can be gained into the role of the main olfactory and vomeronasal 
systems in prey detection by red-backed salamanders.
CHAPTER 3

The Roles of the Olfactory Subsystems of Red-legged Salamanders in Prey Detection

ABSTRACT

Salamanders, like many vertebrates, possess both main olfactory and vomeronasal systems, however the respective roles of each subsystem in detecting chemical cues has not been established. Recent work in the red-legged salamander (*Plethodon shermani*) has shown a high amount of convergence of the olfactory systems at the level of the central nervous system (CNS). In agreement with the Learning Theory of olfaction, which posits learned associations between cues simultaneously detected by both olfactory subsystems, this suggests that the olfactory subsystems could play complementary roles. Immunocytochemistry against c-Fos was used as an indirect marker of neuronal activity to assess which brain regions were activated by olfactory input. Specifically, volatile and soluble prey cues were used for applications directed to the main olfactory and vomeronasal systems, respectively. The salamanders were also stimulated with both types of prey cues simultaneously in order to assess possible effects of CNS convergence. No differences in the number of labelled neurons were apparent between control and treatment animals. Possible reasons for the lack of differences are discussed.
INTRODUCTION

Olfaction plays a role in prey detection by plethodontid salamanders (David & Jaeger 1981; Placyk & Graves 2001, 2002; Chapter 2 of this thesis). Salamanders will use chemical cues emitted from live or dead prey to aid in active searching of prey items. They will also preferentially choose an area where prey cues are found and increase chemoinvestigation of that area. Placyk & Graves (2002) implicated that the vomeronasal system was used in prey detection after cauterizing the nasolabial grooves of *Plethodon cinereus*. Salamanders showed reductions in their foraging ability, particularly in their latency to attack, number of attacks, and number of prey caught. While this method helped to gain insight into which olfactory subsystem was being used, there is still much to be learned. There was no attempt of impairment of the main olfactory system, which may play a role as well. In garter snakes, the vomeronasal system plays an important, but not exclusive, role in recognizing and following prey scent trails via tongue flicking. The main olfactory system is also used; main olfactory lesions diminished the increased tongue-flick rate usually present when snakes are exposed to airborne prey odourants. Without a functional vomeronasal system, snakes lack discrimination between odours of biological significance and will not follow a trail of earthworm extract odour (Kubie & Halpern 1979; Halpern et al. 1997; Zuri & Halpern 2003). Rodents also show complementary use of the two olfactory subsystems for many purposes (Fleming et al. 1979; Powers et al. 1979; O’Connell & Meredith 1984; Pankevich et al. 2004; Keller et al. 2009; Martínez-García et al. 2009). It is also possible that both subsystems are involved in prey detection in plethodontid salamanders.

Evidence of an interdependence of the two olfactory subsystems has long existed, but is only recently being thoroughly considered. The Equivalency Theory of vomeronasal function, which stated that vomeronasal system function was equivalent to pheromone detection has
largely fallen out of favour (Trinh & Storm 2003; Baxi et al. 2006; Hashiguchi & Nishida 2006). The vomeronasal system has been shown to be important in actions not involving pheromones, like prey detection (Halpern & Kubie 1983). As well, the MOS has been shown to detect pheromones (Dorries et al. 1997a; Swann et al. 2001; Xu et al. 2005). The Volatility Theory introduced by Wysocki et al. (1980) suggested that the main olfactory system was for detection of volatile odourants and the vomeronasal system for non-volatiles. This theory was supported in numerous studies (O’Connell & Meredith 1984; Wysocki et al. 1985; Li et al. 2005). However, evidence arose that both systems can detect both soluble and volatile cues (Meredith 1991; Trinh & Storm 2003; Xu et al. 2005). Also alluded to in the literature since the mid-80’s (Beauchamp et al. 1985; Meredith 1986), the Learning Theory of olfaction has attracted interest recently (Moncho-Bogani et al. 2002; Kelliher 2007; Martínez-García et al. 2009; Martínez-Marcos 2009). It suggests that stimulation of the vomeronasal system is required for initial learning of odourants detected concomitantly by the main olfactory system, but after this initial learning event the odourant can be detected and elicit an appropriate response when detected with the main olfactory system alone. This theory requires interdependence of the two subsystems, whereby a cue detected by either system must be able to transmit information about the same response. Thus, one would expect to see one brain regions could produce a behavioural or physiological response by stimulation of either one or the other olfactory subsystems after learning.

In the salamander *Plethodon shermani*, it has been shown that the main olfactory amygdala receives input from the main olfactory bulb and is characterized by a projection to the ventromedial hypothalamus, while the accessory olfactory amygdala (that is, vomeronasal olfactory) receives input from the accessory olfactory bulb and is characterized by projections to
the medial and lateral hypothalamus (Laberge & Roth 2005). The axonal terminal fields of both the main and accessory olfactory bulbs segregate in lateral and medial positions (respectively) in the caudal pole of the telencephalon (Laberge & Roth 2005). Projections of the main olfactory bulb only show a small amount of varicosities around the olfactohabenular tract, which courses in the caudal lateral pallium. However, the projections of the accessory olfactory bulb are clearly identified in the portion of the caudal amygdala. Within the caudal telencephalon, the accessory olfactory component lies just medial to the main olfactory component. Caudally it merges with the dorsal preoptic region, and rostrally it extends dorsal to the striato-pallidum (Laberge et al. 2008). Roth & Laberge (2011) recently showed that amphibians possess a high degree of convergence between the two subsystems at the level of the central nervous system. When the main olfactory nerve and vomeronasal nerve were stimulated separately, they both produced neuronal responses in the striato-pallial transition area, the lateral pallium and the amygdala. The closeness of these areas and possible convergence of the two olfactory systems has been suggested in other reptiles and amphibians as well (see Halpern & Martínez-Marcos 2003) and seems to implicate the co-operation of the two olfactory systems in odourant detection.

By analyzing the response of the central nervous system to prey cues, I hope to elucidate which brain regions are important in prey detection by salamanders. Depending on the pattern of neuronal activation, my findings could support either the Volatility or Learning theories of olfaction. Commonly used in rodents, immunocytochemistry against c-Fos has successfully been used to study neuronal activation in amphibians (Ubink et al. 1997; Cobellis et al. 1999; Tonosaki et al. 2004; Laberge et al. 2008). Experimental application of male courtship pheromones in *P. shermani* elevated c-Fos expression in the extended vomeronasal amygdala, the preoptic area, the ventromedial hypothalamus, and the raphe median. The first three regions
are associated with reproduction in vertebrates (Laberge et al. 2008). When considering prey cue delivery, both soluble and volatile cues need to be delivered, as both modes of cue delivery are likely in the environment. Little is known about how olfactory information courses through the central nervous system of amphibians, but previous work suggests that the main olfactory and vomeronasal bulbs, vomeronasal amygdala, hypothalamus, striato-pallial transition area, caudal telencephalon, lateral pallium and preoptic area should be examined for differences in neuronal activation. Previous work in the laboratory (P. Kelly and F. Laberge, unpublished) was not able to detect Fos-labelled nuclei in the brain of *P. cinereus*, so *P. shermani* was used for this study. Prior to the c-Fos study, behavioural assays were conducted in *P. shermani* to ensure a response to cricket prey cues by the salamanders.

The objective of this study was to analyze how the olfactory input from prey cues is transmitted through the brain. First, I tested salamanders with a behavioural assay designed to analyze their response to prey cues. I hypothesized that if soluble prey cues were attractive to red-legged salamanders, I would see an increase in nosetapping and a side preference for the side containing the soluble prey cue. Next, by analyzing the activity patterns in the brain after experimental application of control and prey cues, I expected to make inferences about the credibility of both the Volatility and Learning Theories. If the two olfactory subsystems are activated by cues differing in volatility, then I would expect to see a difference in neuronal activation in the brain with application of soluble versus volatile cues. Alternately, if both types of cues indiscriminately activate olfactory brain regions, this would provide evidence against the Volatility Theory. Under the assumption that volatile cues will stimulate the MOS while soluble cues will stimulate the VNS (first hypothesis), I also expect that convergence between the two olfactory subsystems in the CNS will result in either synergistic or inhibitory interactions in
some brain regions when both subsystems are stimulated. These findings could help support the Learning Theory, which requires associations between the subsystems. It is important to note that the two theories may not be mutually exclusive; in this case any differences in function should appear based on a factorial treatment plan including both soluble and volatile prey cues.

METHODOLOGY

Animals

A total of 60 \textit{P. shermani} collected at Highlands, North Carolina by the team of Lynne Houck in the early summer 2009 were used for these experiments. Appropriate permits for collection were provided by the North Carolina Wildlife Resources Commission (collection license number 08-SC00362). Twenty-one males and 19 females were used for the c-Fos experiment and 32 males were used in the behavioural assay. Salamanders were held individually in ~1 L, 16.5 x 11.5 x 5.8 cm boxes lined with a moistened paper towel and a crumpled paper towel for enrichment. They were fed two to three crickets once weekly and their bedding was changed following feeding. All methods were approved under University of Guelph animal utilization protocol 08R128.

Behavioural Assay

Testing was conducted by P. Kelly as part of his undergraduate research project course at the University of Guelph. Methods were similar to those listed in Chapter 2 of this thesis, but some differences are noted below. Salamanders underwent a three week long food deprivation period prior to commencement of testing, slightly shorter than the four week deprivation described in Chapter 2. Testing was conducted in the home box of the salamander, with two 11 x
8 cm pieces of filter paper on either side of the testing arena. Treatments were the same as those listed for Series I experiments in Chapter 2: Control Soluble/Control Volatile (CC), Prey Soluble/Control Volatile (SC), Control Soluble/Prey Volatile (CV), and Prey Soluble/Prey Volatile (SV). One mL of a solution was placed on each filter paper according to treatment and 10 mL of air was introduced via syringe into the testing arena. Cues were prepared in the same manner as described in Chapter 2. After filter papers containing soluble cues were placed in the arena, salamanders were returned to the testing arena and volatiles were introduced. Testing then started, where eight animals were tested concurrently and observed for 10 seconds each minute for a total of 60 observations over 80 minutes. This method differed from the four animals being tested concurrently and observed 15 seconds a minute over 60 minutes described in Chapter 2. Observations on quadrant location and whether or not a nosetap occurred were recorded at the end of each 10 second period.

Statistical analysis was performed on three variables by A. Telfer: side preference, number of location changes, and number of scans where nosetapping was observed. Methods followed the statistical analysis performed in Chapter 2. Briefly, the randomization method was used to determine if differences existed based on treatment. A repeated measures ANOVA statistic was calculated for each variable, and the data was shuffled using PopTools without replacement and within rows. A Monte Carlo analysis was then run with 10,000 replicates. Multiple comparison t-tests were run where overall significant differences existed; the number of replicates was 1024 for t-test statistics.
**Fos Protocol**

*Cue Application and Brain Dissection*

Cues were prepared 5 days prior to application, as established in the methodology of chapter 2. Treatments also followed the same 2x2 factorial design of CC, CV, SC, and SV. Experimental applications of soluble cues consisted of 5 µl of solution delivered to the nasolabial grooves by pipette. Volatile cues were delivered by syringe, where 10 mL of air was pushed through tissue containing the cues directly to the nares. Treatments were applied every 5 minutes for 45 minutes in freely-behaving salamanders and followed by a 120 minute survival period. Following treatment application, animals were anaesthetized in 1% MS-222 bath and decapitated. Brain dissection immediately followed and, when completed, the brain was placed in 4% phosphate-buffered paraformaldehyde. All cue applications and brain dissections occurred between December 11, 2010 and January 8, 2011. Brains were stored at 4°C until they were processed for Fos immunocytochemistry.

*Fos Immunocytochemistry*

Brains were removed from the 4% paraformaldehyde and rinsed with phosphate buffer (PB: 0.08 M Na$_2$HPO$_4$, 0.02 M KH$_2$PO$_4$, pH 7.4) before being embedded in 4.4% agar-agar dissolved in PB. A block of agar was trimmed surrounding the brain and then cut using a vibratome (VT 1200S, Leica Microsystems) into 40 µm transverse sections. The sections were transferred sequentially into a multi-well net held in a Petri dish containing 10 mL PB. The remaining steps were all completed on a shaker plate moving at 16 rpm. Endogenous peroxidase activity was blocked by a 15 minute incubation in 0.5% hydrogen peroxide in PB, followed by three 10 minute rinses in PB. A 60 minute incubation in 3% bovine serum albumin (BSA) in PB
followed, and sections were checked to ensure they lay at the bottom of the nets. After this, sections were incubated another 60 minutes in 1.5% normal goat serum (NGS) and 0.1% Triton-X-100 in PB. Sections were then incubated overnight in a solution containing the following: a 1:4000 dilution of the primary antibody rabbit anti-c-Fos (sc-253, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 3% BSA, 0.5% NGS, and 0.5% Triton-X-100. The following day, sections were rinsed three times for 10 minutes each in PB before a 90 minute incubation in a 1:200 dilution of the secondary antibody, biotinylated goat anti-rabbit IgG (Vectastain Rabbit PK-4001 kit, Vector Laboratories, Burlingame, CA, USA) as well as 0.5% NGS and 0.1% Triton-X-100. Three 10 minute rinses in PB followed, and then sections were incubated in an avidin-biotin-peroxidase complex (1% each A and B in PB according to PK-4001 kit) with 0.1% Triton-X-100. After a final round of three 10 minute rinses in PB, sections were mounted to previously prepared glass slides that had been gelatinized with a solution of 0.5% gelatin and 0.05% chrome alum dissolved in distilled water. Then, a diaminobenzidine (DAB) chromogen reaction took place under the fume hood; the solution contained 0.025% diaminobenzidine, 0.03% cobalt chloride and 0.03% nickel sulphate. This was incubated in the dark for 20 minutes, before the addition of 0.0009% hydrogen peroxide, which was allowed to react for 13 minutes. Slides were rinsed twice for 2 minutes each in PB and dipped in distilled water before dehydration in ascending alcohols for 2 minutes at each concentration (70, 80, 90, 96, 100% ethanol). Slides were cleared twice in xylene for 5 minutes each, then coverslipped with Eukitt (Fluka Analytical, Sigma-Aldrich). Control sections were prepared by either omitting the primary antibody from incubation or by adding a 1:400 dilution of c-Fos blocking peptide (sc-253 P, Santa Cruz Biotechnology) to the primary antibody solution 30 min prior to application to
the control sections. Controls were run in tandem with the immunocytochemical procedures on 3-6 sections from selected animals.

Cell Counting

Cell counts were performed visually using a Leica DM 1000 light microscope by Dr. F. Laberge, who was blind to the treatment conditions. Methods follow those described in Laberge et al. (2008). Brains where the majority of sections were badly damaged were discarded from analysis. When a few brain sections were missing, damaged, or used for control sections, the following counting rules were used: 1) a missing half section was attributed the number of immunopositive cells as seen in the opposite side; and 2) when one or more whole sections were missing (as is the case for controls), the number of labelled cells in that regions was calculated as

\[ x = \text{number of labelled cells counted multiplied by total number of sections (intact + missing sections)} \div \text{the number of intact brain sections}. \]

Nomenclature and the extent of brain regions for counting is according to Laberge et al. (2008). The main olfactory bulb (MOB) consisted of the five sections rostral to the beginning of the accessory olfactory, or vomeronasal, bulb (AOB). All sections comprising the AOB were counted. The nucleus accumbens (NA) was counted on the 10 caudal sections following the AOB. Note that this is changed from the previous methodology in Laberge et al. (2008), where the number of sections in that region varied between animals. The migrated medial pallium (MPmig) as well as the ventral medial pallium (MPv) were counted on the ten sections rostral to the anterior commisure. All sections comprising the striato-pallial transition area (SPTA) were counted. The vomeronasal amygdala was counted for 7 sections caudal to the SPTA; thus, there was there was no distinction between the caudal amygdala and vomeronasal amygdala as in Laberge et al. (2008). The dorso-lateral caudal pole of the telencephalon (dlCaudal Pole) was a new area counted in the present study;
the number of sections varied between animals, but all sections caudal of the vomeronasal amygdala were counted. Seven sections rostral of the anterior commissure were counted in the ventral cellular prominence (VCP) and the area below the ventral cellular prominence (Below VCP). For the thalamic eminence and ventral thalamus (TE/VT), all sections displaying Fos-like labelling were counted. All labelled cells were also counted in the preoptic area (POA); the POA begins rostral to the anterior commisure and switches to the rostral hypothalamus (rHYP) above the optic chiasm. All labelled cells in the rostral, lateral and medial hypothalamus (HYP) were counted; the rostral region of the hypothalamus was differentiated from its more caudal regions by a cellular bridge which reached across the dorsal hypothalamus. The 10 most rostral sections including the ventral tegmentum (VTEG) were counted. The torus semicircularis (TOR) and optic tectum (OT) were counted in parallel and comprised 15 sections from their most caudal appearance. The raphe median (RM) and lateral medulla oblongata (latMO) were also counted in parallel, and comprised 15 sections from rostral to caudal beginning at the end of the tectum. The end of this region is somewhat vague and so this was considered the most consistent cut-off point.

Statistical Analysis

Possible differences across treatments in the number of Fos-like labelled cells were analyzed with Prism 4 (GraphPad software, San Diego, CA, USA). First, a D’Agostino and Pearson omnibus normality test was conducted for each region. If the test was significant, the data was square-root transformed before an ANOVA was run. If the normality test was not significant ($\alpha = 0.05$), the ANOVA was run on the raw data. Bartlett’s test for equality of variances was run concurrently with the ANOVA. In some cases, Bartlett’s test determined a
significant deviation from homogeneity of variances ($\alpha < 0.05$) so a Kruskal-Wallis non-parametric analysis was run on the raw data.

RESULTS

Behaviour

A total of 32 salamanders were included in the analysis for this experiment. Salamanders maintained similar levels of movement between all treatments ($F_{(3,31)} = 1.90, p = 0.133$; Figure 1b) and no side preference for the scented side was exhibited ($F_{(3,31)} = 0.477, p = 0.709$; Figure 1a). There was an overall difference in nosetapping by salamanders between treatments ($F_{(3,31)} = 4.60, p = 0.004$; Figure 1c) where salamanders increased nosetapping in the SV, but not SC, treatments compared to the CC treatment ($CC \times SV \ t_{(31)} = 3.25, p = 0.004$; $CC \times SC \ t_{(31)} = 1.67, p = 0.129$). No other significant differences were found between treatments ($CC \times CV \ t_{(31)} = 1.74, p = 0.117$; $CV \times SC \ t_{(31)} = 0.186, p = 0.920$; $CV \times SV \ t_{(31)} = 1.99, p = 0.072$; $SC \times SV \ t_{(31)} = 1.86, p = 0.080$).

Immunocytochemistry

The immunocytochemical methods used showed cell labelling in many brain regions associated with olfaction, such as the main and accessory olfactory bulbs, vomeronasal amygdala, lateral hypothalamus, striato-pallial transition area, and raphe median (Figures 2 and 3). Labelling was very dark in some regions, particularly the lateral hypothalamus. Other regions, such as the torus semicircularis, optic tectum and thalamic eminence, which are not known to receive olfactory input, were also labelled. This was expected as fos-labelling is not specific to olfactory pathways but detects any neuron producing the c-Fos protein. Control
procedures run on a subset of brain sections from each treatment revealed that incubation with a
c-fos blocking peptide eliminated all labelling and that no labelling was present with the
omission of the primary antibody. Statistical analysis showed no significant differences between
prey cue treatments for any brain region (Table 1). Four brains had to be excluded from analysis
due to damage, leaving a sample size of 36 salamanders.
Figure 1. Responses of naïve red-legged salamanders to control and prey cues in a preference/avoidance assay in a 12-15°C room for A) number of scans where salamanders were on the side of prey cues or a randomly chosen side for soluble control treatments, B) number of movements between quadrants and C) number of scans where salamanders nosetapped the substratum. All values reported are mean ± SEM (n = 32 salamanders). The p value of the randomization test is shown above each panel and different letters denote significant differences detected by multiple comparison t-tests that followed an overall significant effect. CC: control soluble, control volatile; CV: control soluble, prey volatile; SC: prey soluble, control volatile; SV: prey soluble, prey volatile.
Figure 2. Micrographs of transverse brain sections from *P. shermani* after c-Fos immunocytochemistry (20x magnification); the micrographs were taken from a control animal. Letters A-D correspond to levels of transverse sections identified on the dorsal brain schematic, from rostral to caudal. MOB = main olfactory bulb, AOB = accessory olfactory bulb, cTEL = caudal telencephalon, LHYP = lateral hypothalamus.
**Figure 3.** Comparison of c-Fos labelling in the four brain areas identified in Figure 1 in salamanders receiving different treatments (100x magnification). Columns in the diagram correspond to treatment, and rows correspond to brain region. CC: control soluble, control volatile cues; CV: control soluble, prey volatile cues; SC: prey soluble, control volatile cues; SV: prey soluble, prey volatile cues; MOB: main olfactory bulb; AOB: accessory olfactory bulb; cTEL: caudal telencephalon; LHYP: lateral hypothalamus.
Table 1. Number of c-Fos labelled cells in salamanders receiving different soluble and volatile control or prey cues.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>CC (n = 9)</th>
<th>CV (n = 9)</th>
<th>SC (n = 10)</th>
<th>SV (n = 8)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOB</td>
<td>166.0 ± 24.1</td>
<td>186.0 ± 26.2</td>
<td>135.4 ± 20.4</td>
<td>136.0 ± 21.8</td>
<td>0.35</td>
</tr>
<tr>
<td>AOB</td>
<td>76 ± 4.7</td>
<td>67.8 ± 15.0</td>
<td>58.0 ± 10.6</td>
<td>63.2 ± 16.8</td>
<td>0.34</td>
</tr>
<tr>
<td>NA</td>
<td>30.1 ± 4.1</td>
<td>38.0 ± 6.6</td>
<td>31.9 ± 4.4</td>
<td>29.3 ± 2.7</td>
<td>0.58</td>
</tr>
<tr>
<td>Mpmig</td>
<td>35.9 ± 3.1</td>
<td>28.7 ± 2.7</td>
<td>36.3 ± 2.6</td>
<td>31.4 ± 5.0</td>
<td>0.32</td>
</tr>
<tr>
<td>MPv</td>
<td>173.6 ± 26.1</td>
<td>153.0 ± 25.4</td>
<td>139.9 ± 13.8</td>
<td>171.9 ± 22.2</td>
<td>0.65</td>
</tr>
<tr>
<td>SPTA</td>
<td>62.7 ± 10.6</td>
<td>52.4 ± 6.1</td>
<td>46.8 ± 6.6</td>
<td>49.9 ± 6.4</td>
<td>0.49</td>
</tr>
<tr>
<td>vomAMY</td>
<td>45.8 ± 9.7</td>
<td>34.9 ± 4.5</td>
<td>34.6 ± 4.5</td>
<td>44.4 ± 5.3</td>
<td>0.46</td>
</tr>
<tr>
<td>dlCaudal Pole</td>
<td>92.6 ± 17.3</td>
<td>73.3 ± 10.7</td>
<td>75.9 ± 5.1</td>
<td>81.5 ± 11.9</td>
<td>0.66</td>
</tr>
<tr>
<td>VCP</td>
<td>10.6 ± 3.3</td>
<td>16.3 ± 6.2</td>
<td>14.3 ± 3.9</td>
<td>15.5 ± 4.6</td>
<td>0.82</td>
</tr>
<tr>
<td>Below VCP</td>
<td>28.6 ± 1.9</td>
<td>24.7 ± 4.1</td>
<td>26.3 ± 2.7</td>
<td>35.6 ± 3.9</td>
<td>0.12</td>
</tr>
<tr>
<td>TE/VT</td>
<td>70.3 ± 8.4</td>
<td>119.1 ± 23.6</td>
<td>93.4 ± 15.2</td>
<td>103.3 ± 10.0</td>
<td>0.17</td>
</tr>
<tr>
<td>POA</td>
<td>54.5 ± 11.5</td>
<td>76.2 ± 18.7</td>
<td>65.4 ± 11.8</td>
<td>75.7 ± 15.3</td>
<td>0.70</td>
</tr>
<tr>
<td>rHYP</td>
<td>44.7 ± 10.3</td>
<td>41.3 ± 6.5</td>
<td>49.1 ± 8.9</td>
<td>51.9 ± 7.9</td>
<td>0.83</td>
</tr>
<tr>
<td>lHYP</td>
<td>75.0 ± 11.3</td>
<td>117.3 ± 23.2</td>
<td>133.8 ± 23.7</td>
<td>128.0 ± 19.6</td>
<td>0.18</td>
</tr>
<tr>
<td>mHYP</td>
<td>41.9 ± 10.7</td>
<td>35.8 ± 12.5</td>
<td>40.2 ± 7.9</td>
<td>49.0 ± 9.2</td>
<td>0.84</td>
</tr>
<tr>
<td>vHYP</td>
<td>53.6 ± 13.1</td>
<td>36.6 ± 6.9</td>
<td>43.8 ± 6.2</td>
<td>54.7 ± 14.4</td>
<td>0.61</td>
</tr>
<tr>
<td>VTEG</td>
<td>11.2 ± 5.4</td>
<td>25.4 ± 8.5</td>
<td>13.7 ± 3.2</td>
<td>22.9 ± 5.6</td>
<td>0.27</td>
</tr>
<tr>
<td>TOR</td>
<td>121.2 ± 11.8</td>
<td>123.6 ± 10.5</td>
<td>102.6 ± 11.3</td>
<td>99.5 ± 10.1</td>
<td>0.31</td>
</tr>
<tr>
<td>OT</td>
<td>42.2 ± 5.5</td>
<td>42.8 ± 4.9</td>
<td>40.4 ± 6.2</td>
<td>40.3 ± 4.8</td>
<td>0.98</td>
</tr>
<tr>
<td>RM</td>
<td>15.1 ± 4.9</td>
<td>41.1 ± 10.2</td>
<td>34.3 ± 9.8</td>
<td>38.6 ± 8.0</td>
<td>0.10</td>
</tr>
<tr>
<td>latMO</td>
<td>42.1 ± 4.8</td>
<td>40.3 ± 6.0</td>
<td>38.0 ± 3.8</td>
<td>40.6 ± 4.7</td>
<td>0.94</td>
</tr>
</tbody>
</table>

No statistical differences were detected between treatments in any brain region. Values reported are mean ± SEM. AOB: accessory olfactory bulb; Below VCP: region below the ventral cellular prominence; dlCaudal Pole: dorso-lateral caudal pole of the telencephalon; latMO: lateral medulla oblongata; lHYP: laterodorsal hypothalamus; mHYP: mediodorsal hypothalamus; MOB: main olfactory bulb; Mpmig: migrated part of the medial pallium; MPv: ventral periventricular medial pallium; NA: nucleus accumbens; OT: optic tectum; POA: preoptic area; rHYP: rostral hypothalamus; RM: raphe median; SPTA: striato-pallial transition area; TE: thalamic eminence; TOR: torus semicircularis; VCP: ventral cellular prominence; vHYP: ventromedial hypothalamus; vomAMY: vomeronasal amygdala; VT: ventral thalamus; VTEG: ventral tegmentum.
DISCUSSION

The present study was unable to detect any differences in the number of labelled neurons based on prey cue treatment. This experiment was not undertaken without background to suggest it may be successful, as responses in the CNS to food stimuli have been documented in the past. An fMRI study in humans found more activity in the dorsomedial amygdala when food-deprived subjects were exposed to food odours than when they were satiated (Gottfried 2003). Neurons in the hypothalamus of hungry squirrel monkeys increased their firing rate to food, but not non-food, sights and tastes (Burton et al. 1976). Mitral cells of the olfactory bulb of food-deprived rats showed enhanced electrical activity to food odours (Pager et al. 1972). Our method of a month-long food deprivation period prior to experimental application of prey cues should have been sufficient to interest salamanders in the prey cues presented; as well, their increased chemoinvestigation during behavioural assays suggests that they were interested in cricket prey. The number of animals used was appropriate, as differences have been shown in salamanders and other amphibians with similar sample sizes (Calle et al. 2006; Laberge et al. 2008).

The Olfactory Bulbs

The lack of a clear response in the olfactory bulbs is quite surprising, as olfactory information must be encoded in these regions. While a high number of cells were counted in the main olfactory bulb, significant differences did not exist between control and prey cue treatments. The high amount of labelling in the main olfactory bulb suggests that c-Fos immunocytochemistry could be an appropriate measure of activity, but our treatments did not affect c-Fos activation in the main olfactory bulb. Cell counts were extremely low across all treatments in the accessory olfactory bulb. Laberge et al. (2008) also did not see much labelling
in the accessory olfactory bulb of *P. shermani*, though the vomeronasal system has been shown
to mediate responses to courtship pheromones and increased labelling was seen in the
vomeronasal amygdala. As chemoinvestigation via nosetapping is part of the foraging activity of
salamanders, labelling was expected in the accessory olfactory bulb. It was suggested that
different transcription factors than the c-Fos-like protein may be at work in the AOB (Laberge et
al. 2008). Other immediate-early genes showing upregulation of transcription following
stimulation include those in the *jun* family and egr-1 (Morgan & Curran 1991; Brennan et al.
1999). When females were exposed to components of male mouse urinary proteins, Brennan et
al. (1999) saw a greater mean density of neurons that were expressing egr-1 in different areas of
the accessory olfactory bulb. It may be beneficial to explore other functional neuroanatomy
methods to reveal activity in the accessory olfactory bulb of *P. shermani*.

*Familiarization and Survival Period*

The idea that familiarity with odours could have diminished the neuronal response is
plausible. Montag-Sallaz & Buonviso (2002) found that when rats were familiarized with an
odour by repeated 20 minute exposures to test odours over six days, the mitral and tufted cells of
the olfactory bulb showed decreased c-Fos expression compared to those rats with no
familiarization upon exposure on the seventh day. This pattern was also present in the cingulate
cortex and anterior piriform cortex. However, it is unlikely that familiarization is what caused a
lack of response in this study. Salamanders had not been exposed to prey cues for at least a
month prior to testing, though prior to the beginning of their food deprivation period they had
ended a behavioural assay where they had been exposed to soluble and/or volatile prey cues at
least three times within a week. The length of time following odour exposures and the length of
the food deprivation period make it unlikely that familiarization with the odours was responsible for a lack of response to prey cues in this case.

The survival period after odour delivery may have affected neuronal activation in the olfactory bulbs. Though preliminary testing by Laberge et al. (2008) showed that a 120 minute survival period was adequate to view Fos-like immunolabelling, as opposed to other survival periods between 90 and 360 minutes; this could possibly explain why differences in labelling were not seen in the bulb. Neuronal activation would be expected to have fairly high normal levels in the olfactory bulbs, as the background environment of sensory cues is constantly changing. In a study on rats, animals sacrificed after 15 and 30 minutes after delivery of various odours showed comparable increases in c-fos mRNA levels, but after 45 minutes the levels were lower and increases were not visible by 60 minutes post-odour delivery (Guthrie & Gall 1995). This time period is much shorter than that used in salamanders by Laberge et al. (2008); however, the metabolic rate of rodents is much higher and a much slower protein turnover rate would be expected in salamanders. Note that most studies do use a longer survival period between 90 and 120 minutes. Labelling was still seen in the main olfactory bulb, but perhaps the survival period after odour delivery will need to be shortened to see treatment-specific differences.

*Intrinsic Reward of Chemical Cues*

The identity of a chemical cue may be key part of how rewarding it is to an organism, and thus how strong a response the organism displays towards it. Behavioural responses are driven by three primary motivations to survive: defensive, ingestive and reproductive (Swanson 2000; Vaccaro et al. 2009). The heightened importance of avoiding or obtaining one can lead to
decreased attention to the others; for instance, female salamanders receiving a male pheromone treatment suppressed their feeding response to live prey (Vaccaro et al. 2009). However, startle responses (attributed to defensive behaviours) were unaffected. In theory, the period of food deprivation salamanders were exposed to in this study would suggest that the desire to feed would be strong. It is not likely that the extended period of food deprivation had negative effects on olfactory ability, as starvation in *C. elegans* was shown to increase olfactory discriminatory abilities (Colbert & Bargmann 1997). As well, many salamanders were observed snapping at syringes during cue delivery (personal observation), similar to the manner in which they would snap at live prey. However, this does not necessarily translate into increased Fos expression in the brain areas associated with the olfactory system or the reward pathways.

Pheromones are considered to be a rewarding olfactory stimulus. Laberge et al. (2008) showed Fos-like labelling after experimental application of male mental gland extract to females in the extended vomeronasal amygdala, the preoptic area, ventromedial hypothalamus, and raphe median. Courtship is a prolonged procedure in *P. shermani*, requiring females and males to participate in a tail-straddling walk which can last up to 90 minutes. The rewarding properties of the cue delivered, especially when the signal reaches the amygdala, may contribute to the motivation of salamanders to continue courtship. In mice, Moncho-Bogani et al. (2002, 2005) have shown that male non-volatiles are intrinsically rewarding to females, activating the reward system in the CNS. c-Fos expression in the basolateral amygdaloid nucleus was higher in experienced females than control or inexperienced females, supporting other studies finding a role of the amygdala in reward (Moncho-Bogani et al. 2002, 2005; Beaver et al. 2006; Murray 2007).
In this study, levels of c-Fos expression were very high across all treatments in the main olfactory bulb, with an average of 155.6 neurons per salamander in the main olfactory bulb displaying fos-like labelling. Guthrie & Gall (1995) also report basal levels of c-Fos expression in the olfactory bulbs of rats to be relatively high. Despite a great deal of literature on c-Fos expression in relation to phenomena like food entrainment, response to feeding, and the relation of peptides like ghrelin to feeding, a thorough search was not able to find articles related to brain responses to food odour only. Brain responses seen in other studies of food odour under different circumstances have more to do with the physiological state of the organism or the reward offered than the scent of the food itself. Food odours anticipate feeding, but there is a big difference between smelling a prey item and its actual ingestion. As the reward from foraging is not guaranteed, the scent of a prey item alone may not activate brain regions associated with reward. To confirm if this were the case, a pilot study could be undertaken to see if levels of c-Fos expression increased in salamanders recently fed after a similar period of food deprivation.

**Prey Identity**

Salamanders have a varied diet, and it is possible that the lack of response could have something to do with the identity of the prey presented. While salamanders readily accept crickets in the laboratory, they may not normally be a preferred prey type. The behavioural response of increased chemoinvestigation shows a piqued interest in the cricket, but this may not develop into an active foraging behaviour in the wild. Discrimination between types of prey can allow an organism to use less energy in the consumption of or foraging for their prey. The chitinous exoskeleton of crickets would make them harder to digest than the soft skin of earthworms or the envelope of insect eggs. It is possible that there is not a large response to cricket cues in the brain of salamanders because they would not normally forage for them.
However, the behavioural response of increased nosetapping in the presence of cricket cues suggests that salamanders will search for crickets if they present themselves. As plethodontid salamanders are broad generalist predators (Burton 1976; Maerz et al. 2005), they would be required to respond to a large number of prey items throughout their foraging period. There must be a neuronal response to the cricket cues presented, but our current methods have not been able to visualize it.

Next Steps

As no differences between treatments were discovered, no evaluation of how the olfactory subsystems are used in detection of soluble versus volatile cues was possible. Studies in rodents have had greater success and suggest that, in mammals, the Learning Theory of olfaction may more accurately describe olfactory function. In Kang et al. (2009), male mouse urine volatiles increased the number of Fos-labelled cells as compared to other odours and controls in typical vomeronasal regions as well as main olfactory regions such as the medial amygdala, anterior and posterolateral cortical amygdaloid nucleus, and the medial preoptic area. Anterograde tracing revealed a convergence of axonal projections from both MOB and AOB mitral and tufted cells at the medial amygdala and the authors suggested information about these social cues may be transferred from the main olfactory to the vomeronasal system in this area via synaptic interactions. Martínez-García et al. (2009) used behaviour to illustrate how the vomeronasal system was essential in initial learning of biologically relevant cues for subsequent main olfactory recall in mice. Many researchers are actively trying to discover the function of the two olfactory subsystems and what kind of functional advantage having two systems offers to organisms. Below I suggest some alternatives to the methods used in this study which could
show differences between soluble and volatile prey cue detection in the salamander brain, and contribute to the discussion on olfactory function.

First, a closer look is required at the labelling present in the lateral hypothalamus. Preliminary investigation suggests that prey cue treatments may have produced darker labelling than the control treatment in this region. The intensity of neuronal activation, not necessarily the number of activated neurons, may reveal a more nuanced effect of the different treatments. Using the program NIS Elements AR 3.10 (Nikon Laboratory Imaging), neurons will be compared for the intensity of their labelling against the background across all treatments. A more darkly stained neuron would be indicative of a greater expression of the c-Fos protein, and thus a stronger response to the prey cue. The lateral hypothalamus receives input from the amygdala via the main olfactory bulb; this tract is associated with feeding (Sheldon 1912; Laberge & Hara 2001).

Thus far, only manual counts of labelled neurons have been undertaken. Automated counts will also be attempted in a few specific regions of interest, namely the caudal pole of the telencephalon, the lateral hypothalamus, and the main and accessory olfactory bulbs. Though the data obtained from manual counts is reliable, a second count may provide new insights. Another option for brain imaging is the use of thallium autometallography (Goldschmidt et al. 2004). Reuptake of potassium ions follows depolarization, and can be exploited in another method that indirectly assays neuronal activity. Thallium ions can be used as analogues for potassium and then detected by histochemistry. This method would be a way to view neuronal activity without relying on immunohistochemistry against immediate-early genes. This procedure has high sensitivity and may reflect differences in neuronal activity following prey cue delivery. Further, it could be used in *P. cinereus*, a species more readily available here in Ontario.
As with the behaviour portion of this Thesis mentioned above, future testing can be attempted using different prey items. Insect eggs and larvae have been shown to be a highly preferred prey and it is possible salamanders would respond more positively to them. Both larvae and adults belonging to the Coleoptera and Diptera orders of insects, as well as mites (Oribatida), are also highly preferred (Burton 1976; Placyk & Graves 2002). Further analysis of the current data will continue as well. All behaviours lead back to the brain and all olfactory information courses through it. I am confident that with additional experimentation we will be able to elucidate the roles of the olfactory system and the brain in mediating salamander responses to prey cues.
CHAPTER 4

GENERAL DISCUSSION

Chemical cues are important in active foraging by salamanders. David and Jaeger (1981) and Placyk and Graves (2001, 2002) established the role of chemical cues and suggested prey detection was mediated by the vomeronasal system. These studies were completed using only one prey type, *Drosophila melanogaster*, and only presented live prey. The results presented in this thesis furthered knowledge by showing prey cues alone can cause increased chemoinvestigation via nosetapping as well as establish a side preference. This study was the first to show that red-backed and red-legged salamanders will increase chemoinvestigation when presented with prey cues from the common house cricket. Our results of increased nosetapping agree with those of Placyk and Graves (2001, 2002), who also found increases when live prey were introduced. This further implicates the vomeronasal system in prey detection, though attempts to elucidate the role of the main olfactory system were unsuccessful. Attempts to visualize responses in the olfactory system using immunocytochemistry against c-Fos were so far inconclusive.

The use of two different plethodontid salamanders offered a unique view of behaviour in closely related species. Red-backed and red-legged salamanders displayed very similar reactions to the presence of prey cues in behavioural assays, where they both increased their chemoinvestigation via nosetapping in the presence of soluble and volatile cues at 12-15°C temperatures. The high similarity between these species and their behaviour suggests that the neurobiological response to the cues presented would be very similar. I am unsure why immunocytochemistry against c-Fos is effective in *P. shermani*, yet does not label neurons in the
brains of *P. cinereus*. Regardless, I expect that further investigation using either species would achieve similar advances in understanding how plethodontid salamanders use olfaction to mediate prey detection.

*Increased Chemoinvestigation to Prey Cues*

In chapter 2, I showed that salamanders will increase chemoinvestigation to prey cues presented in an experimental setting. When naïve animals were tested with a soluble prey cue substrate on one side an increase in nosetapping was seen at two different temperatures used in the test. Salamanders appeared to learn or habituate to the behavioural assay and repeated testing with the same animals failed to reproduce results. The ability of salamanders to learn in response to food has been documented (Crane & Mathis 2011). The assay in the current study provided no reward and thus no motivation for experienced salamanders to persistently explore the surroundings. Future testing conducted should only use salamanders naïve to the assay. The effect of volatile prey cues appeared to be heightened in warmer temperatures; in Series I Experiment One salamanders increased nosetapping when presented with both soluble and volatile prey cues. As well, naïve salamanders tested in lower temperatures did not show a side preference when prey cues were present. How temperature affects the dispersion of cues should be considered further.

A path of future research currently being considered is using different species commonly preyed upon by salamanders in behavioural assays. Burton (1976) and Placyk and Graves (2002) both performed analyses on the stomach contents of red backed salamanders and in both cases, a large portion of the contents found consisted of insect eggs. Both soluble and volatile cues are likely to be very attractive to salamanders for this stationary type of prey, and it is likely that
exhibiting a preference for areas containing prey cues would be an advantageous behaviour. How salamanders respond to other preferred prey items, such as mites, weevils, and true flies can also be examined. This will provide an opportunity to discover how salamanders respond to a diverse array of insects, as their foraging methods may change to suit each prey type.

Future work could also attempt to test whether salamanders would follow a scent trail from their prey, as is seen in garter snakes (Kubie & Halpern 1975). Salamanders are challenging to view in behavioural assays; they are spooked easily by the appearance of their observers and can remain motionless for long periods of time. This can be advantageous as an antipredator defence, yet does not make it easy to observe them exhibiting other normal behaviours. Their nocturnal nature and requirements for moisture add to the challenges. Early work with garter snakes involved presenting a cotton swab with either distilled water or earthworm extract on the tip, and observing how many snakes attacked each swab (Halpern & Frumin 1979). Perhaps a similar assay for salamanders could be designed.

The Role of Olfactory Subsystems in Prey Detection

The brain imaging study described in Chapter 3 did not display any differences between control and prey cue treatments. As a behavioural response to the cues similar to that shown by *P. cinereus* was exhibited by *P. shermani*, this was quite surprising. I had hoped to see one of two patterns: either that areas specific to either the main olfactory or vomeronasal systems were separately activated, or that areas from both subsystems were activated, perhaps with some synergistic or inhibitory responses. While there was a high number of activated neurons in the lateral hypothalamus, on a tract associated with feeding, the control treated animals also exhibited a high amount of labelling.
Previous work by Laberge et al. (2008) showed that c-Fos immunolabelling was a valid indicator of neuronal activity in the salamander brain. Their results confirmed evidence in the literature (Wirsig-Wiechmann et al. 2002, 2006) that the vomeronasal system mediates female response to male courtship pheromones. Courtship pheromones are intrinsically very rewarding to salamanders, and so observing a brain response to this type of cue may be easier than for a more common odourant like a prey cue. As mentioned in Chapter 3, thallium autometallography is promising as an alternative method of brain imaging. Its high specificity, as opposed to that of c-Fos immunolabelling, may help more clearly illustrate the pathways through which prey cue information travels through the brain. Another method which will be attempted is to re-analyze the existing data for differences in the intensity of Fos labelling. Preliminary investigation of the lateral hypothalamus suggests more darkly stained neurons may exist in salamanders exposed to prey cues, suggesting the intensity of staining may be more indicative of a response than the number of neurons labelled.

Unfortunately, this study was not able to contribute any new knowledge to the debate on the function of the two olfactory subsystems. Studies could also be attempted using other types of cues, like social signals, predator cues or alarm cues. Other studies have implicated the use of both main and accessory olfactory systems in the detection of social signals (Madison 1975; Tristam 1977; Martin et al. 2005; Dantzer & Jaeger 2007a,b), though thus far salamanders courtship pheromone detection at least appears to be primarily vomeronasal-mediated (Wirsig-Wiechmann et al. 2002, 2006; Laberge et al. 2008). With recent advances in our understanding from the work of Roth & Laberge (2011), however, a more in-depth analysis is warranted.
Conclusion

As studies on olfactory-mediated foraging by plethodontid salamanders are only in their beginning stages, there remains much to be learned in this field. This study adds to the literature by establishing the response of salamanders to prey cues without the immediate presence of a prey item, as is likely to be the case when foraging takes place in the wild. Evidence points toward the vomeronasal system as being primarily responsible for cue detection, but this assertion cannot safely be made until responses have been documented from salamanders with main olfactory deficits. Future research involving various prey items and using different neurobiological methods is necessary so that a greater understanding of salamander olfaction can be obtained.
REFERENCES


98


*Behaviour* 140: 635-648.
