AN ABSTRACT OF THE DISSERTATION OF

Karen M. Kiemnec for the degree of Doctor of Philosophy in Zoology presented on June 22, 2009

Title: Chemical Cues and the Molecular Basis of Olfactory Chemoreception in Caudate Amphibians

Abstract approved:

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Lynne D. Houck

Chemoreception is one of the dominant sensory modalities for many species of salamanders (reviewed in Chapter 2). At least seven of the ten currently recognized salamander families are known to respond to some sort of chemical cue. These responses are as varied as delaying hatching, seeking refuge, or initiating aggressive behaviors. However, a major limitation to our understanding of many salamander chemoreception systems is that the specific chemical eliciting these responses has not been isolated. However, in one family of salamanders, the Plethodontidae, several pheromones have been isolated. Plethodontid salamander males deliver protein pheromones to females during stereotyped courtship interactions. In these salamanders, a male will deliver pheromones if the female is not immediately receptive to mating. Female receptivity to a particular male may profoundly affect male mating success, and thus acts as an agent of sexual selection and sexual isolation.

In the majority of plethodontid species, a male delivers pheromones, produced in a specialized mental gland, to a female's dorsal skin (transdermal delivery). A deviation from this ancestral mode of delivery occurred in one clade that evolved a delivery method that directly stimulates the female's accessory olfactory system (olfactory delivery). In Chapter 3, I report that female *Plethodon shermani* (a species with olfactory delivery) do not respond behaviorally to pheromone delivery via the

ancestral mode (transdermal delivery). In Chapter 4, I addressed the question of whether the pheromone composition is different in males of species that use transdermal verses olfactory delivery. Evolutionary shifts in pheromone composition were determined by expressed sequence tag (EST) analysis of pheromone-gland RNA from three distantly related plethodontid species. Two of the species use the transdermal delivery mode, *Desmognathus ocoee* and *Eurycea guttolineata*, and have some proteins in common, including two previously identified pheromone components, Plethodontid Modulating Factor and Sodefrin Precursor-like Factor. However, these species also express other unique components that may act as pheromones by changing female physiology. Another pheromone protein, Plethodontid Receptivity Factor (PRF), is the dominant RNA transcript in my focal species with olfactory delivery, *P. shermani.* This protein is related to four-helix bundle cytokines and so it may stimulate cytokine receptors. In Chapter 5, isolation of a common cytokine receptor from *P. shermani* females reveals that this receptor is expressed in the female olfactory system and may interact with PRF. However, since multiple proteins are delivered during courtship, I also investigated the classical families of receptors in vertebrate olfactory organs.

Chemical cues are generally detected by two olfactory sensory organs, the main olfactory and accessory olfactory (vomeronasal) epithelia. I used polymerase chain reaction to survey for molecular components of the signaling cascade used to mediate neuronal stimulation in response to chemical signals in *P. shermani* (Chapter 6). Salamander sensory tissue expressed homologues to (1) mammalian odorant and pheromone receptors, (2) olfactory-specific ion channels, and (3) three different families of G proteins. The molecular conservation between amphibian and mammalian olfactory systems suggests that reception of salamander courtship pheromones (and other social signals) is mediated by pheromone receptors expressed in the accessory olfactory epithelium.

Chemical Cues and the Molecular Basis of Olfactory Chemoreception in Caudate Amphibians

by

Karen M. Kiemnec

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Karen M. Kiemnec, Author

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CONTRIBUTION OF AUTHORS

Lynne Houck assisted with the writing of Chapter 3. She also assisted with experimental design of the courtship trials, data collection during the experiments, and revisions for Chapters 3 and 6. Pamela and Richard Feldhoff purified the pheromones used for the behavioral trials and immunocytochemisty in Chapter 3. Sarah Woodley contributed the immunocytological data in Chapter 3 and some of the *in situ* hybridization data in Chapter 6. Richard Watts assisted with the experimental design of Chapters 4, 5 and 6. Stevan Arnold helped with the interpretation of the DNA sequence data for Chapters 4, 5 and 6. Ronald Gregg and Donald von Borstel assisted with the generation of the cDNA libraries used in Chapter 4. All co-authors also contributed to writing, editing and revising the manuscripts.

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CHAPTER 1. GENERAL INTRODUCTION

Chemical communication is likely the most ancient form of communication. Despite the long-standing awareness of its potential importance, this form of communication has been historically difficult to study because of the specialized equipment necessary to isolate, quantify and synthesize chemical cues (Wyatt 2003). Entomologists pioneered the field of intraspecific chemical communication, both coining a name for this type of signal (pheromones) and isolating the first pheromone compound, bombykol (Butenandt et al. 1959; Karlson and Luscher 1959). However, advances in the last 50 years have revealed that chemical signals are important to many taxa for both inter- and intraspecific communication (Wyatt 2009). Chemicals are used to relay information about species identity, reproductive status, and location in both vertebrates and invertebrates (Wyatt 2003; Müller-Schwarze 2006). These signals evoke a multitude of behavioral responses that are often crucial to the survival and reproduction (e.g., mate attraction and sexual arousal) of an organism (Melrose et al. 1971; Kikuyama et al. 1995). A substantial increase in the number of studies of chemical communication has occurred within the last 20-30 years and the field is advancing rapidly (Wyatt 2003). However, many productive areas of research in chemical ecology still remain. Vertebrates, for example, are a taxonomic group that has complex social interactions and hierarchies that can be organized via chemical communication between individuals. In my research, I have focused on the chemical communication used by a particular clade of *Plethodon* salamanders, and also make comparisons among other families of salamanders (Order Caudata).

Chemical cues in salamanders

In general, salamanders are excellent models for investigating chemical communication. Chemical signals are a vital part of communication between salamanders because most of these amphibians cannot vocalize, and are nocturnal, which reduces the effective transmission of visual information (Palmer and Houck 2005). Most salamanders examined to date use some form of chemical

communication. In addition, predator avoidance and feeding behavior are known to be influenced by chemical cues in the environment. I have addressed the overarching theme of olfactory chemoreception in salamanders by first summarizing general functions of chemical cues in amphibians. I then concentrate on specific aspects of an emerging model system for amphibian chemoreception.

In Chapter 2, I have synthesized the current literature on how salamanders respond to chemical signals in their environment. Over 200 articles have described the use and the diversity of chemical cues in salamanders. One goal of my review has been to examine which functions are conserved across families, and which vary between families. Another goal has been to determine what ecological factors contributed to the differences between groups. For many salamander species, some accounts exist in the literature, but these finding are not often cited by other research groups working on a similar area. I assembled these references into a comprehensive summary that illustrates the commonalities of salamanders that are distributed worldwide and studied by multiple research groups.

Courtship in plethodontid salamanders

One generalization that arises from research summarized in Chapter 2 is that chemical cues are common and used in a variety of contexts. However, pheromones have only been isolated and biochemically characterized in two salamander families, Salamandridae and Plethodontidae; these pheromones are used in male-female courtship interactions. The chemical signals that a male delivers to the female only during courtship are of particular interest because these pheromones affect female reproductive behavior and can increase the male's insemination success (Houck and Arnold 2003). The plethodontid model system is ideal for investigating the behavioral and molecular aspects of pheromone delivery. In the majority of plethodontid species, a male has specialized pheromone-producing gland on its chin, suggesting that pheromone delivery in this group has been selectively favored for millions of years (Houck and Sever 1994). Courtship pheromones are delivered by the male via different courtship behaviors that vary across the Plethodontidae. Within this family, two distinct

modes of pheromone delivery have been described. The proteinaceous pheromone is delivered by the male to the female via one of two distinct delivery modes: "transdermal" delivery and "olfactory" delivery. An evolutionary transition in behavior arose in one clade of animals in which the derived olfactory delivery behaviors replaced the behaviors associated with the ancestral transdermal mode used by the rest of the species in the family. The transdermal mode has been observed in all four major lineages of the Plethodontidae, while olfactory delivery is restricted to a clade of ~28 congeneric species (Houck and Arnold 2003). During courtship with transdermal pheromone delivery, the male scratches the skin on the female's dorsum with his elongated premaxillary teeth, and simultaneously rubs his mental gland on the abraded area. The pheromones are thought to diffuse across the skin into the circulatory system and stimulate centers in the brain (Houck and Reagan 1990). In the derived olfactory mode (used by one species group within an eastern *Plethodon* clade), courtship pheromones are delivered by the direct touch of the male's gland to the female's nares (Houck and Sever 1994; Figure 1.1). The pheromones enter the nasal cavity and are shunted to the female's vomeronasal organ and stimulate the accessory olfactory system (Wirsig-Wiechmann et al. 2002; Wirsig-Wiechmann et al. 2006), resulting in a behavioral response.

Courtship behavior in species characterized by transdermal delivery occasionally involves head-to-head contact in which the male and female are facing each other; contact is made between the male's chin and the female's snout. This contact might result in pheromones being delivered to the female's nares. Whether this behavior is a precursor to olfactory delivery is unclear. One hypothesis is that the head rubbing observed in some transdermal delivery species may have evolved into the olfactory delivery mode (Picard 2005). However, the evolutionary forces that promoted the transition from transdermal to olfactory delivery are not well understood. In Chapter 3, I investigated this evolutionary transition in behavior using a focal species, the red-legged salamander (*Plethodon shermani*). I used *P. shermani* as a model of pheromone reception for several reasons. Pheromones of this species are well-characterized, readily

available, and at least one pheromone has been biochemically synthesized a level of resolution is unusual in vertebrate pheromone systems. At the same time, well-characterized pheromones provide a way to investigate reception and transduction of chemical signals. Second, the specialized pheromone gland of each male is relatively large, easy to ablate, and requires minimal post-operative healing time. Furthermore, *P. shermani* are found in large numbers during the courtship season and their courtship has been well-described (Arnold 1972; Arnold 1976).

In order to fully understand how pheromones trigger specific responses, such as those described in Chapter 3, the specific compound that produces the response must be identified. Progress in identifying pheromones and then matching these pheromones to receptors has been difficult in most vertebrate systems: pheromone blends can be complex, important pheromone elements may not be identified easily, and large carrier molecules may mask the chemical properties required for identification of a given pheromone (Johnston 2003; Rodriguez 2004). In fact, a review of identified vertebrate pheromones revealed the characterization of only three amphibian pheromones (Kikuyama et al. 2002). One of the few study systems that has progressed in this level involves the use of courtship pheromones by plethodontid salamanders.

Male *P. shermani* produce the pheromone delivered to a female in specialized glands that hypertrophy during the breeding season. Three proteins have been purified from male pheromone-producing glands using biochemical methods. Two of these proteins increase female receptivity, while one decreases female receptivity when delivered alone (Rollmann et al. 1999; Houck et al. 2007; Houck et al. 2008). However, the male gland produces a complex mixture that includes multiple proteins. In Chapter 4, I used a comparative approach – involving both molecular and bioinformatic methods – to determine courtship pheromone composition. I used molecular methods to survey the RNA from glands of three plethodontid species that vary in male pheromone delivery behaviors. Two of these species (*Desmognathus ocoee* and *Eurycea guttolineata*) use transdermal delivery, while the third species (*P. shermani*) uses olfactory delivery. I used random sequencing of expressed sequence tags (ESTs) from

pheromone gland cDNA libraries as a proxy for protein concentration in the glands. I then used bioinformatics to identify components that might function to influence a female's behavior or physiology.

Reception of chemical cues

Most tetrapod vertebrates have a dual olfactory system, consisting of the main olfactory system (primarily for volatile cues) and the accessory olfactory system (for non-volatile cues, such as salamander pheromones; Wyatt 2003). In Chapters 5 and 6, my objectives were to elucidate how responses to male courtship pheromones and other chemical cues are mediated in the olfactory systems of plethodontid salamanders. Amphibians were the first vertebrate group to possess a distinct vomeronasal organ, which is the chemoreception organ of the accessory olfactory system. Because the common ancestor of salamanders and mammals existed at a time close to the origin of the vomeronasal organ, the salamander system may cast light on the early evolution of tetrapod chemoreception. The mechanisms of chemoreception are complex, so I focused the final portion of my dissertation research on two specific aspects of the neural pathways involved in pheromone reception.

Plethodontid Receptivity Factor (PRF) is the most-studied plethodontid pheromone protein. The reception of PRF may involve cytokine receptors because its structure is very similar to that of cytokines (a four-helix bundle structure). PRF also exhibits signatures of positive selection at putative receptor binding sites (Watts et al. 2004). Chapter 5 describes the investigation of the expression and variation of a cytokine receptor (Interleukin 6 signal transducer) which may interact with PRF. Chapter 5 also reports the expression of this receptor in plethodontid salamanders.

I also surveyed several key components of the olfactory and accessory olfactory systems in the main study species, *P. shermani* (Chapter 6). In the classical model of chemoreception, responses to chemical cues are mediated by multiple families of G-protein-coupled receptors of independent evolutionary origin (Halpern and Martinez-Marcos 2003). In the main olfactory system, two families of G-protein-coupled receptors are expressed, olfactory receptors (ORs) and trace amine-associated receptors

(TAARs; Buck and Axel 1991; Liberles and Buck 2006). Pheromone receptors in the vomeronasal organ are also G-protein-coupled receptors, and each of the three types is recognized as a distinct protein family: V1R, V2R or formyl peptide receptors (FPRs; Liberles et al. 2009; Rivière et al. 2009). The V1R receptors are characterized by a short extracellular domain and presence in the apical layer of the vomeronasal organ (Dulac and Axel 1995). In contrast, the characterization of V2R receptors (Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997) revealed long extracellular domains and presence in the basal layer of the vomeronasal organ. Both the V1R and V2R receptors have been reported in an anuran (*Xenopus*), fishes, and a variety of mammals and thus are thought to be present in most vertebrates (Rodriguez 2004; Shi and Zhang 2007). The FPRs are likely a group of receptors used mainly by rodents (Liberles et al. 2009).

My survey provides the first evidence that olfactory chemoreception in amphibians is likely mediated by the same mechanisms that mediate pheromone reception in mammals. Using polymerase chain reaction, cloning, and *in situ* hybridization, I compared the expression patterns of *P. shermani* receptors (and their associated G proteins) with homologous families in other vertebrates. I also compared the diversity of salamander ORs and V2Rs to the diversity found in other vertebrates. The extraordinary molecular diversity of these multigene families among vertebrates presumably reflects the evolutionary importance of appropriate responses to a broad spectrum of complex chemical cues that may mediate intraspecific, interspecific, and even environmental interactions.

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Figure 1.1. A *Plethodon shermani* male turns to deliver pheromones to the female's nares during tail-straddling walk. Arrow indicates the pheromone-producing gland. Photo used with permission from Stevan J. Arnold.

CHAPTER 2. THE FUNCTIONS OF CHEMICAL CUES IN CAUDATE AMPHIBIANS

Karen M. Kiemnec

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Abstract

Communication via chemical signals has been documented in many amphibian taxa. In particular, salamanders respond to a wide variety of chemical cues from predators, prey items, and potential mates. Here, I review the diversity and function of chemical cues in caudate amphibians. My broad survey reveals that some chemosensory functions are conserved across salamander families, while others appear to be limited to specific taxonomic groups. Common functions across families include general recognition of conspecifics, predator avoidance and species or sex recognition. Families like Dicamptodontidae, Hynobiidae, and Rhyacotritonidae have not been well studied, but may be promising avenues for future research. Investigations of chemical cues, for a number of logistical reasons, have been heavily concentrated on focal species such as *Plethodon cinereus* and *Notophthalmus viridescens*. In general, many species of plethodontid and salamandrid salamanders respond to volatile and non-volatile cues. In a few cases, peptide/protein pheromones that elicit behavioral responses have been isolated. The diverse behavioral and physiological responses that salamanders have to chemical cues illustrates that chemoreception plays a pivotal role in the ecology and reproductive biology of these animals.

Introduction

Chemical cues are detected by a wide variety of vertebrate and invertebrate taxa (Wyatt 2003). Vertebrates can be highly attuned to certain signals, even at low concentrations, and that these signals can elicit substantial behavioral and physiological changes in the recipient animals (e.g., lampreys [Li et al. 2002] and goldfish [Sorensen et al. 1995]). In mammals, chemical signals are widely used for scent marking and social cues (reviewed in Johnston 2003; Müller-Schwarze 2006). Social interactions in birds are predominately mediated by visual and auditory cues, but chemical cues also can play a role in these interactions (Hagelin et al. 2003). Reptiles use odors to track prey and potential mates (Kubie and Halpern 1979; Mason et al. 1989). Although many advances in understanding the functions of, and responses to, vertebrate chemical

stimuli have been made, vertebrate chemical signals are still a dynamic topic of research, especially in non-model organisms.

The use of chemical cues by amphibians is particularly interesting because these vertebrates often have biphasic life histories. The ecological and developmental changes associated with metamorphosis are likely accompanied by changes in sensitivity to certain chemical stimuli. Responses to chemical stimuli have been documented in many amphibian taxa, although relatively few reports exist on chemical communication in the elusive caecilians. However, female rubber eels, *Typhlonectes natans*, can discriminate between male and female waterborne odors and juveniles prefer female scents over male scents (Warbeck et al. 1996; Warbeck and Parzefall 1997; Warbeck and Parzefall 2001).

Although auditory communication is presumed to be the dominant modality for intraspecific signaling in anurans; adult frogs and toads use chemical cues in a variety of contexts, including mate attraction (Wabnitz et al. 1999; Pearl et al. 2000; Asay et al. 2005; Byrne and Keogh 2007), kin recognition (Waldman and Bishop 2004), and aggression (King et al. 2005). Larval and adult anurans can detect predator chemical cues and conspecific alarm pheromones to avoid predation (e.g., Pfeiffer 1966; Hews 1988; Chivers et al. 1999). Detection and avoidance of predators is probably an important strategy for most aquatic amphibian larvae.

Herein, I review larval, juvenile and adult chemoreception responses in caudate amphibians for several reasons. First, chemical communication between salamanders is generally thought to be the most dominant sensory modality for communicating information. Many species are nocturnal, or live in aquatic environments that lessen the efficacy of visual information. Second, vocalization is minimal (or absent) in most salamander families. Third, salamanders have diverse life histories and ecologies. Salamanders in the family Plethodontidae have both direct development and biphasic life history strategies. Some life history stages may be adapted to detect certain chemicals that are relevant to that life history stage, such as aquatic or terrestrial predators. In addition, the natural habitats of plethodontids range from semi-fossorial to

arboreal to aquatic. The composition of chemical cues may vary dramatically depending on the type of environment in which the cues are transferred (Alberts 1992). I aim to highlight both the commonalities and differences between the different groups of salamanders. Several reviews have detailed particular functions of communication in salamanders (e.g., territoriality [Mathis et al. 1995] and reproductive pheromones [Kikuyama et al. 2002]). In contrast, I present a wide survey of the functions of chemical cues across species. My review is not exhaustive, but compiles the diversity of functions and types of chemical signals used by salamanders. In particular, I focus on synthesizing the findings of those studies that have used experimental, rather than observational, approaches to determine how certain chemical stimuli evoke responses from salamanders. These animals are potentially useful comparative models of chemoreception for vertebrates, especially since several salamander chemical signals have been biochemically characterized. I emphasize these well-studied systems and suggest new areas of research for which salamanders may be suited.

Functions of chemical cues in salamanders

Terms such as kairomones, allomones, and pheromones have been used to refer to specific types of chemical cues. The definitions of these terms, however, are sometimes disputed (Beauchamp et al. 1976). To avoid ambiguity, I discuss chemical cues simply as intra- and interspecific cues. Within these two broad groups, I sorted studies into categories, based on the function of the signal as inferred from the animals' responses (Tables 2.1 and 2.2). Organisms can obtain many types of information from chemical signals. Responses to the signals may be easy to document, but determining what specific information the animal gains from the cue is more challenging (Mason et al. 1998). Predator avoidance, species and sex recognition, and general recognition of conspecifics were the most common types of functions in the articles I reviewed. I found fewer studies of mate stimulation and/or attraction, mate quality assessment, and avoidance of injured conspecifics via chemical cues. Reports of chemical cues involved in life history shifts, territorial assessment, and feeding were relatively rare.

I examined whether different life history stages (i.e., larval, juvenile or adult) have been studied. Life history stages were subject to varying environmental conditions, and different cues may be of primary importance during these stages. For example, predator cues would most likely be almost universally advantageous for animals to detect, yet I was only able to identify reports that documented the detection of predators via chemical cues in three of the ten families of salamanders (Tables 2.1 and 2.2).

The majority of studies of how salamanders respond to chemical cues used a type of choice test, either via a Y-maze or an enclosure with different scents on opposing sides. These tests, or variations thereof, have been used on larvae, juveniles and adults. For aquatic species, scented objects have been placed on either side of test chambers, as well as experimental designs with water flowing through a Y-maze (Dawley 1984; Parzefall et al. 2000). Tests with terrestrial species typically involved a substrate moistened with a cue or a substrate taken from an animal's enclosure. Cues were as diverse as feces, predator odors or macerated tissue (Table 2.3).

Predator avoidance

Salamanders appear to be very sensitive to both intra- and interspecific cues from potential predators (Tables 2.1 and 2.2). Responding to predator cues appears to be a widespread phenomenon in salamanders. These cues can function as both behavioral and physiological cues for salamanders. Avoidance of snake, fish, heterospecific and cannibalistic conspecific salamander cues have been reported in various salamandrid and plethodontid species (Sih and Kats 1994; Chivers et al. 1996; Mathis and Vincent 2000; Sullivan et al. 2004). Larvae may be especially sensitive to conspecific cues, because cannibalism from adults may exert a strong selective pressure on larvae (Chivers et al. 1997).

Responses to predator cues may be plastic responses, in the sense that behavioral responses may be highly variable. Adult grey-bellied salamanders (*Eurycea multiplicata)* distinguished between chemical stimuli from predatory and nonpredatory fish and adjusted their foraging behavior based on olfactory cues and their own level

hunger (Whitham and Mathis 2000). Adult eastern red-backed salamanders (*Plethodon cinereus*) were able to determine whether snakes had consumed conspecifics or heterospecifics and avoided the snake odors from snakes that had consumed conspecifics (Sullivan et al. 2004). Adult *P. cinereus* altered their avoidance response to cues from stressed conspecific in as quickly as 15 minutes (Graves and Quinn 2000) and lost avoidance responses to predatory snakes cues when kept in the laboratory environment (Madison et al. 2005). These changes in behavior suggest that salamanders may assess tradeoffs between predator avoidance and other activities such as foraging or mate seeking (Rohr and Madison 2001).

Species and sex recognition

Both immature and sexually mature animals benefit from being able to ascertain the sex and species of another animal via chemical signals. Recognition of a conspecific of the opposite sex is essential for identifying potential mates in salamanders. A potential mate must be of the correct species, especially in areas where multiple congeners are sympatric. As illustrated in Table 2.1, species in the Ambystomatidae, Plethodontidae, and Salamandridae can discriminate sex and or species cues in direct choice tests. Males in a closely related species complex, the *P. glutinosus* group, can discriminate between conspecific and heterospecific females, as well as between males and females (Dawley 1984; Dawley 1986; Palmer and Houck 2003) Some studies suggest that salamanders can even recognize their own individual chemical cues from those of conspecifics (Jaeger and Gergits 1979). Recognition of conspecifics may be useful when animals need to defend territories, especially breeding territories (Simon and Madison 1984).

Indentifying the sex of individuals is not just important for potential mates. Cues related to sex can also be useful for juveniles, for example to identify whether a male or female has established a territory: in some species, one sex may be much more aggressively territorial. In Lanza's alpine salamander (*Salamandra lanzai*), younger juveniles chose to associate with either sex, but older juveniles chose their own scent over that of an adult female (Gautier et al. 2004). In contrast, juvenile *P. cinereus* were

attracted to the cues of adults, and males were less aggressive towards intruding juveniles than to other adult males (Jaeger et al. 1995).

General recognition

The general recognition category is broad and includes any type of study documenting that salamanders respond to conspecific odors. This category encompasses different tests of chemical cues, such as secretions from particular glands or odors from stressed individuals. The preference for, or avoidance of, conspecific odors was one of the most widespread responses to chemical cues and was documented in four of the ten salamander families (Tables 2.1 and 2.2). Most studies tested a chemical cue against a blank substrate, and it may have been difficult to determine what information animals were getting from a signal. Although an animal may show a simple behavioral response, such as avoidance or preference of a chemical cue, its motivation is more difficult to interpret. The multitude of studies documenting recognition certainly argues that salamanders do assess the presence of conspecifics and that such identification is generally advantageous. For more territorial species such as *P. cinereus*, avoidance of conspecifics may mean avoiding potentially harmful injuries (Jaeger 1981). In other species, such as the salamandrid Luschan's salamander (*Mertensiella luschani*), a shelter marked with conspecific scent may be indicative of a safe refuge (Gautier et al. 2006).

Mate stimulation and/or attraction

Many salamander species use chemical cues to interact with a potential mate. A number of reviews discuss the ecological and evolutionary significance of these cues (e.g., Houck 1986; Kikuyama et al. 2002; Houck and Arnold 2003; Houck 2009), hence I only briefly discuss this topic here.

Salamanders often possess male glands that produce secretions which are delivered to a female either via diffusion through water or applied directly to her body during courtship (Arnold 1972; Houck 1986; Noble 1929). The functional significance of these glands, primarily documented in plethodontids and salamandrids, is that these specialized secretions may increase female receptivity or mediate close contact between

females and males (Kikuyama et al. 1995; Rollmann et al. 1999; Yamamoto et al. 2000). However, some receptive adults will perform courtship behaviors solely in response to water that has housed the opposite sex. Whether or not this is a reaction to a particular gland secretion is not known. Such chemically-mediated initiation of courtship behaviors has been seen in both newts *Taricha granulosa* and axoltls *Ambystoma mexicanum* (Thompson et al. 1999; Park et al. 2004).

Mate quality assessment

The ability of salamanders to detect differences between members of the opposite sex may allow them to assess the quality of potential mates. Such an assessment is one of the most intriguing uses for chemical cues in this taxonomic group. Male western red-backed salamanders (*Plethodon vehiculum*) and male Dunn's salamanders (*P. dunni*) both spent significantly more time on substrates previously occupied by conspecific gravid females than on those of non-gravid females of similar size (Marco et al. 1998). Male eastern newts (*Notophthalmus viridescens*) chose the odor of large females over small females in Y-maze trials, and some data suggest male smooth newts (*Triturus vulgaris*) may also make this distinction (Verrell 1985; Verrell 1986). Conversely, female Pyrenean brook newts (*Euproctus asper*) spent more time near cues emitted by a smaller male than a larger male (Poschadel et al. 2007).

Avoidance of injured conspecifics

The avoidance of odors produced by injured conspecifics is another common behavior, documented in four salamander families. Avoidance of injured conspecifics is commonly interpreted as advantageous because this action presumably permits avoidance of areas with actively foraging predators (Chivers et al. 1996). Studies examining the response of animals to injured conspecific often use a macerate of a whole animal as the stimulus. However, some species are sensitive to much milder stimuli that still indicates a conspecific animal is in danger. *Plethodon cinereus* avoided chemical stimuli from other conspecifics that were subjected to a stimulated predation event (Mathis and Lancaster 1998). Most studies have examined the responses to

injured conspecifics (Table 2.1), but some species avoided cues from injured heterospecific cues as well (e.g., *Rhyacotriton variegatus*; Chivers et al. 1997).

Life history shifts

Reports of larval shifts in patterns of ontogenetic development in response to chemical cues are relatively rare, but have been documented in several species (Tables 2.1 and 2.2). These physiological and morphological changes can be induced by cues from either conspecifics (e.g., *Hynobius retardus*; Kohmatsu 2001) or heterospecifics (*A. barbouri*; Sih and Moore 1993). Larger head growth in larvae, for example, likely functions as a defense against potential cannibalism in *H. retardus* (Kohmatsu 2001). Larvae that were raised in water with conspecific larval chemical cues grew larger head sizes than did those reared in solitary conditions without exposure to cues. Also, the hatching of streamside salamander eggs (*A. barbouri*) was delayed when they were reared in the presence of predatory flatworm chemicals. Delaying hatching may increase survival for larvae that emerge at a larger size when flatworms are present (Sih and Moore 1993).

Territorial assessment

The degree of territoriality that a salamander species exhibits can vary widely (Mathis et al. 1995). In only a few species of salamanders, individuals that encountered the territory of a conspecific responded to chemical cues. Residents usually responded with aggressive postures, while intruders typically displayed submissive behaviors. When an adult male *P. cinereus* encountered a burrow marked with another male's fecal pellet, they significantly increased the number of submissive behaviors they displayed (Jaeger et al. 1986). Female *P. cinereus* adults engaged in significantly more behaviors—both aggressive and submissive—when exposed to a conspecific burrow marked with fecal matter (Horne and Jaeger 1988). The chemical cues emitted by feces were apparently used as territorial markers in *P. cinereus* and *P. vehiculum* (Jaeger and Gergits 1979; Jaeger et al. 1986; Ovaska and Davis 1992). Salamanders may also have been able to detect skin gland secretions (Jaeger and Gabor 1993). In addition, *P. cinereus* males may also assess the size of other males through chemical cues because

they were more aggressive in the presence of chemical signals from similarly sized individuals, and were more submissive when exposed to cues from larger individuals (Mathis 1990).

Feeding

In most salamanders, visual stimulation is most likely required for optimal prey acquisition. For example, in three distantly related taxa, *A. tigrinum*, *N.viridescens*, and *P. cinereus,* feeding was most successful when both visual and chemical cues are presented (Martin et al. 1974; Lindquist and Bachmann 1982). Chemical cues delivered alone usually elicited a reduced behavioral response, olfactory stimuli may have been used primarily to identify the palatability of the food item or aid in prey-catching in low-light conditions (Roth 1976; Lindquist and Bachmann 1982). For other species, however, such as the blind cave salamander, *Proteus anguinus*, chemical cues played a larger role in prey capture (Uiblein et al. 1992).

Composition of chemical cues emitted by salamanders

The only compounds that have been isolated and behaviorally validated in salamanders are composed of amino acids. The first compound to be identified was sodefrin, a decapeptide used by males in some species of newts to attract female newts that have come to a pond to mate and oviposit (Kikuyama et al. 1995). In two species of Japanese newts, *Cynops pyrrhogaster* and *C. ensicauda*, males used the decapeptide to attract mates (Kikuyama et al. 1995; Yamamoto et al. 2000). Rohr et al. (2005) discovered that a protein fraction containing a 33 kDa protein (produced by male cloacal glands) was used by *N. viridescens* males as an indication of the sex of a conspecific. Plethodontid salamanders use mixtures of proteins as courtship pheromones, three of which have been characterized and tested (Rollmann et al. 1999; Houck et al. 2007; Houck et al. 2008). In general, pheromones can yield information on the evolution of these signals because (a) robust phylogenies exist for these species (b) delivery behaviors are well-described and (c) the RNA and DNA that encode for specific proteins can be sequenced for these species.

Many potential pheromones could be emitted from a variety of skin glands that

salamanders possess. All salamander chemical cues that have been isolated to date have been secreted from specific glands, and some of these glands have been retained over millions of years of evolution (Houck and Arnold 2003). Madison (1977) gives a general overview of the types of glands that may excrete substances used in communication, but the composition of most salamander gland secretions is not known. These glands are a promising source of compounds, and animals respond to the extract of clocal, post cloacal, and mental glands (Table 2.3). Large, non-volatile molecules, such as proteins, are probably used by organisms of any species that are able to deliver the signal through water or by direct contact.

The vast majority of studies on terrestrial salamanders have not used experimental designs that show whether salamanders can discriminate between volatile and nonvolatile cues. Animals are usually placed in a chamber some distance away from cues and can be exposed to volatiles being released from substrate, as well as nonvolatiles that are detected by direct contact with the cues. Chemical signals in most terrestrial animals are often a mix of volatile and non-volatile chemicals, depending on the intended longevity of the signal in various environments (Alberts 1992). Non-volatiles are probably used for all of the functions listed in Table 2.1.

Some salamanders are capable of responding to volatile cues alone (Table 2.4). Male *P. aureolus* and *A. jeffersonianum* chose the air flowing from a chamber containing a conspecific female over air from a heterospecific female in a Y-maze (Dawley 1984; Dawley 1986). In *P. glutinosus, P. jordani,* and *P. kentucki*, both females and males could discriminate between volatile cues of conspecific and heterospecific members of the opposite sex (Dawley 1984; Dawley 1986; Dawley and Dawley 1986).

Volatile cues are also used in aggressive interactions. Both *P. cinereus* males and females acted more aggressively towards same-sex volatile cues than opposite-sex cues and males acted more aggressively towards nongravid females (Dantzer and Jaeger 2007a, b Martin et al 2005). During the non-breeding season, *P. cinereus* males preferred volatile cues from neighbors over non-neighbors. During the breeding season,

however, female *P. jordani* preferred non-neighbors over neighbors (Madison 1975). In *P. vehiculum,* both males and females avoided male scents (Ovaska 1988).

Diversity of species responsive to chemical signals

Species in most salamander families respond to some sort of chemical cue. The lack of life history data makes it difficult to predict what functions chemical signals may play in the biology of some of the less-studied animals. For example, little is known about the courtship behaviors of the dicamptodontids or amphiumids, let alone what cues may be using during these encounters (Houck and Arnold 2003). I found published reports of the use of chemical signals in every family of extant salamanders except Amphiumidae, Cryptobranchidae, and Sirenidae (Table 2.1). The number of studies on species within each family varied dramatically. For example, I located only a few studies conducted on species of Dicamptodontidae, Hynobiidae, Rhyacotritonidae, and Proteidae. In two well-studied families, Plethodontidae and Salamandridae, most studies were conducted on one or a few focal species. *Plethodon cinereus* is a well-studied focal species in the Plethodontidae, while *Notophthalmus viridescens* is one for the Salamandridae. Focal species have not been studied in other, less speciose families (e.g., Hynobiidae and Rhyacotritonidae). A number of attributes make a particular species, like *P. cinereus*, highly amenable to study: distributed through much of the eastern United States and high abdundance where it occurs. In addition, *P. cinereus* makes up a substantial amount of the vertebrate biomass in many parts of its range (Burton and Likens 1975).

Focal species are useful for many reasons. The range and abundance of organisms can be obvious limitations to the types of studies that can be performed. A more comprehensive understanding of the responses of a particular species allows researchers to make predictions about other species that are closely related or that share many similarities with the focal organism. Detailed research on predator avoidance in *P. cinereus*, for example, has shown that adults can (a) detect whether a common snake predator, *Thamnophis sirtalis*, has fed on conspecifics, and (b) will avoid snake cues (Sullivan et al. 2004). The *P. cinereus* even avoided chemical cues from snakes that

had eaten another salamander species (*E. bislineata*), but only if the *P. cinereus* were from a population that was sympatric with *E. bislineata* (Sullivan et al. 2005). Results from studies such as these can be used generate research questions, such as whether the cue elicits the same response in multiple species and multiple life history stages.

Conclusions

Salamanders provide ample examples of behavioral responses to chemical signals released by both hetero- and conspecific animals. Many plethodontid species will respond robustly in direct choice or Y-maze tests. Caudate amphibians use chemical cues to (a) assess information about the status of potential mates, (b) avoid predation, and (c) identify conspecifics. However, a salamander's response to chemical cues may be behaviorally complex, and may also depend on the animal's reproductive status, age, or habitat. Differential popularity of certain life history stages in studies makes it difficult to draw conclusions about what life history stages do and do not use chemical cues for various functions. Tests on responses made during multiple life history stages to a single cue would be one way to reveal whether sensitivities change as animals age (e.g., Mathis and Vincent 2000). Such studies could test individuals as they progress from larvae to adults.

Tests of different life history stages and other deeper-level understanding would not be possible without the data gathered on model organisms such as *N. viridescens* and *P. cinereus*. However, not every specific response to a chemical cue in these species can be assumed to be a generalized response across multiple groups. Most studies concentrate on a single species, but the few studies that incorporate a comparative approach are able to draw broader conclusions about a particular genus. Marvin et al. (2004), for example, found that three species of *Eurycea* did not avoid injured conspecifics, but that two other species might. These types of multi-species congeneric comparisons are particularly well-suited for geographic areas that are speciose, such as the southeastern United States.

Drawing conclusions about the functional conservation of chemical signals between families is also difficult because of the difference in the number of species

from each family that have been studied. For example, plethodontids are extremely diverse in their life histories and habitats, and sensitivity to particular types of cues may be vital for some species and unimportant for others. Generally, there appears to be a rough correlation between the number of species in a family and the number of species studied; I found the most reports on chemical signals in the Plethodontidae and few (or no) scientific papers for families that had fewer than ten species (Table 2.2). These gaps represent opportunities for future research. The family Hynobiidae, for example, contains 51 species, but I only identified two studies pertaining to chemical cues in this group. Even within the Plethodontidae, major lineages have not been studied. The genus *Bolitoglossa* contains ~100 of the 391 plethodontid species, but I did not identify a single study that showed these animals respond to chemical signals.

It may seem surprising that in the hundreds of publications that describe behavioral and physiological responses to pheromones in salamanders, only a handful attempt to identify the chemical structure of the signal. However, the isolation and characterization of chemical signals is a time-consuming process that usually involves iterative fractionation of a mixture into components that must then be tested individually. The necessary effort in this regard may be one of the major reasons few chemical cues are identified in salamanders. Even in very well-studied vertebrate model systems, such as mice, only a handful of intraspecific cues have been isolated that have behavioral or physiological effects (reviewed in Swaney and Keverne 2009). Identification of these compounds, however, provides research avenues into the underlying complexity in the production and reception of these signals within the context of ecological and evolutionary frameworks. In spite of the difficulties associated with isolating compounds, researchers are making remarkable progress in identifying compounds in amphibians. In the last five years alone, researchers identified an anuran peptide that causes intermale aggression (King et al. 2005); two salamander proteins that influence female receptivity (Houck et al. 2007; Houck et al. 2008); and a new population-specific variant of the newt sex attractant, sodefrin (Nakada et al. 2007). Salamanders will no doubt continue to provide critical insight

into what roles chemical cues play in the initiation and modulation of vertebrate behavior and physiology.

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Table 2.1. Functions of chemical cues identified in well-studied salamander families.

	Ambystomatidae	**Plethodontidae**	**Salamandridae**
Predator avoidance	*Ambystoma annulatum* (1) *Ambystoma barbouri* (2) *Ambystoma macrodactylum* (3) *Ambystoma texanum* (4)	*Desmognathus ochrophaeus* (13) *Eurycea bislineata* (14) *Eurycea multiplicata* (15) *Eurycea nana* (16) *Plethodon angusticlavius* (17) *Plethodon cinereus* (18) *Plethodon dorsalis* (13) *Plethodon richmondi* (13)	*Notophthalmus viridescens* (40) *Triturus marmoratus* (41) *Taricha torosa* (42)
Species and/or sex recognition	*Ambystoma jeffersonianum* (5) *Ambystoma macrodactylum* (6)	*Desmognathus fuscus* (19) *Desmognathus imitator* (20) *Desmognathus ochrophaeus* (20) *Desmognathus ocoee* (21) *Plethodon aureolus* (22) *Plethodon cinereus* (23) *Plethodon glutinosus* (22) *Plethodon kentucki* (22) *Plethodon montanus* (24) *Plethodon shermani* (24) *Plethdon teyahalee* (24)	*Notophthalmus viridescens* (43) *Triturus montandoni* (44) *Mertensiella luschani* (45)
General recognition of conspecifics	*Ambystoma maculatum* (7) *Ambystoma opacum* (8)	*Desmognathus fuscus* (25) *Desmognathus ochrophaeus* (26) *Eurcyea longicauda* (27) *Eurcyea wilderae* (27) *Plethodon caddoensis* (28) *Plethodon cinereus* (29) *Plethodon glutinosus* (22) *Plethodon serratus* (30)	*Euproctus asper* (46) *Mertensiella luschani* (45) *Notophthalmus viridescens* (47) *Salamandra atra* (48) *Salamandra lanzai* (48) *Salamandrina perspicillata* (49) *Triturus alpestris* (50) *Triturus boscai* (51)

		Plethdon teyahalee (28) *Plethodon vehiculum* (31)	*Triturus carnifex* (50) *Triturus helveticus* (52) *Triturus italicus* (50) *Triturus montandoni* (45) *Triturus vulgaris* (45)
Mate stimulation and/or attraction	*Ambystoma mexicanum* (9)	*Desmognathus ocoee* (32) *Plethodon shermani* (33)	*Cynops ensicauda* (53) *Cynops pyrrhogaster* (54) *Triturus cristatus* (55) *Taricha granulosa* (56)
Mate quality assessment		*Plethodon dunni* (34) *Plethodon vehiculum* (34)	*Euproctus asper* (57) *Notophthalmus viridescens* (58) *Triturus vulgaris* (59)
Avoidance of injured conspecifics	*Ambystoma macrodactylum* (10)	*Desmognathus ochrophaeus* (35) *Plethodon richmondi* (36) *Plethodon vehiculum* (37)	*Cynops pyrrhogaster* (60) *Notophthalmus viridescens* (60)
Life history shifts	*Ambystoma barbouri* (11)		
Territorial assessment		*Plethodon cinereus* (38) *Plethodon vandykei* (31)	
Feeding	*Ambystoma mexicanum* (12)	*Plethodon cinereus* (39)	

Numbers in parentheses refer to citations: (1) Mathis et al. (2003); (2) Sih and Kats (1994); (3) Murray et al. (2004); (4) Kats (1988); (5) Dawley and Dawley (1986); (6) Verrell and Davis (2003); (7) Ducey and Ritsema (1988); (8) Smyers et al. (2001); (9) Park et al. (2004); (10) Chivers et al. (1996); (11) Sih and Moore (1993); (12) Eisthen and Park (2005); (13) Cupp Jr (1994); (14) Petranka et al. (1987); (15) Hickman et al. (2004); (16) Epp and Gabor (2008); (17) Watson et al. (2004); (18) Sullivan et al. (2004); (19) Uzendoski and Verrell (1993); (20) Verrell (1989); (21) Verrell (2003) ; (22) Dawley (1986); (23) Dantzer and Jaeger (2007); (24) Palmer and Houck (2005); (25) Keen et al. (1987); (26) Evans and Forester (1996); (27) Marvin et al. (2004); (28) Anthony (1993); (29) Jaeger and Gergits (1979); (30) Mathis et al. (1998); (31) Ovaska and Davis (1992); (32) Houck et al. (2008); (33) Rollmann et al. (1999); (34) Marco et al. (1998); (35) Lutterschmidt et al. (1994); (36) Hucko and Cupp Jr. (2001); (37) Chivers et al. (1997); (38) Jaeger et al. (1986); (39) David and Jaeger (1981); (40) Mathis and Vincent (2000); (41) Orizaola and Brana (2003); (42) Elliott et al. (1993); (43) Dawley (1984); (44) Cogalniceanu (1994); (45) Gautier et al. (2006); (46) Parzefall et al. (2000); (47) Rohr et al. (2002); (48) Gautier and Miaud (2003); (49) Romano and Ruggiero (2008); (50) Belvedere et al. (1988); (51) Aragon et al. (2000); (52)

Secondi et al. (2005); (53) Yamamoto et al. (2000); (54) Kikuyama et al. (1995); (55) Malacarne and Vellano (1987); (56) Thompson et al. (1999); (57) Poschadel et al. (2007); (58) Verrell (1985); (59) Verrell (1986); (60) Marvin and Hutchinson (1995)

Table 2.2. Functions of chemical cues identified in less-studied salamander families.

	Dicamptodontidae	**Hynobiidae**	**Proteidae**	**Rhyacotritonidae**
Predator avoidance	*Dicamptodon tenebrosus* (1)			
General recognition of conspecifics			*Proteus anguinus* (4) *Necturus maculosus* (4)	
Avoidance of injured conspecifics				*Rhyacotriton variegatus* (5)
Mate quality assessment		*Hynobius leechii* (2)		
Life history shifts		*Hynobius retardatus* (3)		

Numbers in parentheses refer to citations: (1) Rundio et al. (2003); (2) Park and Sung (2006); (3) Kohmatsu (2001); (4) Parzefall et al. (1980); (5) Chivers et al. (1997)

Table 2.3. Chemical cues emitted by salamanders that are commonly used in assays.

Cue	Species	Reference
Whole body rinse		
Stressed animal	*Plethodon cinereus*	Mathis and Lancaster (1998)
Unstressed animal	*Plethodon shermani*	Schubert et al. (2006)
Glands		
Mental	*Plethodon shermani*	Rollmann et al. (1999)
Post-cloacal	*Plethodon cinereus*	Jaeger and Gabor (1993)
Cloacal	*Cynops pyrrhogaster*	Kikuyama et al. (1995)
Whole animal macerate	*Notophthalmus viridescens*	Marvin and Hutchinson (1995)
Feces	*Plethodon cinereus*	Horne and Jaeger (1988)
Marked substrate	*Plethodon cinereus*	Jaeger and Gergits (1979)
Home tank water from stimulus animal	*Notophthalmus viridescens*	Rohr et al. (2002)
Air flowing over stimulus animal	*Notophthalmus viridescens*	Dawley (1984)

Table 2.4. List of salamander families and types of chemical cues detected.

Family	No. of species in family[1]	Habitats	Volatile cues known to be detected?	Composition of any cues identified?
Ambystomatidae	32	terrestrial and aquatic	no	no
Amphiumidae	3	aquatic	no	no
Cryptobranchidae	3	aquatic	no	no
Dicamptodontidae	4	terrestrial or aquatic	no	no
Plethodontidae	391	aquatic, arboreal, terrestrial, semi-fossorial	yes	yes
Proteidae	6	aquatic	no	no
Rhyacotritonidae	4	semi-aquatic	no	no
Salamandridae	79	terrestrial or aquatic	yes	yes
Sirenidae	4	aquatic	no	no
Hynobiidae	51	terrestrial or aquatic	no	no

[1]number of species current at time of submission (from www.amphibiaweb.com)

CHAPTER 3. TRANSDERMAL TRANSFER OF COURTSHIP PHEROMONES DOES NOT INFLUENCE RECEPTIVITY IN FEMALE RED-LEGGED SALAMANDERS, *PLETHODON SHERMANI*

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Abstract

Communication between the sexes occurs in plethodontid salamanders during courtship via male pheromones that can alter female receptivity by decreasing courtship duration. Pheromone delivery occurs via two distinct types of male behaviors. The transdermal delivery mode is considered ancestral and is used by the majority of plethodontid species. A second mode of pheromone delivery, olfactory delivery, has evolved in one clade of plethodontids. Using two approaches, we tested whether the ancestral pathway of pheromone detection, with pheromones delivered transdermally, is still present in the red-legged salamander (*Plethodon shermani*), a species with the derived olfactory mode. In the first test, we staged courtship trials with male-female pairs and experimentally delivered pheromones in a manner mimicking the ancestral delivery. Contrary to our predictions, we found that females failed to be more receptive to males when pheromones were delivered to the dorsum of the body. We then used immunocytochemistry to determine that pheromones did not activate vomeronasal sensory neurons when delivered to the female dorsum, verifying that response to the transdermal and olfactory delivery is mediated by separate organs. Thus, female *P. shermani* were only stimulated by courtship pheromones delivered directly to the nares and apparently have lost the pathway for pheromone stimulation via the dorsal skin. This pattern is likely to characterize all members of the *Plethodon glutinosus* species group.

Introduction

Communication between two animals requires successful production and transmission of a signal to a receiver (Johnstone 1997). Communication also requires that the neural pathway mediating a response is functional in the recipient animal. As signaling systems evolve, the recipient animals and their neural pathways may either become more or less attuned to certain signals (Johnstone 1997). Whether signals become redundant as (a) a new stimulatory pathway evolves or (b) whether a new pathway replaces the existing signaling channel is significant in determining if animals may extract information from one or both signals (Endler and Basolo 1998). The

evolution of an alternative signaling system that affects courtship and mating may also contribute to speciation (Møller and Pomiankowski 1993).

Males in many plethodontid species communicate with a potential mate via protein pheromones (Houck and Arnold 2003). Males produce these courtship pheromones in the mental gland, a specialized gland located on the chin (Arnold 1977; Houck and Arnold 2003). Two dramatically different behaviors are used by males to deliver these mental gland pheromones during courtship and the corresponding stimulatory pathways in the female appear to represent two distinct ways that a male can influence female receptivity. Depending on the species, a male will use one of two types of behaviors to deliver pheromones: either via a "transdermal" or "olfactory" delivery (cf Arnold 1972; Arnold 1977).

In the majority of plethodontid species, a male rubs his mental gland over the female's dorsum; the pheromones are presumed to diffuse through the skin and into the female's superficial circulatory system (Houck and Reagan 1990; Organ 1961). The target organ is presumed to be the brain, but little is known about the sites of action and neural pathways for these pheromones. This transdermal delivery may be facilitated by "scratching" behavior, whereby a male uses his elongated premaxillary teeth to scratch the female's dorsum immediately after he rubs his gland on her skin (Arnold 1977: Noble 1929). However, simply mimicking pheromone delivery to the dorsum (without scratching) is sufficient to increase female receptivity in that courtship duration is decreased (Houck and Reagan 1990; Houck et al. 2008). The "transdermal" mode has been observed in four major lineages of plethodontids (Bolitoglossini, Desmognathinae, Hemidactyliini, and Plethodontini; Figure 3.1) and is clearly the ancestral mode of pheromone delivery in this family (Houck and Arnold 2003; Houck and Sever 1994).

In the derived "olfactory" mode (used by only 28 out of approximately 390 species in the family Plethodontidae), courtship pheromones are delivered by the direct application of the male's gland to the female's nares (Arnold 1976). From the nares, the pheromones enter the female's nasal cavity where they stimulate sensory neurons of the vomeronasal organ and activate the accessory olfactory system (Laberge et al. 2008;

Wirsig-Wiechmann et al. 2002; Wirsig-Wiechmann et al. 2006). Courtships are more rapid (mating is completed more quickly) when a female experimentally receives the male pheromone rather than a saline control (Houck et al. 2008; Rollmann et al. 1999; Rollmann et al. 2003).

The evolutionary transition from transdermal to olfactory delivery is not well understood, and is made more complex by the presence of a group of species phylogentically intermediate to the transdermal and olfactory species groups (Figure 3.1). These intermediate species probably use olfactory delivery, based on their pre-maxillary tooth and mental gland morphologies (Highton 1962). However, some males exhibit extensive head rubbing behavior in addition to delivering pheromones via olfaction (Picard 2005). In particular, Picard (2005) suggested that male *P. dorsalis* (a species in the *P. welleri* group; Figure 3.1) deliver pheromones via diffusion through the skin, even though this species predominately uses olfactory delivery. In fact, in some well-documented olfactory delivery species, the male still rub its mental gland on the female's dorsum during courtship (Arnold 1976; Marvin and Hutchison 1996; Organ 1958).

The information on pheromone composition in plethodontids is primarily based on surveys of pheromone RNA. The closely related small eastern and large eastern *Plethodon* both express three pheromone proteins (Plethodontid Receptivity Factor [PRF], Plethodontid Modulating Factor [PMF], and SPF [Sodefrin Precursor-like Factor]). In *P. shermani*, PRF and PMF make up 85% of the proteins secreted by the mental gland (Feldhoff et al. 1999). The presence of PRF, PMF and SPF has been documented in the gland secretions of small eastern *Plethodon* species, but the relative compositions of their gland secretions are not known. The presence of the same components in both transdermal and olfactory species suggests that the same proteins might act as pheromones via both pathways. These observations led us to hypothesize that the ancestral stimulatory pathway via diffusion through the skin might still exist in olfactory delivery species.

We chose *P. shermani* as our focal species because the behavior and neurobiology of pheromone delivery during their courtships is well described (e.g., Houck et al. 2008; Laberge et al. 2008; Rollmann et al. 1999; Rollmann et al. 2003; Wirsig-Wiechmann et al. 2002; Wirsig-Wiechmann et al. 2006). Our first objective was to determine if male pheromones solely delivered via diffusion through the skin might evoke a behavioral response in *P. shermani* females. Our second objective was to use immunocytochemical methods to determine whether a fluid solution of male pheromones applied to the dorsum of the female could result in activation of sensory neurons of the vomeronasal organ (perhaps by spreading across the skin from the dorsum to the nares). We wanted to test whether a behavioral response to the pheromone applied to the dorsum was or was not due to pheromones acting through the olfactory pathway.

Materials and methods

Behavioral trials

Adult female and male *P. shermani* were collected from Macon County, North Carolina, USA (035°10′48″N 083°33′38″W) during August 2006. Animals were collected with the appropriate permits from North Carolina Department of Wildlife. The salamanders were housed at Oregon State University for the duration of the behavioral trials, which were staged from 7-24 September 2006. Methods and animal care were approved by Oregon State University's Institutional Animal Care and Use Committee (LAR 3007 to L.D. Houck). Animal care followed the protocols of Houck et al. (1998). Animals were individually housed, kept on a natural photoperiod, and fed waxworms (*Galleria mellonella*) weekly. In addition, to ensure that females only received pheromones experimentally delivered by the researchers, we anesthetized each male to be used in the courtship trials and surgically ablated its mental gland (see Houck et al. [1998] for methods). De-glanded males included in the experiment were given at least two weeks to recover before being used in behavioral trials. Males fully recover and court normally after this procedure (Houck, personal observations).

Before the behavioral trials began, males and females were prescreened to determine their propensity to mate in the laboratory, as described in Houck et al. (2008). Once reproductively active animals had been identified, they were randomly reassigned to different male-female pairs before the onset of the courtship trials. Pairs remained matched in the trials until they had mated a single time. Individual salamanders were used only once in the data set analyzed for behavioral trials.

The experimental protocol used in the behavioral trials was a modified version of a previously described protocol that successfully increased female receptivity in *D. ocoee*, a species with transdermal delivery of courtship pheromones (Houck et al. 2008). Females were placed in courtship boxes (9 cm x 17 cm x 30 cm) with a damp paper towel substrate before the male was introduced. A 2 x 4 mm piece of low-protein-binding filter paper (Whatman glass microfiber filter) was held with fine tweezers and 5 µl of one of two treatments was pipetted onto the paper which was then placed on the female's dorsum, between the forelimbs (see Figure 20.2 in Houck et al. [2008] for a more detailed description). A female either received (a) a piece of filter paper containing 6 µg/µl male pheromone (in 0.5 X PBS = phosphate buffered saline) extracted from the mental gland using standard procedures (Houck et al. 1998) or (b) a control saline (0.5 X PBS) solution. As in Houck et al. (2008), these small filter paper patches were used as a substrate to hold the treatment solutions in place during the entire observation period and to allow the solution to diffuse slowly into the female's circulatory system. We chose not to experimentally mimic the male's scratching behavior because Houck et al. (2008) showed that scratching was not necessary to elicit a behavioral response in *D. ocoee*. Researchers observing male and female courtship behaviors were blind as to which treatment was placed on a given female. The treatments were randomized, except that half of the females received pheromone and half received the saline control during each night of courtship observations.

The filter paper rectangle remained on a female's back for 15-20 minutes before a single, randomly assigned male was introduced to that female's box. The rectangles then remained in place for the three-hour observation period. Behavioral observations

took place under dim red light and commenced as soon as a male had been placed in each box. Trials were staged during the time of night when the animals normally would be found courting in the field (2200 h to 0100 h EST; Houck, personal observations). These three-hour courtship periods were used to standardize duration of male-female interactions. During each trial night, we recorded courtship behaviors using instantaneous scan sampling and focal animal sampling (Altmann 1974). For all pairs (pheromone- and saline-treated), we recorded: (a) the initiation of courtship, defined as when the female first entered a tail-straddling walk; a distinct courtship behavior characteristic of all plethodontids (Arnold 1972); (b) each attempt by a male to deliver pheromone to the female by touching his chin to the female's nares; (c) the completion of courtship, defined as the time when the male deposited a spermatophore; and (d) if the female was inseminated. We chose these behaviors (described by Arnold 1976) because they are unambiguous and easily scored by all observers.

Data on the mean duration of courtship were analyzed using a one-tailed *t*-test with $\alpha = 0.05$. We used a one-tailed test based on results from several prior behavioral experiments showed that pheromones significantly reduced courtship duration (e.g., Rollmann et al. 1999; Rollmann et al. 2003). Also, based on earlier experiments, our prediction was that pheromone delivery would decrease average courtship duration by 15-20%. Data on the average number of times the male attempted to apply pheromone to the female's nares (males attempted pheromone delivery even though each had been deglanded) were analyzed using a two-tailed *t*-test with $\alpha = 0.05$. Normality of the courtship duration and pheromone delivery attempt data was confirmed before the analyses were conducted. Data on insemination success for pheromone-treated and saline-treated females were analyzed using a 2 x 2 contingency table with $\alpha = 0.05$. We did not expect number of delivery attempts and insemination success to vary between treatment groups (based on previous experiments) but considered these variables to be indicators of normal courtships.

Immunocytochemistry

Our experimental delivery of pheromones to the female's dorsal skin might also result in the flow of pheromones across the surface of the skin to the female's head and then into the nasal cavity. Thus, the objective of the immunocytochemcial assay was to assess the level of vomeronasal cell activation that resulted from application of male courtship pheromones to the female's dorsal skin versus to her nares. We used 15 adult female *P. shermani* that were collected from the same locality as those in the behavioral trials for the immunocytochemistry assay. Each treatment group experimentally received one of three different combinations of male pheromone or saline control (0.5 X PBS) to the dorsal skin and/or to the nares. The three treatments were: (1) saline to the nares and saline to the dorsal skin (n=5), (2) saline to the nares and male pheromone to the dorsal skin (n=5) and (3) saline to the dorsal skin and male pheromone to the nares (n=5).

The method of agmatine (AGB) uptake was used to assess whether pheromones would stimulate neurons in the vomeronasal organ. AGB is a guanidium analogue that, when co-delivered to the vomeronasal organ with a chemosensory stimulus, enters activated sensory neurons. Vomeronasal sensory neurons that have taken up AGB can be visualized with standard immunocytochemical methods. The method of AGB uptake previously had been used to show that male pheromones from the mental gland activated vomeronasal sensory neurons of female *P. shermani* (Schubert et al. 2008; Wirsig-Wiechmann et al. 2002; Wirsig-Wiechmann et al. 2006).

To deliver chemosensory stimuli to a female via diffusion through the dorsal skin, a 2 x 4 mm piece of the low-protein-binding filter paper was placed on the dorsum of each female salamander at the level of the forelimbs, as in the behavioral trials. Either one µl of male pheromone (10 µg/µl concentration) or a control saline solution was applied to the filter paper three times, with an interval of 10 min between applications. This dose (30 µg) equaled the dose delivered to the dorsum during the behavioral experiment described above.

Chemosensory stimuli (pheromones) were mixed 1:1 in a 6mM solution of AGB dissolved in PBS and applied to the nares. To deliver pheromones to the nares, 2 µl of

either male pheromone (1.5 µg/µl concentration) or saline were delivered to the nares of the female using a micropipette every 2 minutes for a total of 21 applications per female. The pheromone and control (PBS) solutions contained 3 mM of AGB. After the final application of the AGB solution, an application of 3 x 5 µl PBS followed to rinse away any excess AGB.

Animals were sacrificed via decapitation 45 min after the first application of the chemosensory stimulus to a female's back. The upper jaw was fixed overnight in 4% paraformaldehyde-2.5% glutaraldehyde, pH 7.4. Then, the jaw was decalcified in DeCal (DeCal Corporation) for 2 days and cryoprotected in 30% sucrose in PBS. Upper jaws were embedded in OCT (Fisher Scientific) and sectioned at 20µm using a cryostat. Sections were collected on polylysine-coated superfrost plus slides and stored at -80°C until immunocytochemistry. Every 4th section underwent immunocytochemistry for AGB (following the methods of Schubert et al. [2006] and Schubert et al. [2008]). Cells with darkly stained cytoplasms were considered to be AGB-immunoreactive (AGB-IR) and were counted. The numbers of AGB-IR cells in the VNO in both the left and right nasal cavities were summed to give the total number of AGB-IR cells. The investigator was blind to the treatment of each animal.

The number of AGB-IR cells was log-transformed so that error variances were homogeneous and the data were normally distributed. The numbers of AGB-IR cells were analyzed with a 1-way ANOVA followed by Student-Newman-Keuls *post hoc* pairwise comparison tests.

Results

We obtained behavioral data for 89 courtships in which each female had a treatment delivered to her dorsum: 47 in which the female was treated with pheromone, and 42 in which the female was treated with the saline control. Contrary to our predictions, no difference in courtship duration was found between the pheromone- and saline-treated behavioral groups (t_{88}=1.02, p=0.31). The mean duration was 38.7 min (SE=3.4) for the saline-treated group and 36.2 min (SE=3.6) for the pheromone-treated group. For both groups combined, the overall mean insemination success was 88% and

did not differ between groups (p=0.98), nor did the number of times a male attempted the olfactory delivery of pheromones (p=0.36).

In the immunocytochemistry experiment, treatment groups differed in the number of activated vomeronasal sensory neurons ($F_{2,14} = 17.2$, $P < 0.001$). More vomeronasal cells were activated by delivery of male pheromone to the nares than by delivery of PBS to the nares (Figure 3.2). Application of male pheromone to a female's dorsum did not activate significantly more vomeronasal sensory neurons than did the application of the saline control to the dorsum (Figure 3.2).

Discussion

We hypothesized that species of plethodontid salamanders that use the transdermal delivery mode to deliver male courtship pheromones affect female behavior via a different signaling pathway than do the large eastern *Plethodon* salamanders that use the olfactory delivery of pheromones. Experimentally delivering pheromones in a modified ancestral manner (dorsal application) did not reveal the decrease in courtship duration that was found in earlier behavioral assays that tested the typical (olfactory) manner of pheromone delivery in *P. shermani* (Rollmann et al. 1999; Rollmann et al. 2003). In addition, the experimental mimicking of the transdermal delivery did not stimulate neurons in female *P. shermani* vomeronasal organs. In two previous studies of a different plethodontid salamander, *D. ocoee*, experimental delivery of male courtship pheromones to the dorsal skin of the females did increase female receptivity to male mating (Houck and Reagan 1990; Houck et al. 2008). However, males of this species use the ancestral transdermal mode of pheromone delivery. Therefore, we conclude that female *P. shermani* have lost the ability to be stimulated by the ancestral transdermal mode of courtship pheromone delivery.

The courtship encounters we observed appeared to be normal with respect to the average courtship duration when compared to previously published data for *P. shermani*. Courtships lasting approximately 30 minutes appear to be within the normal range of courtship variation recorded for this species and closely related species such as *P. kentucki* under laboratory conditions (Arnold 1976; Marvin and Hutchison 1996;

Rollmann et al. 2003). It is conceivable that the pheromones stimulated females via diffusion but we failed to achieve statistical significance due to our sample size. If this were the case, the dorsal skin pathway would be much less effective at eliciting a response compared to when courtship pheromones were delivered to the nasal cavity because previous experiments using olfactory delivery have achieved statistical significance with smaller sample sizes (Houck et al. 2008; Rollmann et al. 1999).

The *P. shermani* salamanders have apparently lost all vestiges of the ancestral pheromone delivery mode. The ineffectiveness of pheromone delivery via dorsal patches may be due to several factors. The composition of pheromone proteins varies between distantly related species using transdermal and olfactory delivery (Kiemnec-Tyburczy et al. 2009; Palmer et al. 2007), so it is possible that, during the evolutionary transition in pheromone delivery behavior, males lost the expression of carrier proteins that allowed the pheromone components to pass through the skin and enter the female's circulatory system. Males could have also lost or varied the expression of particular pheromone components, such as variants of PRF. An alternative hypothesis is that the females have lost their sensitivity to the pheromones in the organs targeted by pheromones introduced to the dorsal skin. Also, male *P. shermani* and males of other olfactory delivery species may have retained the head rubbing behavior simply because this contact provides tactile stimulation during courtship (Beachy 1997).

Our experiment suggests the need to examine some of the intermediate species of eastern *Plethodon* salamanders that are more closely related to transdermal delivery species than is *P. shermani*. Males within the intermediate group (which includes the *P. welleri* and *P. wehrlei* species groups; Figure 3.1) may use both stimulatory pathways (both olfactory and transdermal) to modify female behavior because these intermediate groups are more closely related to the transdermal delivery *Plethodon* species than is *P. shermani*. A greater understanding of the behaviors in these intermediate species would provide insight into the evolution of alternate pheromone delivery modes. These groups are a therefore a promising avenue for future study, but behavioral experiments may be difficult because of the low numbers of observed courtships under laboratory conditions

(e.g., *P. dorsalis*, n=2 complete courtships [Picard, 2005] and *P. angusticlavis*, n=2 [Dyal, 2006]).

In addition, the physiological basis for the behavioral response to pheromones is not well understood, and further investigation is needed to reveal the physiological pathway(s) that are associated with the unique stimulation of transdermal delivery. At the same time, this study has illustrated that the evolution of signaling pathways used during plethodontid salamander courtship has shown that (a) male pheromone signals act through the olfactory pathway in *P. shermani* and (b) a loss of the ancestral (transdermal) pathway of stimulation for *P. shermani* male courtship pheromones.

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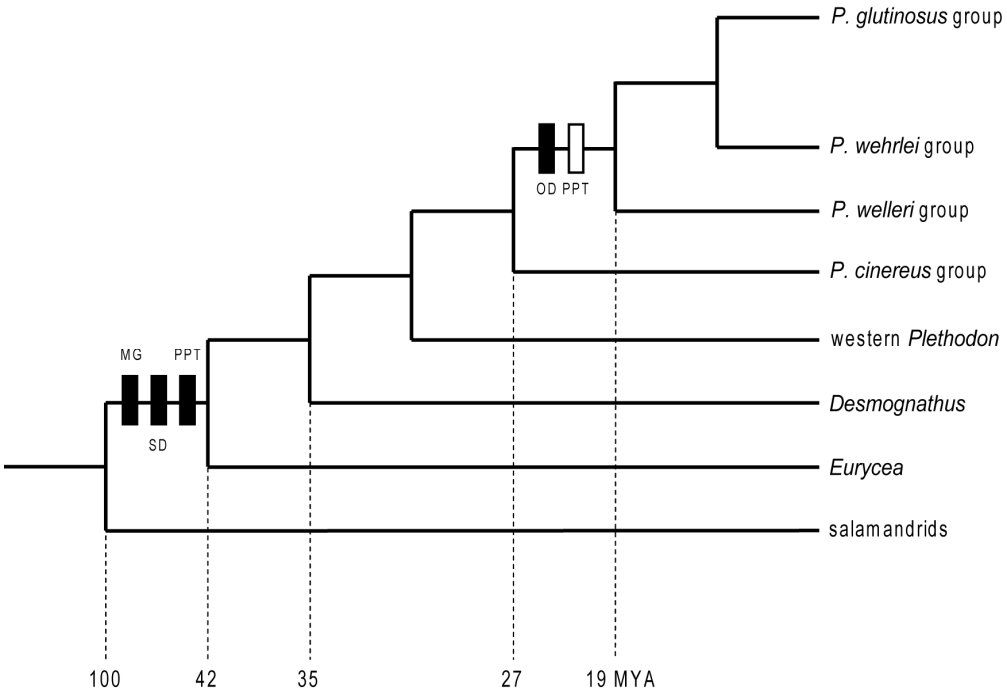

Figure 3.1. Cladogram showing the relationships of particular clades of plethodontid salamanders and the evolution of characters involved in courtship pheromone delivery (modified from Palmer et al., 2007). Salamandrid salamanders are used as an outgroup. Representative species are listed for each group (described by Highton 1962); others species have been omitted for simplicity. Relationships at the generic level are concordant across studies using both morphological and molecular characters (Chippindale et al. 2004; Macey 2005; Mueller et al. 2004). Approximate divergence times (shown at bottom) are based on albumin immunology (Larson et al. 2003). Rectangular boxes show the point of origin (solid) or loss (open) of particular characters: mental gland (MG), protruding premaxillary teeth (PPT), transdermal delivery of courtship pheromones (SD), and olfactory delivery of courtship pheromones (OD).

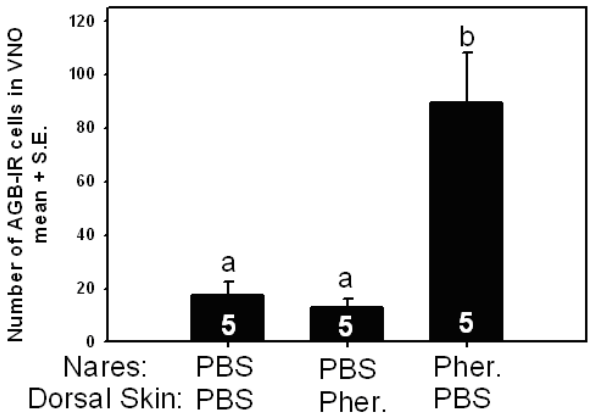

Figure 3.2. The mean number of AGB-immunoreactive cells in the vomeronasal organ (VNO) of female *P. shermani* receiving male pheromone to the nares or to the dorsal skin. PBS indicates control (saline) treatment and Pher indicates pheromone treatment. Sample sizes are included in the bars. Bars that do not share a letter are significantly different ($P < 0.05$, Student-Newman-Keuls post hoc comparison tests).

CHAPTER 4. EVOLUTIONARY SHIFTS IN COURTSHIP PHEROMONE COMPOSITION REVEALED BY EST ANALYSIS OF PLETHODONTID SALAMANDER MENTAL GLANDS

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Abstract

Courtship behavior in salamanders of the family Plethodontidae can last more than an hour. During courtship, males use stereotyped behaviors to repeatedly deliver a variety of proteinaceous pheromones to the female. These pheromones are produced and released from a specialized gland on the male's chin (the mental gland). Several pheromone components are well characterized and represented by high frequency transcripts in cDNA pools derived from plethodontid mental glands. However, evolutionary trends in the overall composition of the pheromonal signal are poorly understood. To address this issue, we used random sequencing to survey the pheromone composition of the mental gland in a representative species from each of three distantly related plethodontid genera. We analyzed 856 high-quality expressed sequence tags (ESTs) derived from unamplified primary cDNA libraries constructed from mental glands of *Desmognathus ocoee*, *Eurycea guttolineata*, and *Plethodon shermani*. We found marked differences among these species in the transcript frequency for three previously identified, functional pheromone components: Plethodontid Receptivity Factor (PRF), Sodefrin Precursor-Like Factor (SPF), and Plethodontid Modulating Factor (PMF). In *P. shermani* mental glands, transcripts predominately encoded PMF (45% of all ESTs) and PRF (15%), with less than 0.5% SPF. In contrast, in *D. ocoee* and *E. guttolineata* the proportions were ~20% SPF, 5% PMF, and PRF was absent. For both *D. ocoee* and *E. guttolineata*, peptide hormone-like transcripts occur at high frequency and may encode peptides that change the physiological state of the female, influencing the female's likelihood to complete courtship. These and previous results indicate that the evolution of courtship pheromones in the Plethodontidae is dynamic, contrasting with the predominant mode of evolutionary stasis for courtship behavior and morphology.

Introduction

The best understood pheromones of amphibians are proteinaceous compounds that mediate sexual communication in salamanders (Kikuyama et al. 1995; Rollmann et al. 1999) and mate attraction in anurans (Wabnitz et al. 1999). Several proteinaceous

pheromones of amphibians have been characterized and range in size from 10 to over 200 aa (reviewed in Kikuyama et al. 2002). Some of these pheromones are prone to rapid diversification via amino acid substitutions, which may promote speciation (Palmer et al. 2005, 2007a, 2007b; Watts et al. 2004). In contrast, pheromones used by salamanders in other sexual and nonsexual contexts (e.g., species and sex identification) have been detected by behavioral assays but have not been characterized (Dantzer and Jaeger 2007; Dawley 1986).

Plethodontid salamanders use protein pheromones during an intricate courtship that accomplishes indirect sperm transfer via a spermatophore that is attached to the substrate (Houck and Arnold, 2003). In most of the 300+ species of plethodontid salamanders, the male's chin (mental) gland seasonally hypertrophies and produces multiple proteins during a lengthy courtship season (Houck and Sever 1994; Lanza 1959; Sever 1975). During courtship, males deliver these mental gland proteins to the female using one of two, stereotyped behaviors (Arnold 1977). In 'scratching delivery', the male swabs the female's dorsum with his mental gland and then abrades the swabbed site with specialized premaxillary teeth, apparently introducing phermones into the superficial vessels of her circulatory system. In 'olfactory delivery', the male repeatedly touches the secretory surface of his mental gland to the female's nares, with the consequence that pheromones are introduced into the female's nasal cavity and reach her vomeronasal organ (VNO). Scratching delivery is the ancestral delivery mode, found in all major clades and a majority of species, while olfactory delivery is a derived mode, restricted to a clade of about 30 species in the genus *Plethodon* (Fig. 1; Houck and Arnold 2003).

Partial biochemical characterization of the major gland proteins has been accomplished for two plethodontid species: *Plethodon shermani* (olfactory delivery) and *Desmognathus ocoee* (scratching delivery). Analyses using NH_2-terminal protein sequencing, SDS-PAGE and HPLC revealed three structurally unrelated proteins that constitute the major components of the male courtship pheromones in these two species (Feldhoff et al. 1999; Houck et al. 2008b; Rollmann et al. 1999). The first pheromone

that was isolated, Plethodontid Receptivity Factor (PRF), is related to the interleukin-6 family of cytokines and reduces the duration of courtship in *P. shermani* (Rollmann et al. 1999). PRF expression appears to be limited to a single genus, *Plethodon* (Palmer et al. 2007).

The second pheromone, Plethodontid Modulating Factor (PMF), is expressed in *Aneides*, *Desmognathus*, and *Plethodon,* based on RT-PCR isolation from mental glands (Palmer 2004). Plethodontid Modulating Factor (a 7 kDa protein) has an effect on female courtship behavior opposite that of PRF. When delivered alone to *P. shermani* females, PMF increases total courtship duration. Since these components are delivered together during courtship, as part of the total pheromone mixture, this apparently enigmatic effect may be an experimental artifact. In the natural mixture, PMF may have synergistic interactions with other pheromone components. For example, PMF may calm the female and enhance the effects of PRF on the female's courtship behavior (see Houck et al. 2007 for additional discussion).

The third protein pheromone identified for plethodontid salamanders, Sodefrin Precursor-Like Factor (SPF), is similar to the uncleaved precursor protein of the salamandrid (newt) sex attractant, a decapeptide termed sodefrin (Palmer et al. 2007b). Like PRF, SPF decreases total courtship time when it alone is delivered to the female (Houck et al. 2008). Sequences of SPF have been amplified from the cDNA of mental glands of four genera of plethodontid salamanders: *Aneides*, *Desmognathus*, *Eurycea* and *Plethodon* (Palmer et al. 2007). To date, SPF has been validated behaviorally only in *D. ocoee* (Houck et al. 2008). Studies using codon-substitution models to estimate the nonsynonomous/synonomous substitution rates of PMF, PRF and SPF show that all three have experienced positive selection across lineages at a subset of amino acid sites (Palmer et al. 2007). These sites may be co-evolving with binding sites on receptors in females (Watts et al. 2004).

In sum, previous work demonstrated that proteins secreted by plethodontid mental glands influence female behavior and have experienced rapid evolution. However, HPLC and SDS-PAGE analyses have shown that many proteins expressed in

male mental glands remain uncharacterized in *P. shermani* (Feldhoff et al. 1999) and in *D. ocoee* (Houck et al. 2008b). Thus, the complex protein profiles revealed by initial analyses may include other molecules that persuade courting females to mate with particular males. Unfortunately, evolutionary trends in the overall composition of the mental gland secretions are poorly understood. It is unknown whether overall pheromone composition is evolutionarily conserved or prone to rapid diversification that parallels documented diversification of particular pheromone components (PMF, PRF, and SPF). PCR isolation has revealed a shift from SPF to the dominant use of PRF within the genus *Plethodon* (Palmer et al. 2007b), but no other shifts have been documented. Evolutionary inferences are also limited because protein screening is partial and limited to only two plethodontid species. To address these broader evolutionary issues, we used an EST approach to compare the mental gland expression profiles of three distantly-related plethodontid genera. We predicted that mental gland proteins corresponding to high frequency transcripts are likely to have functional effects during courtship because specialized glands are known to contain large amounts of pheromone RNA in both vertebrates (newts; Iwata et al. 2000) and invertebrates (sea hares; Fan et al. 1997).

We compared the ESTs from mental glands of three diverse members of the family Plethodontidae: *D. ocoee*, *P. shermani*, and *E. guttolineata*. These three taxa represent three of four major plethodontid lineages (Chippindale et al. 2004; Macey 2005; Mueller et al. 2004). The genera *Desmognathus* and *Plethodon* represent two sister clades, while *Eurycea* is more distantly related (Fig. 1). Our primary goals were to (1) determine the overall composition of the mental gland secretion for each species, (2) examine the complexity of expression in the male mental glands (including investigating novel high-frequency transcripts that might encode functional proteins) and (3) compare the composition of the mental gland proteins between the three species. We hope that these comparisons will begin to elucidate the evolutionary history of pheromone composition in this family. In addition, because previous RT-PCR was unable to distinguish low and high frequency transcripts, our final aim was to establish

whether differences in sequences previously obtained by RT-PCR reflect actual sequence variation in mental gland transcripts.

Materials and methods

Tissue collection and RNA isolation

Adult males in breeding condition (indicated by enlarged premaxillary teeth and/or a visible mental gland) were collected for each of the three study species. Because previous studies of courtship pheromones have been focused on *D. ocoee* and *P. shermani* (e.g., Houck et al. 2008; Rollmann et al. 1999), one of our goals was to compare the EST pheromone sequences to those obtained by PCR from these focal species. Male *D. ocoee*, *E. guttolineata,* and *P. shermani* were collected from Macon County, North Carolina (35°02'20"N 083°33'08"W, 35°02'40"N 083°10'17"W, and 35°10'48"N 083°33'38"W, respectively).

To collect gland tissue, all animals were anesthetized in 4% ethyl ether in water. The mental gland of each male was then surgically ablated with iridectomy scissors (Houck et al. 1998). Males fully recover from this procedure in approximately one week (Rollmann et al. 1999). For each species, glands from multiple males were stored as pooled samples in RNA*later* (Ambion, Austin TX). RNA from the *D. ocoee* glands (n=20) and *E. guttolineata* glands (n=7) was extracted using Trizol reagent (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. RNA from *P. shermani* (n=10 glands) was extracted from the pooled glands using an mRNA isolation kit (Stratagene, La Jolla CA).

cDNA library construction and plasmid sequencing

The *P. shermani* library was constructed using the ZAP Express XR Library Construction Kit (Stratagene, La Jolla CA) that utilized the Lambda-ZAP Vector. The library was generated using standard protocols supplied by the manufacturer. The *D. ocoee* and *E. guttolineata* gland cDNA libraries were synthesized using the Creator Smart cDNA Library Construction Kit (BD Biosciences Clontech, Palo Alto CA) according to the manufacturer's instructions. Briefly, long-distance PCR was used to generate cDNA inserts that were run through column fractionation to exclude small

products (≤500 bp). The size exclusion biased the libraries toward larger sequences, deemed appropriate because these smaller sequences were assumed to be mostly 3' and 5' untranslated regions. Although the use of differing methodologies to construct libraries may have biased the representation of some ESTs in the libraries, general information about the percentage of transcripts is nevertheless informative for our purposes.

In order to obtain a representative sample of ESTs from each library, primary clones were chosen randomly from each of the three libraries. Plasmid DNA was isolated from each clone using the Qiaquick miniprep kit (Qiagen, Valencia CA) and the DNA was then sent away for sequencing. Clones from the *P. shermani* library were sequenced using the T3 primer at the DNA core facilities at the University of Louisville. Plasmid DNA from the *D. ocoee* and *E. guttolineata* libraries were sequenced with the M13 Forward primer at the Nevada Genomics Center. An additional screening step was used on the primary clones from *E. guttolineata* library because unlike the other two libraries, this one had a smaller proportion of clones containing inserts. Primary clones were screened by isolating plasmid DNA from each clone using the Qiaquick miniprep kit (Qiagen, Valencia CA) and digesting with *Sfi*I. Agarose (1%) gel electrophoresis followed by ethidium bromide staining was used to visualize the digested DNA under UV light. Clones containing inserts over 500 bp were then sent for single-read sequencing.

EST assembly and identification

SeqManII (DNAstar Version 5.0) was used to identify and exclude low quality sequences, cluster sequences, and assemble contigs. High-quality sequences were sorted into contigs of 80% identity. The consensus sequence from each contig was then compared to known sequences in GenBank using translated BLAST searches (ww.ncbi.nlm.nih.gov/BLAST) against the translated sequence database. GO (Gene Ontology)-slim functions were recorded as a way to classify the salamander ESTs into putative functional categories based on their similarity to previously identified genes (e.g., Wagstaff and Harrison 2006). The ESTs were sorted into GO-slim categories if

they had a BLAST e value <e^{-10} to a known gene product. ESTs from all three species were deposited in the NCBI EST database (http://www.ncbi.nlm.nih.gov/dbEST). The *D. ocoee* ESTs were archived under the accession nos. **FK700083**-**FK700535**, the *E. guttolineata* ESTs under **FG985087**-**FG985271**, and the *P. shermani* ESTs under **FK253129**-**FK253344**, **FK703776** and **FK703777**.

Our primary interest in constructing these libraries was to identify cDNAs (generated from RNA) that likely transcribed proteins secreted into the lumen of the male mental gland that might act as functional pheromones. We had two criteria for identifying a contig as a potential pheromone component. First, it had to contain more than three ESTs (and was therefore relatively highly transcribed). Second, it had some region of homology to gene products that were physiologically active (but the BLAST score was not necessarily below the e-value of e^{-10}). Therefore, highly transcribed contigs not identified in the initial BLAST search (described in previous paragraph) were subjected to an additional screening process that looked more closely at the BLAST matches to determine if there were any regions of homology to physiologically active gene products. Once potential candidates were identified, NCBI Unigene gene expression profiles were examined to determine whether the gene was normally expressed in the blood, skin, or connective tissues of other vertebrates. Those predicted ORFs that were determined to be pheromone candidates were aligned with those of other vertebrates using BioEdit version 7.0.9 (Hall 1999). Additionally, each ORF was checked for the presence of a signal peptide using SignalP 3.0 (Bendtsen et al. 2004) and biochemically relevant information about cleavage sites was used to predict whether the salamander ORF might transcribe a functional peptide.

Calculation of pheromone sequence dissimilarity

To compare the DNA sequence diversity of PMF, PRF, and SPF between our ESTs and the previously published sequences, we compiled and aligned our ESTs and those sequences in GenBank separately. We used ClustalW implemented in MegAlign (DNAstar Version 5.0) to align the pheromones from each species. To maximize the number of ESTs included in the analysis, we used the first 114 bp of each pheromone

ORF to estimate nucleotide dissimilarity. The same 114 bp fragment was used from published pheromone sequences in order to directly compare sequence diversity. Average nucleotide sequence dissimilarity was measured using the Tamura-Nei method (calculated in MEGA, Version 4.0; Tamura et al. 2007) as the number of unique substitutions per nucleotide site for a pair of sequences with a correction for multiple hits.

Comparison of unidentified ESTs in all libraries

We also checked for similarity of the unidentified sequences between the three libraries. Because it is known that *P. shermani* and *D. ocoee* both share the pheromone component PMF, the three species may have other proteins in common. To investigate this possibility, we compiled all ESTs from all three species that did not have an identified function and used SeqManII to cluster these unknown ESTs into contigs of 75% identity. Each of these contigs was checked manually to determine whether ESTs from multiple species were present in the same contig.

Results

Overview of mental gland libraries

The average EST length for all male mental gland library sequences was approximately 500 bp. The total number of high-quality ESTs obtained was 856 (453 from *D. ocoee*, 185 from *E. guttolineata*, and 218 from *P. shermani*). As expected, transcripts encoding previously identified pheromones were the most prevalent transcripts in all of the libraries (Table 4.1). In fact, all the libraries were roughly equivalent in general composition. Aspects similar in every library included the number of sequences assigned to multi-sequence or single-sequence contigs, number of sequences grouped into all GO-slim categories (except unclassified), and average length of ESTs (Fig. 2). However, there were also some striking differences among the three libraries. When the ESTs were classified into GO-slim functions, the number of ESTs grouped into the general pheromone and unclassified categories differed among the salamander taxa. The percentages of ESTs identified as pheromone transcripts in the *P. shermani* library was about double that found in the *D. ocoee* and *E. guttolineata*

libraries (Fig. 2). In comparison, the *D. ocoee* and *E. guttolineata* libraries had a much higher percentage of unclassified ESTs.

Comparison of pheromone ESTs across the three plethodontid genera

The three species showed distinct differences in the number of known pheromone ESTs (PMF, PRF, or SPF) found in each of the male gland libraries (Fig. 3). In *P. shermani*, the pheromone ESTs were predominately identified as PMF and to a lesser extent, PRF. The *D. ocoee* and *E. guttolineata* mental gland EST compositions were similar, each having approximately equal proportions of SPF and PMF ESTs. The DNA sequence variation present in the ESTs was generally higher in the libraries than that obtained by RT-PCR. In *P. shermani*, the average nucleotide dissimiliarty was the same (0.01%) for the PRFs obtained by both methods, but the ESTs showed a higher dissimilarity (0.25%) for PMFs than did the sequences obtained by RT-PCR (0.01). In *D. ocoee* the dissimilarity was higher in the ESTs in both PMF (0.08% vs. 0.01%) and SPF (0.23% vs. 0.02%). Because so few sequences were obtained by RT-PCR for *E. guttolineata*, we were unable to do any dissimilarity comparisons with that species.

Identification of novel highly-expressed transcripts

ESTs that were identified at low levels (less than three ESTs) and that were similar to genes expressed in blood, skin, or connective tissue were considered to be part of the normal housekeeping repertoire of gland cells and associated tissues. An EST from *E. guttolineata* had short regions of identity to relaxin, a hormone with pleiotropic effects produced by primarily reproductive organs in male and female animals (Bani 1997). This hormone may be secreted and delivered to the female during courtship, but because there was only a single EST, it is unlikely that it is highly transcribed by the mental gland. Other highly expressed transcripts were mostly attributable to general cellular maintenance and did not appear to function as pheromones. A few of the highly expressed transcripts may instead encode enzymes used to process excreted pheromone components. For example, in both *D. ocoee* and *E. guttolineata*, several of the ESTs encoded a transcript that showed identity to the M3 Thimet peptidase family, a family that cleaves circulating peptides (Lew 2004). The *P.*

shermani and *E. guttolineata* libraries both contained ESTs that were identified as cystatins. Some members of the cystatin family have cysteine protease inhibiting functions. PMF and SPF have multiple conserved cysteines. Thus, these cystatins may function to protect these pheromones from being degraded in the lumen of the mental gland.

One of the two candidate pheromone transcripts represented ~7% of all ESTs from the *E. guttolineata* library and encoded a predicted protein that showed similarity to the natriuretic peptide family. This family of small hormones (~29 aa) that mainly stimulate sodium excretion and vasorelaxation (Bovy 1990; Matsuo 2001) . The predicted ORF from *E. guttolineata* appears to be about 50 aa shorter than natriuretic peptide precursors produced in the mammalian heart, but contains multiple sites known to be necessary for functionality (Fig. 4). These sites include two cysteine residues that disulfide bond to form a cysteine ring (salamander residues 7 and 23), and a phenylalanine at residue 8 (Bovy 1990). The ORF also contained a predicted signal peptide of 22 aa and four basic amino acids that may be used as a mono- or dibasic cleavage site to generate the mature peptide from the precursor. Since the length of the peptide is unknown, we cannot be certain of the cleavage site. We defined the first arginine as a likely cleavage site and used this assumption to generate the predicted bioactive peptide in Fig. 4. The predicted ORF from the consensus sequence has been deposited in GenBank (accession no. **EU797453**).

The second predicted ORF was encoded by 5% of all ESTs from the *D. ocoee* library (Fig. 3). The consensus sequence was similar to that of the glucagon superfamily, which are 28-38 aa hormones primarily expressed in the pancreas and intestine. The glucagon-like peptide from the salamander (Fig. 4) contained the conserved residues known to be necessary for the glucagon-like peptide 1 to fully function: 1 (Histidine), 4 (Glycine), 6 (Phenylalanine), 7 (Threonine), 9 (Asparagine) 22 (Phenylalanine), and 23 (Isoleucine; reviewed in Kieffer and Habener 1999). As in mammalian glucagon, the predicted glucagon-like ORF from *D. ocoee* contained a single arginine that is likely the N-terminal cleavage site (Irwin 2001) and a predicted

signal peptide (22 aa). We hypothesized that the lysine at position 28 was the last aa on the c-terminal and functions as the cleavage site since glucagon-like peptides in *Xenopus* have lysine as their final aa (Irwin et al. 1997). The *D. ocoee* glucagon-like ORF has been deposited in GenBank (accession no. **EU797454**).

Similarity of unidentified ESTs in all libraries

When we compiled all ESTs (from all three species) that showed no significant similarity to known genes in the initial BLAST search, we found that only a single contig contained ESTs from two species. Some of these unknowns were present at relatively high levels (up to 5% of total ESTs in *D. ocoee* and 7% in *E. guttolineata*) and later identified as potential pheromone components. Thus, each species apparently had a large proportion of unique transcribed sequences, even though they all transcribed the known pheromone components PMF and SPF.

Discussion

Our EST analysis revealed extensive differences in mental gland pheromone composition between genera, based on the assumption that message frequency can be used as a proxy for protein frequency in the pheromone blend itself. The changes in pheromone composition inferred from the EST data contrast with the evolutionary conservatism of the behavioral and morphological components of the courtship pheromone delivery system. Plethodontid salamanders are an ancient but morphologically and behaviorally conservative group of salamanders that contain many cryptic species described recently with genetic techniques (Highton 2000; Houck and Arnold 2003). Against this generally static background, an important transition in behavior – from scratching delivery to olfactory delivery – occurred about 19 myr ago within the genus *Plethodon* (Houck and Arnold 2003; Palmer et al. 2005; Picard 2005). Studies of the process of molecular evolution of the three pheromone components (PRF, PMF, SPF) revealed that although there is stasis in morphology and delivery behavior, the pheromone components have evolved rapidly (Palmer et al. 2007a, 2007b; Watts et al. 2004). Our EST data confirm that the variation seen in PCR amplification of the previously identified pheromone components is present in transcripts in the male mental

gland. Our results also demonstrate considerable differences in mental gland ESTs underlying the apparent conservatism in behavior in the two genera with scratching delivery, *Desmognathus* and *Eurycea*.

Both SPF and PMF are expressed by members of all three divergent clades of plethodontid salamanders, but SPF appears to be the primary component in *D. ocoee* and *E. guttolineata*. The PMF and SPF components apparently originated early in the plethodontid lineage (Palmer et al. 2007b). Palmer et al. (2007b) proposed that sodefrin evolved as a pheromone before the split of salamandrids and plethodontids, but a shift occurred within the plethodontids to a SPF/PMF blend. It appears that at least three distinct shifts in pheromone composition have occurred after the evolution of the SPF/PMF blend, during the differentiation of *P. shermani*, *D. ocoee*, and *E. guttolineata.* Two shifts have occurred during the differentiation of *D. ocoee* and *E. guttolineata*. Each appears to have independently recruited a hormone-like component as part of their pheromone secretion. It appears that in the 42 myr since these two genera diverged (Fig. 1), each has evolved different pheromone components while the morphology of the animals has remained relatively static. Both of these lineages may have recruited hormone-like compounds because the males' scratching delivery facilitates rapid entry of gland secretions into the female circulatory system. Finally, our data suggest there was a shift from the SPF-dominated pheromone to a PMF/PRF-dominated pheromone in the eastern *Plethodon* lineage, as originally postulated by Palmer et al. (2007b) by showing that *P. shermani* male mental glands express predominately PMF and PRF.

The unanswered question about mental gland expression of natriuretic- and glucagon-like peptides is whether these peptides affect the sexual behavior of the plethodontid female. Such effects are plausible given the diversity of physiological effects that these peptides are known to possess. In particular, such physiological effects may mediate a change in female behavior during courtship. For example, natriuretic peptides are known to regulate pathways that reduce stress in rats (Franci et al. 1992) and so might make a female less prone to startle and exit courtship. There is precedent

for expression of this peptide family in exocrine glands. Natriuretic-like peptides isolated from snake venoms are functionally and structurally similar to peptides expressed in the heart of mammals (e.g., Fry et al. 2005; Schweitz et al. 1992).

Members of the glucagon superfamily, helodermin and helospectins, are secreted in the venom of a lizard and reduce the blood pressure of rats (Grundemar and Högestätt 1990). Novel glucagon-like peptides identified in anuran amphibians are related to the well-known proglucagon-derived hormone found in mammals, glucagon-like peptide 1 (GLP-1; Irwin et al. 1997). These anuran peptides act as potent agonists to the human glucagon receptor, even though there are nine aa differences between the anuran glucagon-like peptides and human glucagon. The efficacy of the frog glucagon-like peptides suggests that even though the salamander glucagon-like peptide has numerous aa substitutions, it may still be physiologically active. Because glucagon mediates satiation in other vertebrates (Chelikani et al. 2005), it is possible that glucagon-like peptides in plethodontid pheromones decreases a female's perceived hunger or otherwise affect her sexual behavior.

To understand how different pheromone components evolve, one can use a simple resource allocation model to describe different selective pressures that may affect a female's likelihood to mate. The probability of a male inseminating a female will not only be a function of her reproductive status and how attractive she finds the male, but will also be affected by her need to forage and her need to engage in predation avoidance (such as moving to a less exposed environment). A first order model of the probability of insemination (P_i) given encounter between sexual partners can be written as

$$P_i = \delta(M)(1- P_d)$$

where P_d is the probability of the female departing prior to insemination and $\delta(M)$ describes the relative ability of the male to persuade the female to mate. Thus, P_d is the sum of the probabilities of the female departing to forage or avoid predators, and can be thought of as the balance of her time allocation trade-off. Male persuasiveness (M) ranges from 0 to some maximum, M_{max}, and so $\delta(M) = M/M_{max}$. The equation describes

one quadrat of a hyperbolic paraboloid, a surface which contains no local minima or maxima so two pheromone functions could evolve independently. This model suggests that males have two independent and nonexclusive ways to increase insemination success. First, males can increase their persuasiveness by manipulating females such that δ(M) increases. This aspect of persuasiveness was investigated in behavioral assays that manipulated pheromone composition (Houck et al. 2007, 2008b; Rollmann et al. 1999). A second strategy is to manipulate females such that P_d decreases. A male might use courtship pheromones (in particular the natriuretic- and glucagon-like peptides) to alter the balance of the trade-off the female makes towards courtship by reducing female vigilance or increasing her perceived satiation. In other words, the female might be more likely to invest time in courtship and insemination because she is less likely to depart from a sexual encounter.

The resource allocation model and the presence of the natriuretic- and glucagon-like transcripts in the male gland raises the possibility of a previously unrecognized pheromone action - that a courting male can modify female likelihood to successfully complete courtship by influencing the factors that determine whether a female engages and remains in courtship. However, although it is plausible that the natriuretic- and glucagon-like peptides we identified affect female behavior, either directly or in combination with pheromones, such effects remain to be verified. To date, the only direct tests of the effects of male pheromones on female plethodontids during courtship have used courtship duration as an indicator of female receptivity. Tests for behavioral and physiological effects of these peptides should be a focus of future efforts conducted with plethodontids.

In conclusion, our study has (1) provided a framework for conceptualizing how males use chemical communication to influence the sexual response of potential mates and (2) expanded our understanding of how the composition of mental gland secretions evolves. At the same time, our EST analysis highlights both the need to survey mental gland composition in a broader array of plethodontid taxa and to assay the behavioral effects of additional pheromone components.

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Table 4.1. Summary statistics of mental gland EST libraries from three plethodontid salamander species.

	D. ocoee	***E. guttolineata***	***P. shermani***
Total no. of sequences in multi (2+) sequence contigs	283	112	181
Known pheromone	127	52	135
Function classified	21	11	9
Function unclassified	135	49	37
Total no. of single sequence contigs	170	73	37
Known pheromone	0	0	1
Function classified	19	33	8
Function unclassified	151	40	28
Totals	453	185	218

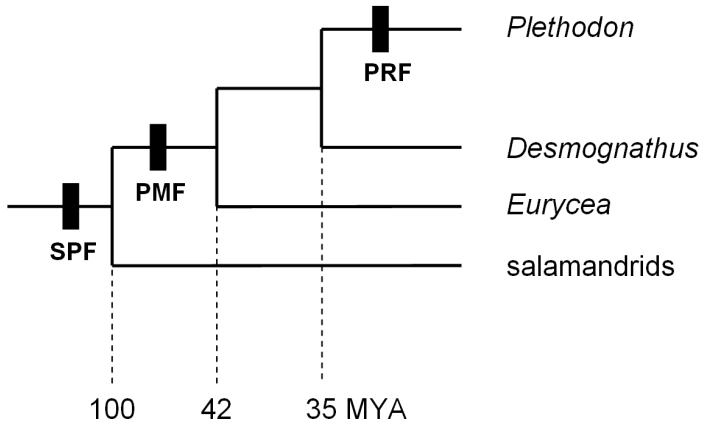

Figure 4.1. Cladogram showing the relationships of three genera of plethodontid salamanders (*Eurycea*, *Desmognathus*, and *Plethodon*) and origin of three courtship pheromones. The outgroup (salamandrids) represent a second family of salamanders (the Salamandridae) that includes *Cynops* and *Salamandra*. Relationships shown here are concordant across studies using both morphological and molecular characters (Chippindale et al., 2004; Macey, 2005; Min et al., 2005, Vieites et al., 2007). Approximate divergence times (shown at bottom) are based on albumin immunology (Larson et al., 2003). Small rectangular boxes show the point of origin of three pheromones: Sodefrin-like Precursor Factor (SPF), Plethodontid Modulating Factor (PMF), and Plethodon Receptivity Factor (PRF). Basal clades within the genus *Plethodon* (not shown) do not possess PRF (see Palmer et al., 2007b for details).

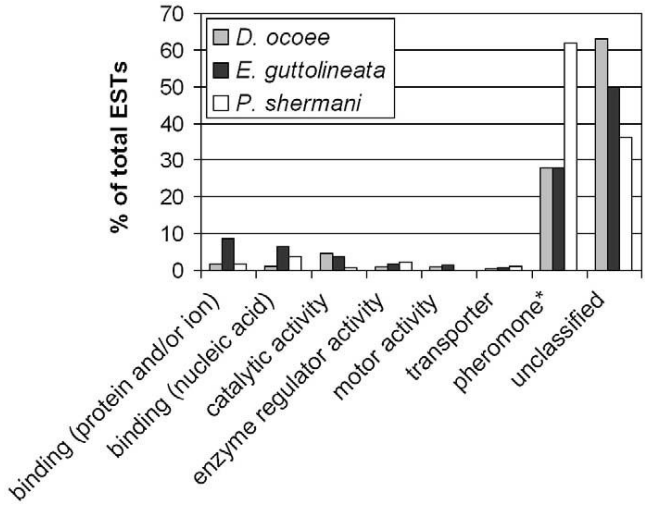

Figure 4.2. Summary of the GO-slim molecular functions of ESTs from *D. ocoee*, *E. guttolineata*, and *P. shermani*. *Pheromone is not a GO-slim function, but this term is used for convenience.

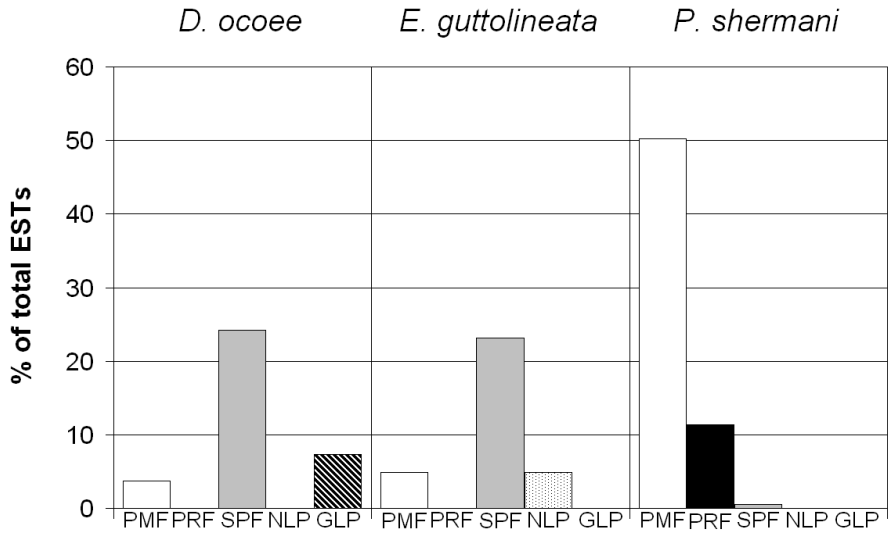

Figure 4.3. The precentage of total ESTs that were identified as the three known pheromones (PMF, PRF, or SPF), natriuretic-like peptides (NLP), or glucagon-like peptides (GLP).

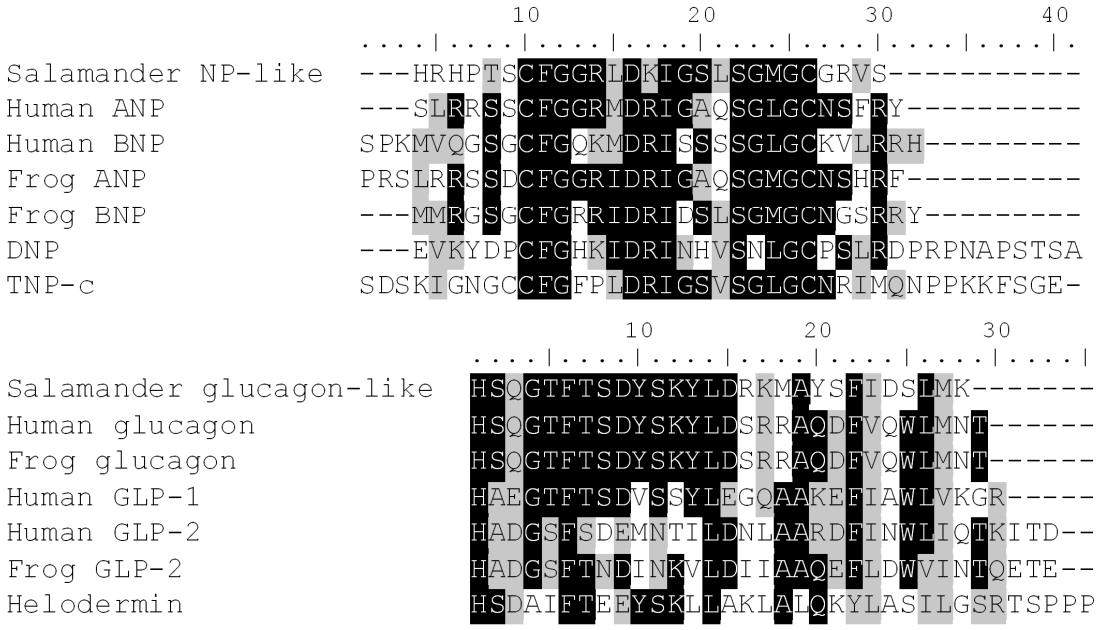

Figure 4.4. Comparison of the mature hormone-like peptides aligned using ClustalW implemented in MegAlign. Upper panel contains the aligment of salamander (*E. guttolineata*) natriuretic peptide, human atrial natriuretic peptide (ANP; GenBank accession no. **NM_006172**), human peptide precursor type B (BNP) (**NM_002521**), African clawed frog ANP (**AF287050**), anuran BNP (**AY660659**), *Dendroaspis* natriuretic peptide (DNP; Schweitz et al., 1992), and taipan natriuretic peptide (TNP) -c (**P83230**). The lower panel contains the salamander (*D. ocoee*) glucagon-like peptide, human glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2; **NP_002045**), African clawed frog glucagon and GLP-2 (**NP_001079142**), and helodermin (**P04204**). Black shading indicates identical aa and gray shading indicates similar aa that are conserved in two or more peptides (shading executed in BioEdit; Hall, 1999).

CHAPTER 5. MOLECULAR CLONING AND CHARACTERIZATION OF A PUTATIVE CYTOKINE RECEPTOR, INTERLEUKIN 6 SIGNAL TRANSDUCER, FROM CAUDATE AMPHIBIANS

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Abstract

Cytokines initiate immune responses through interactions with transmembrane receptors that signal via intracellular transduction mechanisms. Interleukin-6 signal transducer (IL6ST) is a common signaling receptor for several multi-functional cytokines in vertebrates. We amplified a full-length putative IL6ST sequence from vomeronasal tissue of the red-legged salamander (*Plethodon shermani*). The open reading frame was predicted to contain many of the conserved features found in mammalian IL6STs, such as the cytokine binding homology region and the three intracellular domains necessary for Jak/STAT signaling. Phylogenetic analysis showed that the *P. shermani* putative IL6ST grouped with the IL6STs from other vertebrates, rather than with other members of the class I cytokine receptor family. Using PCR, we showed that IL6ST RNA was present in most *P. shermani* tissues, including muscle, brain and intestine. Fragments of IL6ST amplified from three other salamander species revealed that the receptor was highly conserved among these taxa. The expression profiles, along with the structural predictions, support our hypothesis that *P. shermani* expresses a functional IL6ST and that it likely signals through a Jak/STAT pathway. Our study of this newly identified caudate IL6ST indicates that the roles that cytokines and their receptors play in amphibians may be similar to those documented in mammals.

Introduction

Cytokines and their receptors are central to the health and survival of vertebrates because they act predominately as initiators of immune responses, cell proliferation and differentiation (reviewed in Arai et al. [1990]). Cytokines can also regulate pathways as diverse as bone metabolism and placental gonadotrophin secretion (Kishimoto et al. 1995). Despite their functional diversity, cytokines share general functional and structural similarities. In particular, the four-helix bundle (or hematopoietic) family of cytokines is one of the most well-studied families because of their regulatory function and because some cytokines in this family are models for the study of receptor-ligand interactions (Boulanger and Garcia 2004).

Recent research has focused on features of hematopoietic cytokines that might promote multifunctionality. The functional redundancy and pleiotropy of the four-helix bundle cytokines is likely caused by the specificity and binding capabilities of their receptors (Ishihara and Hirano 2002). These receptors can cross-signal and co-regulate signaling cascades (e.g., Jak/STAT), allowing specialized and overlapping responses in different tissues. Cytokines in the four-helix bundle family bind to a hematopoietic superfamily of cytokine receptors that is characterized by a cytokine binding homology region on the amino terminal of the receptor (Wells and de Vos 1996). When the cytokine approaches the cell surface, it binds to one (or more) receptors and forms a multi-subunit complex. These complexes transduce an intracellular signal by incorporating a signaling receptor that activates second messenger cascades. One of the most common signal transducing cytokine receptors is the interleukin-6 signal transducer (IL6ST), a receptor activated by multiple hematopoietic cytokines and expressed in most human cell types (Bravo and Heath 2000).

Mammals have been the primary research system for the study of cytokine/IL6ST receptor interactions. For example, targeted mutation of IL6ST results in embryonic lethality and single nucleotide polymorphisms in IL6ST have been linked to several diseases in humans including heart attacks and diabetes (e.g. Benrick et al. 2008; Wang et al. 2005; Yoshida et al. 1996). Nevertheless, little is known about cytokines or their receptors in other vertebrate systems, even though cytokines are likely to be important for generalized immune responses. Comparative analyses have provided insights into the evolution of other vertebrate cytokines on a site-by-site basis (Boswell et al. 2006; Krausea and Pestka 2005). Information on the function and evolution of the IL6ST may also help elucidate the roles that conserved amino acids play in receptor activation (Jones and Rose-John 2002).

Studies of *Xenopus laevis* and *Ambystoma mexicanum* have shown that amphibians have the majority of the elements found in mammalian immune systems, including cytokines (Carey et al. 1999). Salamanders are basal tetrapods and are especially useful for comparative analysis because they can help define early tetrapod structure and function. Work on other amphibians and mammals suggests that

cytokines may be responsible for initiating immune responses to pathogens and other stressors that threaten salamanders in their natural environment (Carey et al. 1999), but to date, knowledge of cytokine receptors in amphibians is very limited. The only published information available on IL6ST for amphibians are putative *X. laevis* IL6ST sequences in GenBank and a sequence of the *X. laevis* IL6ST cytokine binding homology domain (Bravo et al. 1998).

We used the red-legged salamander, *Plethodon shermani*, to investigate the expression of IL6ST in caudate amphibians. We chose this particular species because the males use a four-helix cytokine-like protein as a pheromone during courtship (Rollmann et al. 1999). Because this male pheromone is delivered to and activates the olfactory system (Wirsig-Wiechmann et al. 2006), we wanted to determine whether the IL6ST was expressed in that tissue. Consequently, our major objectives for this study were to characterize the IL6ST receptor and its expression in various tissues of *P. shermani*. We also identified IL6ST fragments in other salamander species so that we could compare divergence of this receptor in plethodontids and in a related salamander family, the Salamandridae.

Materials and methods

Animal and tissue collection

Adult *P. shermani* were collected from a natural population (see Table 5.1 for locality information). To investigate the expression levels in different *P. shermani* tissues, approximately 50 mg of each tissue was collected from one female and one male and immediately placed in 500 µl of RNAlater (Ambion; Austin, TX). Animals were sacrificed by decapitation before tissue collection, in accordance with the standards of the Institutional Animal Care and Use Committee at Oregon State University (ACUP 3549 to Lynne D. Houck). A variety of tissues were tested because IL6ST is ubiquitously expressed in mammals (reviewed in Kishimoto et al. 1995). The tissues sampled were olfactory mucosa (containing both olfactory and accessory epthelia), skin (from the neck), brain, muscle, liver, eye, reproductive tissue (testes or oviduct), pancreas, and intestine.

To determine whether IL6ST was present in two related salamander families, we collected genomic DNA from two other plethodontid species (*P. vehiculum* and *Aneides flavipunctatus*) and one salamandrid (*Taricha granulosa*). In all cases, ~ 5 mm of the posterior end of the tail was taken from live animals for genomic DNA extraction by squeezing the tail tip with scissors, causing the tail tip to autonomize at the pressure point.

Nucleic acid extraction

The RNA from all *P. shermani* tissues was removed from RNAlater and immediately submerged in Tri Reagent (Invitrogen; Carlsbad, CA). The RNA was extracted according to the manufacturer's protocol. The purified RNA was resuspended in 30 µl of RNase-free H_2O and stored at -80° until further use.

Genomic DNA was extracted from the tail tips of other salamander species by an overnight digestion with proteinase K, followed by standard phenol/chloroform extraction. DNA was precipitated with 5M NaCl and ethanol, washed with 70% ethanol, dried and redissolved in 100 µL sterile ddH_2O. DNA was stored at -80° until use in PCR.

PCR

Our first objective was to amplify the entire IL6ST receptor sequence from the focal species, *P. shermani*. Because the human IL6ST gene has large introns, we used cDNA as a template to obtain the full-length expressed sequence from *P. shermani.* Olfactory tissue RNA was reverse-transcribed using the ImProm-II™ reverse transcription system (Promega; Madison, WI) into cDNA. The cDNA was used as a template for the subsequent reactions to obtain the full-length cDNA sequence, which were all preformed with GoTaq DNA polyermase (Promega). First-strand 5'RACE-ready cDNA was synthesized with the FirstChoice® RLM-RACE kit (Ambion) and 3' RACE-ready cDNA was made using the ImProm-II™ reverse transcription system (Promega). Degenerate primers were designed to align with two regions of human IL6ST (amino acid residues 778-785 and 903-910; Figure 5.1) that are conserved in other vertebrates and that annealed within the last exon of humans and mice (degenerate IL6ST forward and reverse; Table 5.2). These primers were used to obtain the first

fragment of IL6ST from genomic *P. shermani* DNA. Subsequent overlapping species-specific primers (not shown) were then designed from amplified regions from *P. shermani.* These sets of species-specific primers were used to obtain regions closer to the 5' and 3' ends of the open reading frame (ORF) using a 'primer walking' technique with olfactory tissue cDNA. Once IL6ST 5' and 3' untranslated regions (UTR) sequences were obtained from 5' and 3' RACE, *P. shermani*-specific primers which annealed outside the coding region were designed to amplify the entire coding sequence (*P.s.* forward and reverse; Table 5.2). These primers were used to amplify the coding sequences with the high-fidelity *Pfu* polymerase (Stratagene; La Jolla, CA).

To determine whether IL6ST was present in related salamanders, we amplified a 510 bp region of IL6ST from *P. vehiculum*, *A. flavipunctatus* and *T. granulosa* using GoTaq DNA polymerase (Promega). To avoid potential intron/exon boundaries, we designed primers that sat after the last splice site in mice and humans (salamander IL6ST forward and reverse; Table 5.2). All IL6ST amplicons from all species were cloned using the pGEM®-Teasy vector system (Promega). After propagation of the bacteria on LB (Luria Broth) agar medium containing ampicillin, single colonies were chosen using blue/white screening. These colonies were cultured overnight in ampicillin-containing LB medium. Plasmid DNA was isolated from each culture with the Qiaprep spin miniprep kit (Qiagen; Valencia, CA) and sent to the Nevada Genomics Center for sequencing using the SP6 and T7 universal primers, and then unique sequencing primers to obtain the full length sequence.

RT-PCR analysis of IL6ST expression in P. shermani *organs*

To determine the distribution of IL6ST expression across tissues in *P. shermani*, total RNA from male and female *P. shermani* tissues was used to generate first-strand cDNA with the ImProm-II™ reverse transcription system (Promega). For each tissue type, 175 ng of RNA was added to each reverse-transcription reaction. One µl of the resulting cDNA was used as a template to amplify a 200 bp fragment of IL6ST from the organs with two primers, *P. shermani* fragment forward and reverse (Table 5.2). A ~170 bp region of β-actin (GenBank accession no. FJ824845) was simultaneously amplified from the same cDNA as a control for the efficiency of reverse transcription

using the primers *P.shermani* actin forward and reverse. Products were run on a 1.75% agarose gel and visualized under UV light.

In silico analyses

Several bioinformatics programs were used to determine whether the *P. shermani* IL6ST shared features common in other vertebrate sequences. The length of the signal peptide was estimated using SignalP (Bendtsen et al. 2004) and a search for a transmembrane domain was conducted with TMHMM 2.0 (Krogh et al. 2001). The specialized BLAST for conserved domains (NCBI Conserved Domain Database; Marchler-Bauer et al. 2007) was used to predict domains present in the *P. shermani* IL6ST. Because IL6ST is gylcosylated in humans, potential glycosylation sites were predicted using NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc). The mass of the IL6ST protein was predicted using a protein and peptide molecular weight calculator (www.basic.northwestern.edu/biotools/proteincalc.html). The percent identity between IL6STs of vertebrates was calculated using BLASTP 2.2.19 (Altschul et al. 1997; Altschul et al. 2005).

Full-length DNA sequences were translated to amino-acid sequences and then aligned using the ClustalW algorithm, with minor adjustments made by eye. The MEGA (version 4.0; Tamura et al. 2007) sequence alignment editor was used for constructing and editing all sequence alignments. A tree of vertebrate class 1 cytokine receptors was generated from these alignments using the minimum evolution method (Rzhetsky and Nei 1992). All positions containing gaps and missing data were eliminated from the dataset. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985).

Results

Isolation of a full-length IL6ST from P. shermani

The primer walking strategy, combined with 5’ and 3’ RACE, was successful in amplifying the full-length IL6ST from *P. shermani* olfactory tissue. A contiguous sequence was obtained using the two primers that annealed outside the coding region.

The predicted ORF was 2748 bp and encoded a 915 amino acid protein (GenBank accession no. FJ824846). The length of the *P. shermani* IL6ST ORF (915 amino acids) is comparable to other vertebrates. The longest IL6STs reported are in chickens and humans, each of which has a 918 amino acid IL6ST. The shortest known IL6ST (881 amino acids) is from the frog, *X. laevis*. The predicted molecular weight of the *P. shermani* unglycosylated IL6ST was approximately 102 kDa. The use of 5' and 3' RACE also allowed the estimation of the IL6ST untranslated regions (UTR). The *P. shermani* IL6ST 5' and 3' UTRs were 142 bp and 433 bp, respectively, comparable to the UTRs found in human IL6ST mRNA (Hibi et al. 1990).

As predicted, the *P. shermani* IL6ST open reading frame (ORF) had many features conserved in other vertebrates. The extracellular region of the *P. sherman*i IL6ST was predicted to be 625 amino acids, with a transmembrane domain of 23 amino acids and the remaining 245 residues comprising the intracellular portion of the protein. SignalP predicted a signal peptide of 19 amino acids for the *P. shermani* IL6ST, a size similar to the 22 amino acid signal peptide in human IL6ST (Szalai et al. 2000) and the 26 amino acid peptide in the chicken (Geissen et al. 1998). After cleavage of the signal peptide, mature human IL6ST contains six fibronectin type III (FBN) extracellular domains that each play a role in receptor functionality: one with an immunoglobin-like conformation, two comprising the cytokine receptor homology domain, and an additional three FBN repeats (Bravo and Heath 2000). The *P. shermani* amino acid sequence was predicted to contain the ligand-binding immunoglobulin-like domain, cytokine receptor homology domain and other three fibronectin type III domains. The *P. shermani* IL6ST contained the conserved amino-acid motif WSXWS as well as the four conserved cysteines (*P. shermani* residues 140, 154, 182, 191; Figure 5.1) necessary for the functionality of the cytokine receptor homology domain (Figure 5.1). The extracellular region of IL6ST also contains 11 potential *N*-glycosylation sites, but one predicted sequon contains a proline and is probably not glycosylated.

The conserved intracellular region of IL6ST is responsible for transducing the cellular signal and three cytoplasmic motifs in particular are important for initiating the signal response. The first, Box1, was identical in both human and *P. shermani* except

for a conservative substitution of valine to isoleucine at residue 661 in *P. shermani* (Figure 5.1). The first six amino acids of Box2 were identical in *P. shermani* and human, and only the three of the second six (salamander residues 703-705) were different than the human IL6ST. Similar conservation was seen in Box3; the salamander IL6ST was identical in amino acid composition to the human IL6ST in 8 out of the 11 amino acids. The Box3 region of the salamander also contained the conserved YXXQ amino acid sequence, a motif that interacts with the second messenger, STAT3, in humans (Stahl et al. 1995).

IL6ST appears highly conserved between *P. shermani* and other vertebrates. The gene tree clearly grouped the *P. shermani* sequence with the IL6STs, rather than OSMr, LIFR, or CSF3R (Figure 5.2), suggesting the salamander sequence is homologous to other vertebrate IL6STs. The subtree containing fish, avian, amphibian, and mammalian IL6STs is generally consistent with the accepted consensus vertebrate tree, although the *P. shermani* sequence was not placed as a sister group to *Xenopus* (Figure 5.2). The polytomy involving the two amphibians reflects the fact that *P. shermani* and *Xenopus* shared about the same percentage of amino acid identity as did *P. shermani* and humans. At the amino acid level, the *P. shermani* IL6ST shared 45% identity with *Xenopus* IL6ST, 53% with chicken IL6ST and 49% with human IL6ST.

Tissue distribution of IL6ST expression

The RT-PCR analysis showed that IL6ST was expressed in most tissues in both male and female adult *P. shermani*. We surveyed portions of olfactory tissue, skin, brain, muscle, liver, eye, reproductive tissue (testes or oviduct), pancreas, and intestine. Although RT-PCR can only be used as a rough approximation of the level of RNA present in tissues, we noted some large qualitative differences between some tissues (Figure 5.3). No expression was seen in the skin of the female and only weak expression in the skin of the male. The female also had low expression in its muscle tissue, as compared to the male. Overall, the expression patterns were very similar between the sexes and IL6ST RNA was detected in almost every tissue examined (Figure 5.3).

Comparison of salamander IL6STs

Fragments of IL6ST that spanned a portion of the intracelluar region of IL6ST were isolated from four salamander species: *P. shermani*, *P. vehiculum*, *Aneides flavipunctatus* and *T. granulosa* (deposited in GenBank under the accession nos. GQ176294-GQ176296). These 500-510 bp fragments encode predicted protein fragments that share many of the conserved residues necessary for function in other species. The fragment was located near the C-terminus of the predicted protein and includes the Box3 domain that is important for activation of the signal transduction cascade. The Box3 domain of the other three salamanders was identical to that of *P. shermani* except for a single substitution of serine to threonine (*P. shermani* residue 772) in *T. granulosa*. Amino acid identity in the 510 bp region varied from 93% between closely related *P. shermani* and *P. vehiculum*, to 62% between *P. shermani* and the most distantly related species, *T. granulosa*. A consensus tree of the salamander IL6ST fragments (not shown) generally agreed with phylogenies based on morphology and DNA (mt and nuclear) sequences (Weins et al. 2005; Vieites et al. 2007).

Discussion

We amplified a full-length IL6ST-like sequence from a plethodontid salamander, *P. shermani*. Overall, the high sequence similarity and multitude of conserved regions between salamanders and humans strongly suggest that the IL6ST is functional in salamanders and mediates signaling through a Jak/STAT pathway. Finding such highly conserved regions implies that many of these sites are crucial for correct conformation and binding in all vertebrates. The *P. shermani* ORF contained many predicted regions important for receptor function. It contained a predicted cytokine binding homology domain with four conserved cysteines, as well as three intracellular box motifs necessary for Jak/STAT signaling. The presence of IL6ST in salamanders suggests that the ligands for this receptor are also probably part of the molecular basis for immune response in amphibians. Cytokines such as leukemia inhibitory factor, ciliary neurotrophic factor and oncostatin M all signal via IL6ST in mammals (Bravo and Heath 2000). However, identifying these cytokines in non-model organisms is difficult because of the low sequence homology between these cytokines in distantly related groups (Hill et al. 2002).

IL6ST mRNA was detected in a variety of tissues in *P. shermani*. Both male and female salamanders expressed IL6ST in almost every tissue examined, although the expression levels varied across tissue types. This expression pattern is consistent with the ubiquitous expression of IL6ST in different mammalian cell lines (Hibi et al. 1990). IL6ST in salamanders probably mediates pleiotrophic cytokine effects as it does in other vertebrates (Ishihara and Hirano 2002). Interestingly, IL6ST was expressed in the olfactory tissue, suggesting IL6ST may have a special function in *P. shermani*, one previously unrecognized in tetrapods. Male *P. shermani* use a cytokine-like protein, Plethodontid Receptivity Factor, to increase female receptivity during courtship (Rollmann et al. 1999). Males deliver the pheromone from a specialized chin gland to the nasal cavity openings of the female, resulting in neuronal stimulation in the vomeronasal organ (Wirsig-Wiechmann et al. 2006). The pheromone stimulates receptor neurons, which leads to the opening of membrane channels and action potentials in the receptor cells, but the transmembrane receptors for this pheromone remain uncharacterized (Rollmann et al. 1999). Class 1 helical cytokines such as interleukin-6 can cause membrane depolarization in neurons, although it is not known what receptors are responsible for mediating this response (Xia et al. 1999). The functional redundancy of IL6ST may allow it to bind to a unique cytokine receptor subunit in *P. shermani*. Specificity for a pheromonal function versus an immune response may depend on additional receptor subunits and the localization of subunit expression in various organs.

Partial IL6ST sequences were amplified from the genomic DNA of the four salamander species surveyed in this study. The topology of the phylogenetic tree constructed for IL6ST fragments was congruent with the patterns of plethodontid diversification that have been well-supported by morphological, mtDNA, and nuclear DNA data (Chippindale et al. 2004; Vieites et al. 2007). This wide-spread presence of IL6ST RNA in caudate amphibians broadens our understanding of the evolution of this receptor in basal tetrapods by documenting an ancient pattern of conservation within homologous genes.

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Table 5.1. Locality data for the four salamander species from which the cytokine receptor IL6ST was amplified.

Species	Location	Latitude	Longitude
Aneides flavipunctatus	Jackson County, OR	042°05′18″N	123°01′39″W
Plethodon shermani	Macon County, NC	035°10′48″N	083°33′38″W
Plethodon vehiculum	Benton County, OR	044° 29′49″N	123°34′05″W
Taricha granulosa	Benton County, OR	044° 28′49″ N	123°39′35″W

Table 5.2. Primers used in the IL6ST study.

Primer name	Sequence (5'→ 3')
degenerate IL6ST forward	TCA AGR TCY GAR TCH ACH CAG CC
degenerate IL6ST reverse	ACW CTY TGY GGS AWR TAA CTT TT
P. shermani forward	TCT GGG TGG CAC AGG ATG ACG T
P. shermani reverse	CGA GAG GAT AAC AGG CAG ATG
salamander IL6ST forward	GGV GGV TCI TCR TGY ATG TC
salamander IL6ST reverse	YTG YGG IAD STA ACT TTT
P. shermani tissue forward	GGA TAT TGG AGT GAT TGG AG
P. shermani tissue reverse	CCT AAA ACT TTC CCG TTG GC
P. shermani actin forward	CTG GCA CCT AGC ACA ATG AA
P. shermani actin reverse	TGT TTA GAA GCA TTT ACG GTG

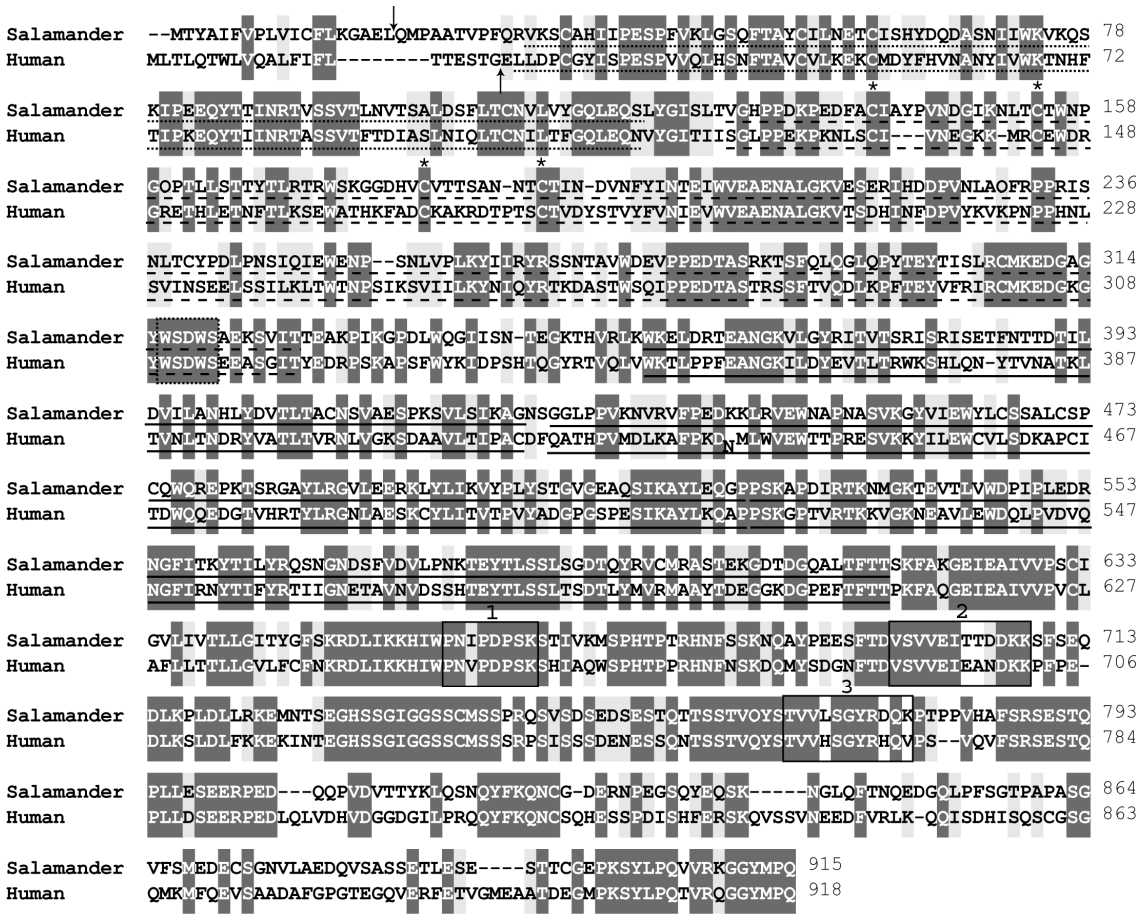

Figure 5.1. Full-length deduced amino acid sequence of IL6ST from a salamander, *P. shermani*, aligned with human IL6ST (GenBank accession no. BC117402). The symbols indicate known and predicted features of human and salamander IL6ST, respectively: vertical arrows represent the signal peptide boundaries; dotted lines highlight the immunoglobulin-like domain; dashed lines underline the cytokine-binding homology region; solid lines indicate the three remaining fibronectin type III domains; dotted box contains the WSXWS motif; box1, box2, and box3 motifs are indicated by solid boxes; and conserved cysteines are marked by asterisks. Black shading indicates identical amino acid and gray shading indicates similar amino acid that are conservative substitutions (shading executed in BioEdit; Hall 1999).

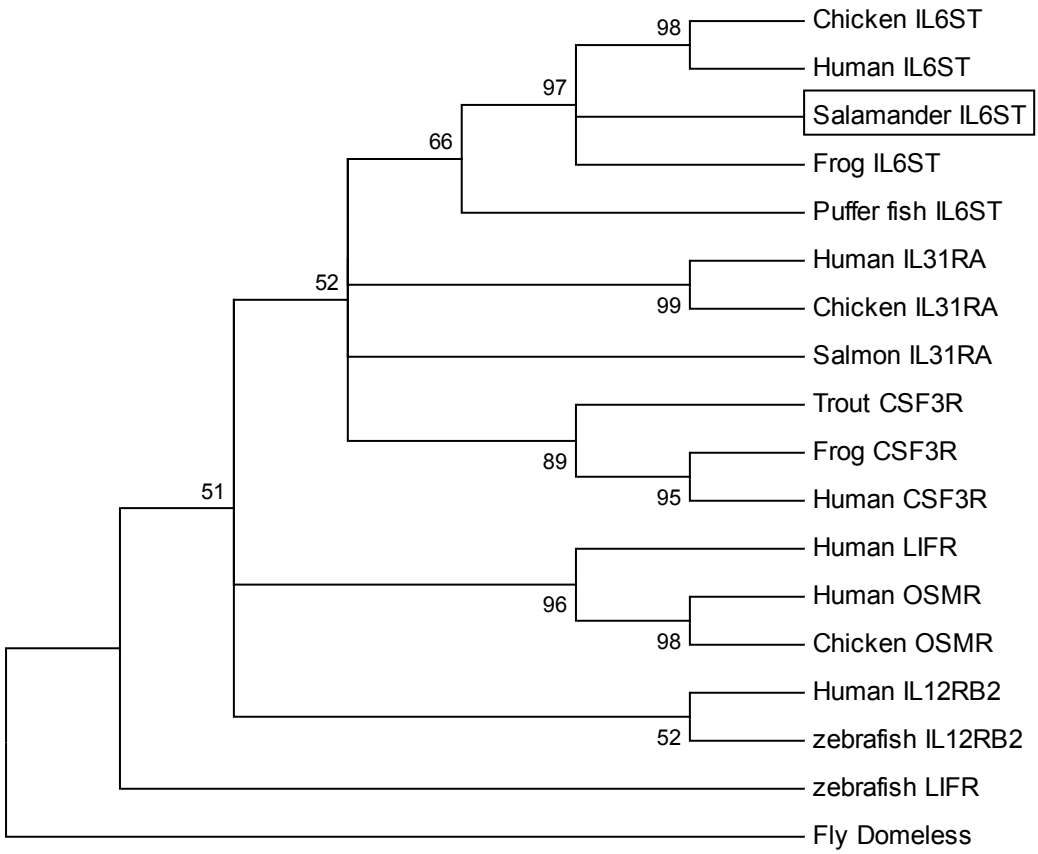

Figure 5.2. Phylogenetic tree of cytokine receptors constructed using the minimum evolution method, rooted with fly (*Drosophila melanogaster*) domeless cytokine receptor. GenBank accession numbers: Salamander (*P. shermani*) IL6ST (FJ824846), Frog IL6ST (AF041845), Chicken IL6ST (NM_204871), Human IL6ST (BC117402), Puffer fish IL6ST (AY374498), Salmon IL31RA (ACN10970.1), Chicken IL31RA (XP_424732.1), Human IL31RA (NM_139017), Zebrafish IL12RB2 (NP_001106977.1), Human IL12RB2 (NP_001550.1), Trout CSF3R (CAE83614), Frog CSF3R (AAH77792), Human CSF3R (Q99062), Human LIFR (NP_001121143), Zebrafish LIFR (NP_001014328.1), Human OSMR (NP_003990), Chicken OSMR (XP_425020.2), and Fly domeless (AAN64333).

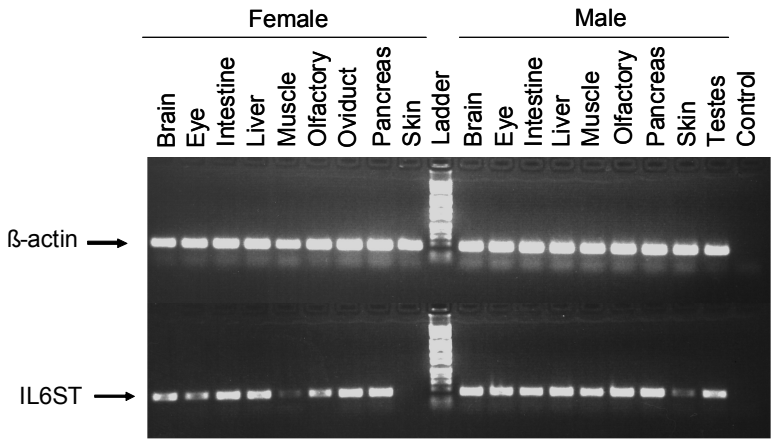

Figure 5.3. Expression of IL6ST (and β-actin) in various female and male *P. shermani* tissues.

CHAPTER 6. EXPRESSION OF OLFACTORY SIGNAL TRANSDUCTION COMPONENTS IN THE MAIN OLFACTORY AND VOMERONASAL ORGANS OF A CAUDATE AMPHIBIAN

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Abstract

Multigene families of G-protein-coupled receptors are responsible for binding to odorants and initiating responses in vertebrate olfactory neurons. We investigated the evolution of the molecular basis of chemoreception in an understudied vertebrate lineage: caudate amphibians. Our focal species, *Plethodon shermani*, is a terrestrial salamander that uses chemical cues during social interactions. In particular, a male delivers protein pheromones to the female's nares during courtship, and the pheromones are then shunted to the vomeronasal organ. We focused on two families of G-protein-coupled receptors that are commonly expressed in either the vomeronasal organ or main olfactory epethelia. First, we used PCR on cDNA from olfactory and vomeronasal tissue to examine the presence and diversity of one family of olfactory receptors (ORs) and one family of vomeronasal receptors (V2Rs). Analyses of isolated sequences showed that ORs were 97-99% similar and all belonged to the class II family of vertebrate ORs. Our sequence analyses of the V2Rs showed that the V2R family appears to be as diverse in *Plethodon* as in other vertebrates. In addition, we demonstrated the expression of other members of the G-protein-coupled receptor signal cascade. We amplified fragments of specific G proteins that are coexpressed with specific receptor families. We isolated G_{olf} (coexpressed with ORs), $G_{\alpha i2}$ (coexpressed with V1R family of vomeronasal receptors), $G_{\alpha o}$ (coexpressed with V2Rs) and a vomeronasal-specific ion channel, transient receptor potential protein C 2 (trpc2), which is necessary for signal transduction of pheromones in mammalian vomeronasal neurons. We verified patterns of expressions in the olfactory and vomeronasal epithelia with *in situ* hybridization using probes for trpc2 and G_{olf}. Our work indicates that the salamander olfactory system contains many elements of olfactory signal transduction identified in other vertebrates.

Introduction

The organizational complexity of olfactory organs varies across vertebrates. In fish, all odorants are detected by a single olfactory organ. In the tetrapod lineage, a separate olfactory organ evolved: the accessory olfactory or vomeronasal organ (VNO). Amphibians are the first group to possess these two olfactory systems, one mediated by

the main olfactory epithelium (MOE) and the other by the vomeronasal organ (VNO). Although secondary losses of the VNO have occurred, functional VNOs are present in many vertebrate taxa and are necessary for prey detection and social interactions (Halpern 1987; Halpern and Martinez-Marcos 2003).

Molecular signal transduction of odorants is mediated by several families of G-protein-coupled receptors that are present in the sensory organs of vertebrate olfactory systems. The three most well-studied families, the olfactory receptors (ORs) and two vomeronasal receptors (V1Rs and V2Rs), are present in all sequenced vertebrate genomes (Niimura and Nei 2005; Shi and Zhang 2007). These receptors are coexpressed with specific G proteins: ORs are coexpressed with G_{olf}, V1Rs with$G_{\alpha i2}$, and V2Rs with $G_{\alpha o}$ (Dulac 2000). In fish, all three receptor families are expressed in the olfactory rosette (Cao et al. 1998; Pfister and Rodriguez 2005). In anuran amphibians, both ORs and V1Rs are expressed in the MOE (Wakabayashi et al. 2007; Date-Ito et al. 2008). Mice and rats express ORs in the MOE and V1R and V2Rs in the VNO, but goats express ORs and V1Rs in the MOE, a pattern similar to that of anurans (Buck and Axel 1991; Matsunami and Buck 1997). Thus, evolutionary recruitment and expression (localization) of ORs, V1Rs and V2Rs and coexpressed G proteins varies across tetrapod taxa and even within the class mammalian. This variation makes it difficult to predict what types of chemicals each receptor family is specialized to detect. Exploring a number of diverse vertebrate taxa may help clarify whether the plasticity of receptor expression is influenced by the ecology and sociality of an organism. The MOE was once thought to be used for detecting odorants, while the VNO was functioned in intraspecific (pheromone) communication between individuals in mammals (Halpern and Martinez-Marcos 2003). However, many exceptions to these generalizations exist suggesting that these categories may not reflect the true specializations of the two organs (Eisthen 1997; Baxi et al. 2006).

In this context, we characterized the presence and localization of genes involved in the mediation of chemical communication in the main and accessory olfactory systems of a caudate amphibian, *Plethodon shermani*. Olfactory receptor expression has been described in three anurans: *Xenopus tropicalis*, *X. laevis, Rana esculenta*, and one

caudate: *Ambystoma tigrinum* (Freitag et al. 1998; Marchand et al. 2004). However, receptor organization in both the VNO and MOE has only been investigated in one amphibian genus, the clawed frogs (*Xenopus*). Key differences exist between *Xenopus* and *P. shermani* that may have differentially shaped receptor expression. *Plethodon shermani* is terrestrial, an ecological adaptation that may influence the V1R and V2R receptor repertoire (Shi and Zhang 2007). In addition, the documented use of chemical cues in plethodontid communication makes them a useful caudate representative for understanding chemoreception in amphibians. In particular, plethodontid salamanders respond to chemical cues that are an important part of their social interactions. These chemical cues are used to relay information about species identity (Dawley 1986), sex (Dawley 1984),and territories (Horne and Jaeger 1988). Plethodontids respond to both volatile and non-volatile chemical cues (Palmer and Houck 2005; Dantzer and Jaeger 2007).

Plethodon shermani is an emerging model system for chemoreception in vertebrates. In particular, *P. shermani* has been the focus of studies of specific pheromones used during courtship. For example, two unrelated proteins have been identified as courtship pheromones that modulate female behavior during courtship in *P. shermani* (Rollmann et al. 1999; Houck et al. 2007). These pheromone proteins activate cells in the VNO, which in turn stimulate higher brain centers (Wirsig-Wiechmann et al. 2006; Laberge et al. 2008). One of the motivations for this study is to better understand receptor organization in order to determine which families of receptors may be mediating the response to courtship pheromones in *P. shermani* salamanders. Our primary goal was to identify and isolate classes of G-protein-coupled receptors (and associated G proteins) that are expressed in the *P. shermani* MOE and VNO. We also investigated the expression of a VNO-specific ion channel, transient receptor potential cation channel, subfamily C, member 2 (trpc2), which is present in most vertebrate genomes and is important in pheromone signal transduction in mammals (Liman et al. 1999; Grus and Zhang 2009).

Materials and methods

Animal collection and RNA extraction

Six adult female *P. shermani* were collected from Macon County, North Carolina (035°10′48″N 083°33′38″W) during their breeding season and with permits from the North Carolina Department of Fish and Wildlife. Animals were sacrificed by decapitation in accordance with the standards of the Institutional Animal Care and Use Committee at Oregon State University (LAR 3549 to Lynne D. Houck). Tissue from the main and accessory (vomeronasal) epithelium was carefully removed by dissection from the nasal cavity. Tissue was immediately preserved in RNA*later* (Ambion, Austin, TX). To extract RNA, the tissue was removed from RNA*later* and immediately homogenized in TRI Reagent (Ambion). RNA was extracted according to the manufacturer's instructions.

RT-PCR isolation and cloning of gene sequences

Degenerate polymerase chain reaction (PCR) was used to isolate *P. shermani* homologs of system-specific genes from the main and accessory olfactory systems. This strategy has been used previously for ORs, V1Rs, and V2Rs in amphibians (Hagino-Yamagishi et al. 2004; Marchand et al. 2004; Date-Ito et al. 2008). Total RNA extracted from the tissues was reverse-transcribed into cDNA using the ImProm-II™ reverse transcription system (Promega, Madison, WI). This cDNA was then used as a template for degenerate PCR. The primers used for each gene/gene family are listed in Table 1. All PCR amplicons were generated with GoTaq (Promega). First-strand 5'RACE-ready cDNA was synthesized with the FirstChoice® RLM-RACE kit (Ambion).

All amplicons were cloned using the pGEM®-Teasy vector system (Promega). After propagation of the bacteria on Luria Broth (LB) agar medium containing ampicillin, single colonies were chosen using blue/white screening. These colonies were propagated overnight in liquid ampicillin-containing LB medium. Plasmid DNA was isolated from each culture with the Qiaprep spin miniprep kit (Qiagen, Valencia, CA).

The length of signal peptides was estimated using SignalP (Bendtsen et al. 2004) and searches for transmembrane domains were conducted with TMHMM 2.0 (Krogh et

al. 2001). The percent identity between genes of *P. shermani* and other vertebrates was calculated using BLASTP 2.2.19 (Altschul et al. 1997; Altschul et al. 2005).

Gene tree construction

The MEGA (version 4.0; Tamura et al. 2007) sequence alignment editor was used for constructing and editing all sequence alignments. Receptor DNA sequences were translated to amino-acid sequences and then aligned using the ClustalW algorithm. Trees were generated from these alignments using the minimum evolution method (Rzhetsky and Nei 1992). Branches corresponding to partitions reproduced in fewer than 50% bootstrap replicates were collapsed. The percentage of replicate trees (if greater than 70%) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985).

In situ *hybridization*

Preparation of riboprobes for the *in situ* hybridization analyses followed a protocol previously described by Butler et al. (2001). Sense and antisense DIG-labeled riboprobes were synthesized with a DIG RNA labeling mix according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany) using SP6 or T7 RNA polymerase (Fisher Scientific, Pittsburgh, PA). The fragments of genes isolated from *P. shermani* cDNA in the pGEM vector were used to synthesize RNA probes. PCR was run to make template for the reverse transcription using 50 ng of plasmid DNA as template. One µg of each PCR product was then used as templates for either to synthesize sense and anti-sense DIG-labeled probes using standard procedures. PCR products were incubated with the appropriate polymerase, digUTP labeling mix (Roche Diagnostics), DEPC water, RNasin, buffer, and DTT (Takara) at 37°C for 2 hours. DNA template was then degraded using 1 unit of DNase1 (Fisher Scientific).

To obtain sections of the nasal cavity, females were decapitated and the heads were fixed in 4% paraformaldehyde (in PBS) overnight. The heads were decalcified in 10% EDTA in DEPC water for 2 days, rinsed with distilled water, and submerged in 30% sucrose in DEPC water in PBS overnight. The heads were embedded in OCT (Fisher) and sectioned using a cryostat. Sections 18 µm thick were mounted onto Superfrost plus slides coated with 10% poly-L-lysine.

Hybridization of the probes was carried out according to Hagino-Yamagishi et al. (2004). The slide-mounted sections were rinsed twice with SSPE, incubated with 20 units of proteinase K for 30 min, and rinsed in 0.3 M NaCl and 0.002 M EDTA (pH ~7.4). The sections were then refixed in 4% paraformaldehyde in PBS for 10 min. The sections were incubated with 0.2N HCL for 15 min, rinsed with SSPE, and incubated with 0.1M triethanolamine (pH 8) for 5 min. After two sequential additions of acetic anhydride, probe hybridization was carried out at 60°C overnight. Hybridization was carried out in a hybridization solution containing 5 ng/ul cRNA probe, 50% formamide, 1% blocking reagent (Omnipure, Caldwell, ID), 5X SSC, 5mM EDTA, 0.5mg/ml Torula RNA (Sigma, St. Louis, MO), 0.1 mg/ml heparin (Fisher Scientific), 0.1% Tween. After the overnight hybridization, sections were incubated with 5 µg/ml RNase A for 30 min at 37°C, incubated in 50% formamide for 45 min at 60°C, and rinsed three times with 100mM Tris (pH 7.5) and 150 mM NaCl. The sections were then incubated for two hours in 100mM Tris (pH 7.5), 150 mM NaCl, and blocking reagent (Roche Diagnostics). After incubation, the slides were incubated with alkaline phosphatase–conjugated anti-DIG Fab fragment antibody (Roche Diagnostics) for 1 hr, washed three times with 100mM Tris (pH 7.5) and 150 mM NaCl, and equilibrated in alkaline phosphatase buffer for 10 minutes before signals were visualized with the BM purple chromogenic substrate (Roche) for 24-48 hrs. The reaction was stopped with a solution of MEMFA fixative in DEPC water.

Digital photography

Slides were photographed using an Olympus DP70 camera. The contrast of the photographs was manually adjusted in Adobe Photoshop and labels were added to the photographs using Microsoft PowerPoint.

Results

Expression of genes in the MOE

We successfully amplified three types of genes from female *P. shermani* olfactory tissue. From olfactory cDNA, we amplified members of one G-protein-coupled receptor family, the ORs, and their associated G protein, G_{olf}. We also amplified the V1R-associated G protein $G_{\alpha i2}$. We sequenced 30 OR clones, resulting in

nine unique amino acid sequences and three sequences with premature stop codons. These 513 bp fragments appeared to be closely related. However, 20 DNA substitutions were found across the 9 unique sequences, resulting in 18 variable amino acid sites. All the *P. shermani* OR fragments showed highest similarity to Class II olfactory receptors (Freitag et al. 1995). The *P. shermani* fragments were most similar to an olfactory mRNA isolated from the two other salamander species for which OR sequences are available, *Necturus maculosus* and *Ambystoma tigrinum* (Figure 6.1).

The G_{olf} fragment amplified from *P. shermani* cDNA was 954 base pairs in length and showed strong similarity to other vertebrate G_{olf} proteins (Table 2). The *in situ* hybridization clearly demonstrated that G_{olf} was expressed solely in the female *P. shermani* main olfactory epithelium and not in the lateral diverticula where the VNO was located (Figure 6.2). The expression of G_{olf} solely in the MOE was consistent across the four females examined (data not shown). A fragment of $G_{\alpha i2}$, which is coexpressed with V1Rs, was also amplified from olfactory cDNA, suggesting that both of these G proteins are expressed in the salamander MOE.

Expression of genes in the VNO

Three types of genes were amplified from the VNO: the V2R family of G-protein-coupled receptors, their coexpressed G protein, $G_{\alpha o}$, and the ion channel, trpc2. We sequenced approximately 60 clones of ~500 bp V2R fragments. The V2Rs ranged from 55%-99% identity included many sequences similar to those in subfamilies from *Xenopus*, fish and mice (Figure 6.3). The *P. shermani* fragments contained only six of the seven transmembrane domains present in V2Rs, because the primers were designed to amplify the conserved transmembrane domains. A divergent group of V2Rs present in fishes did not amplify from *P. shermani*. We also identified multiple sequences that contained frameshift and nonsense mutations, a pattern also noted in some of the *P. shermani* OR sequences.

Using 5' and 3 prime RACE, we were able to amplify full-length V2R receptors. We sequenced four clones, but three of the four appeared to be missing an exon. The transmembrane prediction software (TMHMM) predicted seven domains that align with those found in rat and *Xenopus* V2Rs. The *P. shermani* full-length sequence also

contained a long extracellular domain, one of the defining features of V2Rs. Overall, the *P. shermani* sequence shared 33% identity with *Xenopus* xV2R1 and 32% with *Rattus* Vom2r52. Generally, the N-terminal domain was less conserved than was the transmembrane domain, a pattern consistent among most G-protein-coupled receptors.

The other two gene types to be amplified from VNO cDNA were $G_{\alpha o}$ and trpc2. The portion of $G_{\alpha o}$ (the G protein coexpressed with V2Rs) that was amplified from female *P. shermani* VNO cDNA was very similar to that of other vertebrates (Table 2). The salamander homologue of the ion channel, trpc2 was also very similar to the mammalian trpc2. The salamander trpc2 fragment was one of the largest cDNA sequences we obtained from the VNO (Table 2). *In situ* hybridization revealed the expression of trpc2 RNA in the lateral diverticula of the nasal cavity (VNO; Figure 6.2). Clearly, most sensory neurons in the VNO express trpc2, just as those in the MOE express G_{olf}.

Discussion

We isolated and localized sequences from genes necessary for mediating chemoreception in vertebrates. We showed that G-protein coupled receptors (ORs and V2Rs) and G proteins (G_{olf}, $G_{\alpha i2}$, $G_{\alpha o}$) used for chemoreception were expressed in the nasal cavities of terrestrial salamanders (*P. shermani*). *In situ* hybridization showed that G_{olf} RNA is localized in the MOE, while trpc2 RNA is only seen in the VNO. The expression pattern of these two genes mirrors that in other vertebrate taxa: in rodents, for example, G_{olf} is also expressed in the MOE while trpc2 is expressed in the VNO (Liman et al. 1999). We also presented the first description of trpc2 localization in VNO outside of mammals. Trpc2 is found in the genomes of sea lampreys and elephant sharks, but these taxa lack vomeronasal organs; the function of trpc2 in fishes is not clear (Grus and Zhang 2009). Since amphibians are a sister taxon to the rest of the tetrapod lineage (Kumar and Hedges 1997), our study suggests that trpc2 may be expressed in the VNO of all tetrapods.

The ORs isolated from *P. shermani* olfactory tissue were all closely related. Although we used primer sequences from Marchand et al. (2004), we did not amplify PCR products as diverse as those obtained from *Ambystoma tigrinum*. Since *Xenopus*

expresses both fish-like (class I) and mammalian-like (class II) receptors, the *P. shermani* genome also may contain these two classes of receptors, even though we were only able to amplify class II sequences. The *Xenopus laevis* genome contains 410 putatively functional ORs, along with 478 partial sequences or pseudogenes (Niimura and Nei 2005). Some of these *Xenopus* sequences belong to families that are >90% similar (Freitag et al. 1995). We have likely amplified a subset of ORs from the gamma group from Niimura and Nei (2005), but the primers almost certainly failed to amplify other groups of class II ORs.

The V2R family underwent extensive duplication during the vertebrate radiation (Grus and Zhang 2009). The diversity of V2R fragments from *P. shermani* suggests that these fragments are part of a large repertoire, like that documented in *Xenopus* (Shi and Zhang 2007). The *P. shermani* sequences form some unique groups, but others appear to be newly discovered members of families already identified in other vertebrates (Shi and Zhang 2007).

Shi and Zhang (2007) postulated that aquatic or semi-aquatic animals may primarily use V2Rs instead of V1Rs for chemoreception. V2Rs are known to mediate detection of large, nonvolatile molecules while V1Rs can bind volatile compounds (Boschat et al. 2002; Punta et al. 2002; Chamero et al. 2007). The *Xenopus tropicalis* genome contains a much larger V2R repertoire and a smaller V1R repertoire, probably appropriate for its aquatic life history. Despite extensive effort, we were unable to amplify V1Rs in *P. shermani* and so cannot compare the relative diversity between the V1R and V2R families. However, the expression of V2Rs in VNO tissue suggests that this family of receptors may be used for reception of male courtship pheromones since these pheromones are relatively large proteins and activate only vomeronasal neurons (Wirsig-Wiechmann et al. 2002; Wirsig-Wiechmann et al. 2006). Since V1Rs respond primarily to volatile chemical cues, so these receptors are much less likely to mediate female response to pheromones. Amplification of the $G_{\alpha i2}$ (the G protein associated with V1Rs) from MOE cDNA of *P. shermani* suggests that V1Rs are expressed in the MOE. This is consistent with the localization of V1Rs in *Xenopus* and fish (Pfister and

Rodriguez 2005; Date-Ito et al. 2008). Further work, such as *in situ* hybridization, is needed to confirm the expression of V1Rs in the MOE and/or VNO of salamanders.

Our study has helped to elucidate the evolutionary history of the partitioning of receptors in the two olfactory organs of vertebrates. Salamanders and frogs appear be be similar in many respects, such as in the diversity in their respective V2Rs, but differences between them may become apparent with further work on salamander chemoreception. Future work will investigate localization of additional G proteins, along with V1R and TAAR receptors. These objectives will help elucidate the molecular biology of chemoreceptory neurons and allow to us make comparisons that promote understanding the evolution of these families of important sensory genes.

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Table 6.1. Primers used to amplify *P. shermani* olfactory gene fragments.

Gene name	Forward Primers 5'→ 3'	Reverse Primers 5'→ 3'	Amplicon length(s)
ORs[1]	ATG GCG TAC AGA TAC GTG GCG ATA TG	TAT ATG AAC GGG TTT AGC ATT GG	513 bp
G_{olf}[2]	AGC ACG ATT GTG AAA CAG ATG	TGC ATT CTC TGG ATG ATG TC	954 bp
$G_{\alpha i2}$	CAC CAT YCA GTC YAT YAT GGC	CAG GTT RTT YTT GAT GAT GAC	819 bp
V2Rs	AAG GCC AAY AAY MRS ASY CTG AGC TT	CTG AAK GTR ATG WAC TTG GCY TCA TT	~430 bp
$G_{\alpha o}$	ATG GGM TGY ACH CTG AGC GC	AAS MAY TTG TTG TTR CAG ATG	779 bp
Trpc2	GTG GCH GTG GAC ACM AAC CA	TAV GGS ACR TAG ATR TTG TTG A	1407 bp

[1]Primers from Marchand et al. (2004)
[2]Primers from Hagino-Yamagishi et al. (2004)

Table 6.2. Amino acid similarity between *P. shermani* and other vertebrate signal transduction genes.

		Danio[1]	*Xenopus*[2]	*Gallus*[3]	*Mus*[4]
G_{olf}	*P. shermani*	83%	93%	92%	89%
$G_{\alpha o}$	*P. shermani*	94%	96%	96%	95%
$G_{\alpha i2}$	*P. shermani*	85%	91%	94%	90%
Trpc2	*P. shermani*	76%	not identified	not identified	74%

[1]*Danio* GenBank accession nos. G_{olf}: NP_999968.2; $G_{\alpha o}$: NP_957081.1 $G_{\alpha i2}$: NP_956136.1 ; Trpc2: NP_001025337.1
[2]*Xenopus* GenBank accession nos. G_{olf}: AJ296281; $G_{\alpha o}$: NP_001081529; $G_{\alpha i2}$: AF086606
[3]*Gallus* GenBank accession nos. G_{olf}: NP_001008746; $G_{\alpha o}$: XP_001232687.1 $G_{\alpha i2}$: NP_990733.1
[4]*Mus* GenBank accession nos. G_{olf}: NP_796111.2; $G_{\alpha o}$: NP_001106855.1; $G_{\alpha i2}$: NP_032164.2; Trpc2: NP_035774.1

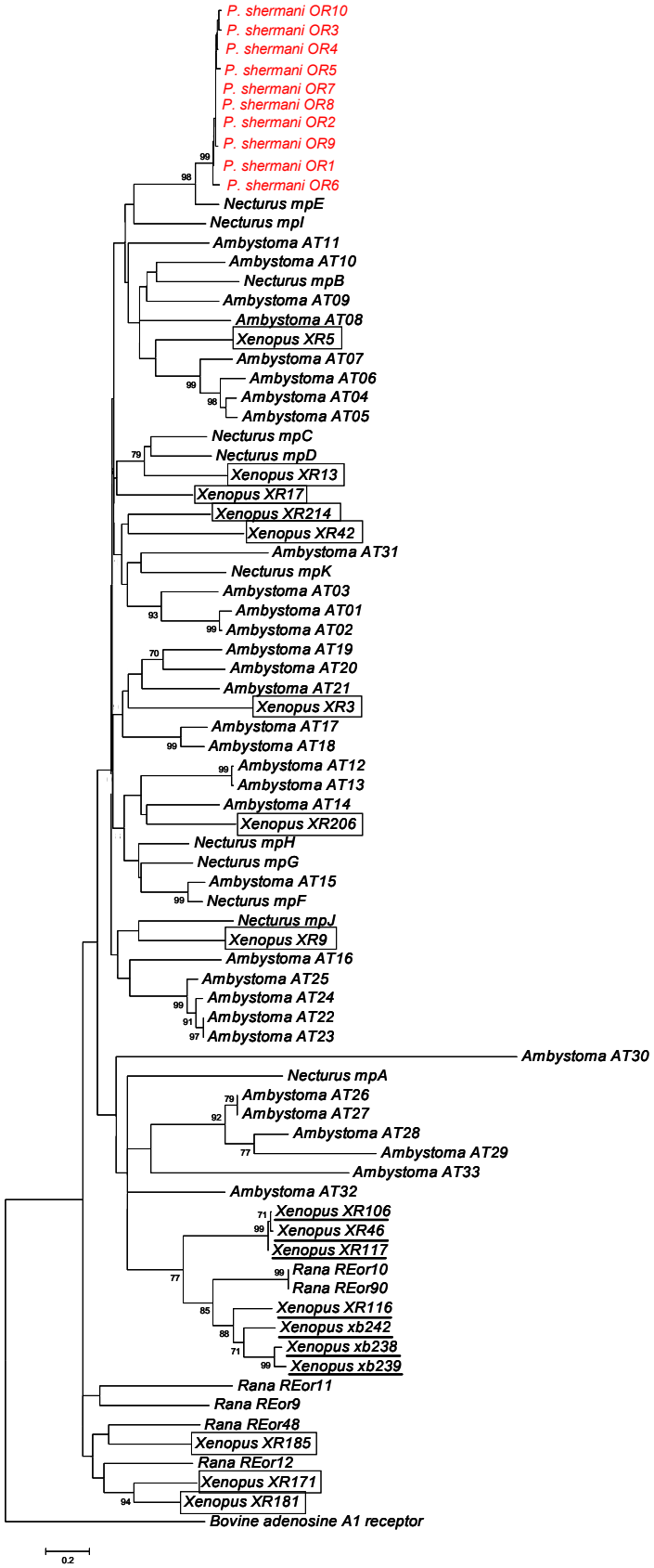

P. shermani OR10
P. shermani OR3
P. shermani OR4
P. shermani OR5
P. shermani OR7
P. shermani OR8
P. shermani OR2
P. shermani OR9
P. shermani OR1
P. shermani OR6
Necturus mpE
Necturus mpI
Ambystoma AT11
Ambystoma AT10
Necturus mpB
Ambystoma AT09
Ambystoma AT08
Xenopus XR5
Ambystoma AT07
Ambystoma AT06
Ambystoma AT04
Ambystoma AT05
Necturus mpC
Necturus mpD
Xenopus XR13
Xenopus XR17
Xenopus XR214
Xenopus XR42
Ambystoma AT31
Necturus mpK
Ambystoma AT03
Ambystoma AT01
Ambystoma AT02
Ambystoma AT19
Ambystoma AT20
Ambystoma AT21
Xenopus XR3
Ambystoma AT17
Ambystoma AT18
Ambystoma AT12
Ambystoma AT13
Ambystoma AT14
Xenopus XR206
Necturus mpH
Necturus mpG
Ambystoma AT15
Necturus mpF
Necturus mpJ
Xenopus XR9
Ambystoma AT16
Ambystoma AT25
Ambystoma AT24
Ambystoma AT22
Ambystoma AT23
Ambystoma AT30
Necturus mpA
Ambystoma AT26
Ambystoma AT27
Ambystoma AT28
Ambystoma AT29
Ambystoma AT33
Ambystoma AT32
Xenopus XR106
Xenopus XR46
Xenopus XR117
Rana REor10
Rana REor90
Xenopus XR116
Xenopus xb242
Xenopus xb238
Xenopus xb239
Rana REor11
Rana REor9
Rana REor48
Xenopus XR185
Rana REor12
Xenopus XR171
Xenopus XR181
Bovine adenosine A1 receptor
0.2

Figure 6.1. Phylogenetic tree of olfactory receptors constructed using the minimum evolution method, rooted with the bovine adenosine A1 receptor (GenBank accession no. X63592). For simplicity, we compared *P. shermani* ORs only to other amphibian sequences. Accession nos.: *Necturus maculosus* (AF019237-AF019247), *Ambystoma tigrinum* (AY485156- AY485188;(Marchand et al. 2004), *Rana esculenta* (AJ233771-AJ233776; (Freitag et al. 1998), and *Xenopus laevis* (Y08340-Y08354, AJ250750-AJ250752; (Freitag et al. 1995). Scale bar represents 0.2 amino acid substitutions per site. *Xenopus* Class I receptors are underlined and Class II receptors are boxed (from Freitag et al. 1995).

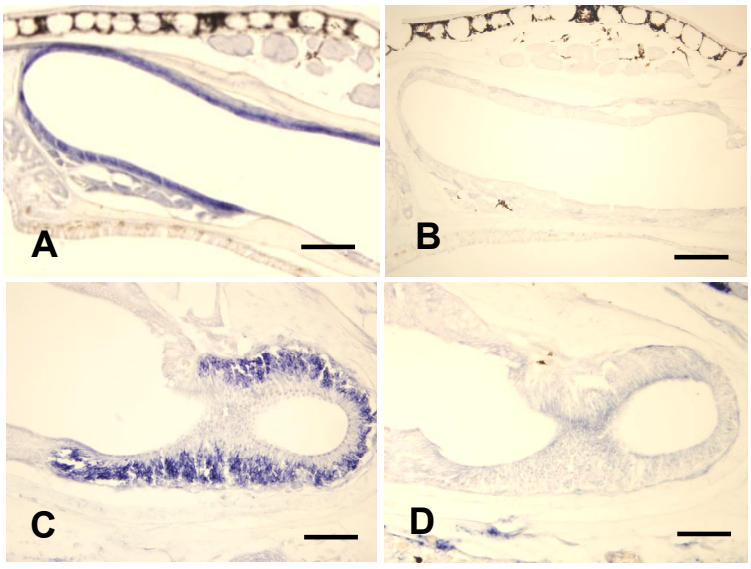
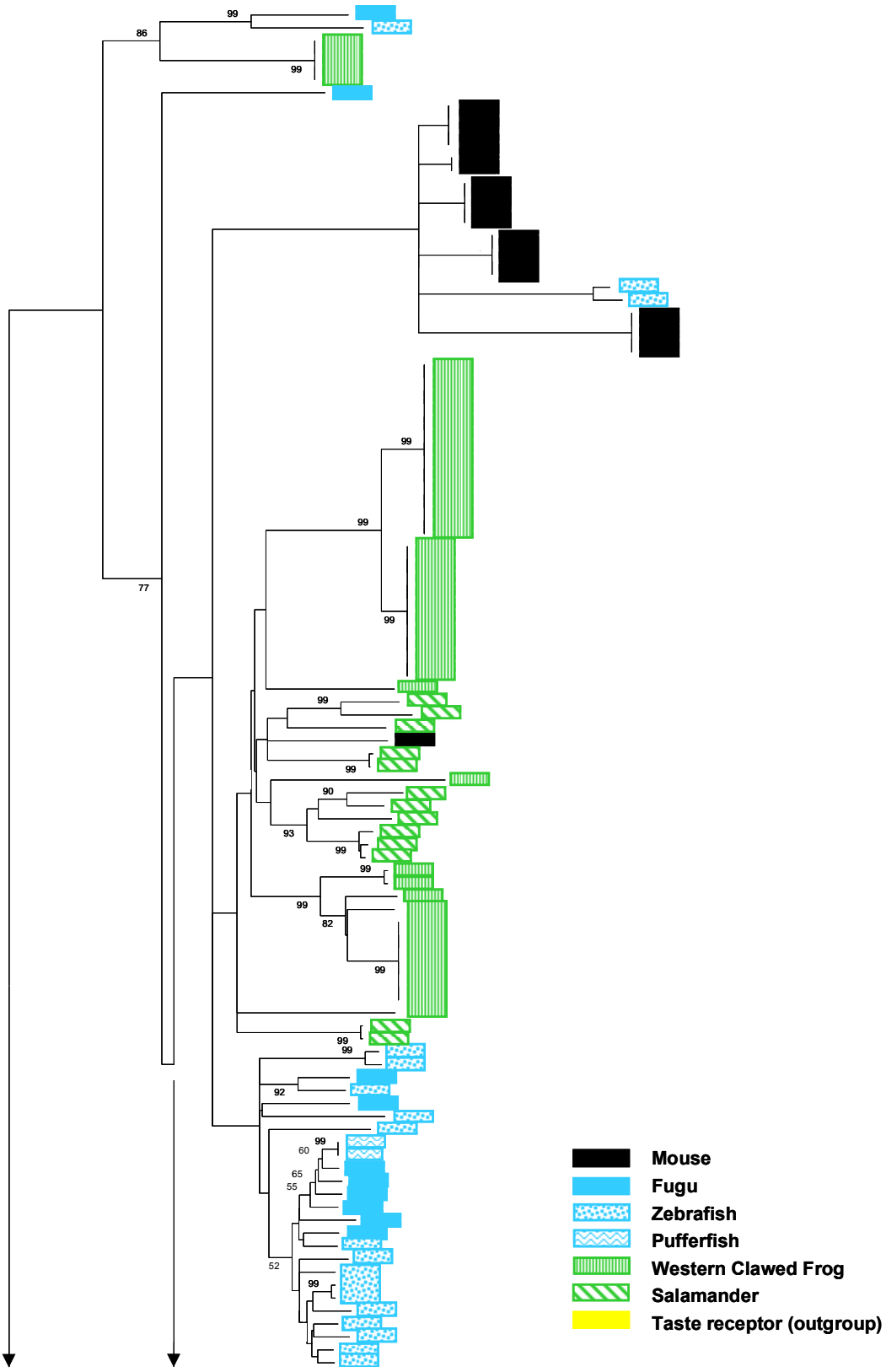


Figure 6.2. Expression of G_{olf} and trpc2 in the nasal cavity of *Plethodon shermani*. Sections of the nasal cavity were hybridized with DIG-labled antisense probes of (A) G_{olf} or (C) trpc2. Control sections were hybridized with sense probes of (B) G_{olf} or (D) trpc2. Scale bars = 200 µm in A and B; 100 µm in C and D.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52
99
Mouse
Fugu
Zebrafish
Pufferfish
Western Clawed Frog
Salamander
Taste receptor (outgroup)

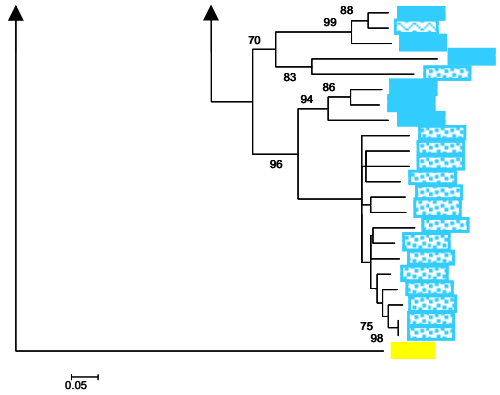

Figure 6.3. Phylogenetic tree constructed with a ~500 bp fragment of V2R receptors from mouse, fugu, pufferfish, zebrafish, western clawed frog and salamander (*P. shermani*). The V2R tree was constructed with a subset of V2R sequences from Shi and Zhang (2007) and rooted with a mouse taste receptor **(**GenBank accession no. AAK39438). Scale bar shows 0.2 amino acid substitutions per site. Fish are denoted by blue boxes, amphibians by green boxes, mouse by black boxes, and outgroup (taste receptor) by a yellow box.

CHAPTER 7. GENERAL CONCLUSIONS

The complexity of pheromone composition, reception, and evolution is documented in this dissertation. My thesis began with a broad survey of chemical communication (Chapter 2) and addressed specific aspects of chemical communication in a focal species of salamander, *P. shermani* (Chapters 3-6). Integration of the *P. shermani* pheromone system with those of other species informs a multi-leveled view of how chemical communication functions in salamanders and provides a better understanding of how chemicals are used as communication signals.

Several key finding of my literature review document the state of the science of salamander chemoreception. First, salamanders use pheromones for a number of diverse functions. Like mammals, salamanders use chemical cues for territorial marking and social interactions (Jaeger et al. 1986). Aquatic salamanders can use chemical cues to attract mates, a phenomenon common in fish of the genus *Carassius* (Sorensen et al. 1995; Sorensen 1996). Salamanders can change their behaviors in response to pheromone signals. In addition, salamanders apparently use chemical signals for a variety of functions that may have not been previously recognized (Chapter 2). Second, some salamander families are more likely to be studied and therefore are disproportionally represented in the literature. For example, more studies have been conducted on a single species of plethodontid, *Plethodon cinereus*, than on all salamanders in the families Dicamptodontidae, Rhyacotritonidae, and Hynobiidae combined (about 59 species). The paucity of information on these families makes it difficult to draw conclusions about the caudates as a group. Third, some research methodologies allow more precise inferences concerning the kind of information salamanders can gain from chemical cues. Avoidance or preference of a chemical cue over a blank substrate is more difficult to interpret than are the results from a direct choice test between two cues. Lastly, animals at different life history stages can respond to different chemical cues. Even larvae at different developmental stages can have different responses to the same stimuli (Mathis and Vincent 2000).

The delivery mode and composition of courtship pheromones are not static traits in plethodontids (Chapters 3 and 4). The kinds and modes of transmission of chemical

cues can vary dramatically across salamander lineages. Stimulatory pathways can change over evolutionary time (Chapter 3). In *P. shermani*, evolutionary shifts have caused the loss of the stimulatory pathway for male courtship pheromones that acted via transport through the female skin, and were replaced by a type of female response that can only be elicited via delivery to the olfactory system.

Complex mixtures of proteins are used as courtship pheromones in plethodontid salamanders (Chapter 4). These protein mixtures may influence female behavior and physiology in ways not yet documented in salamanders. Many pheromones in vertebrates are multi-component signals (Swaney and Keverne 2009), and this pattern may be a theme in salamander pheromones as well. The protein courtship pheromones of plethodontid salamanders can vary from a single protein being the dominant component, to a mix of many proteins (Houck et al. 2008b). Although three proteins, PMF, PRF and SPF have been extensively studied in multiple plethodontid species (Palmer 2004; Watts et al. 2004; Palmer et al. 2007), new cDNA sequences that probably encode functional peptides were revealed in cDNA libraries constructed from *D. ocoee* and *E. guttolineata* mental glands. Thus, there may be multiple forces driving the rapid evolution of male pheromone profiles, even though courtship behaviors are remarkably conserved across the Plethodontidae. In addition, protein components may change a female's behavior in ways not yet explored.

The diversity of male salamander pheromones suggests that females may have a diverse receptor repertoire to detect the pheromones. Plethodontid salamanders express genes that are used by other vertebrate taxa for detecting chemical signals. *Plethodon shermani* express two families of G-protein-coupled receptors, the ORs and V2Rs (Chapter 6). The ORs were isolated from olfactory tissue and the V2Rs from vomeronasal tissue. *Plethodon* also express the vomeronasal-specific ion channel, trpc2, as well as the two G proteins associated with vomeronasal receptors, $G_{\alpha o}$ and $G_{\alpha i2}$. The expression patterns of G_{olf} (the G protein associated with ORs) and trpc2 show that *Plethodon* express these components in the main and vomeronasal olfactory epithelia, respectively. The expression patterns of these two genes in *P. shermani* mirror those found in mammals. These molecular components are likely responsible for

mediating the response to chemical cues, including courtship pheromones. Our overall understanding of the vertebrate vomeronasal organ and the mechanisms underlying pheromone communication has been predominately based on research in mammalian systems (Brennan and Zufall 2006) and my research shows that there is robust conservation among vertebrates in specific receptor organization.

Future directions

My dissertation findings have generated many avenues of potential research. I concentrate here on the three areas that are most directly applicable to my dissertation work. I also highlight areas that are feasible and have the ability to generally inform our understanding of chemical communication.

The two hormone-like peptides that are encoded by RNA found in *P. shermani* mental glands (described in Chapter 4) belong to hormone superfamilies for which many types of physiological and behavioral assays have been established. These proteins may belong to subfamilies within the larger superfamilies that have evolved for use in courtship interactions. The next research step is to synthesize and test some of these proteins for functionality. Synthesis of short peptides can now be performed commercially for a reasonable cost. Some of these peptides could be tested on species such as *D. ocoee*, which are plentiful and have been used for many courtship assays (e.g., Houck and Reagan 1990; Houck et al. 2008b). Females from this species could be used to test whether feeding is reduced when the salamander glucagon-like peptide is delivered to the dorsum, mimicking the natural transdermal delivery mode. Assays for other tests of pheromone effects are already being developed at Oregon State University, and may help us determine multi-dimensional effects of courtship pheromones on females.

Determining the expression patterns of more olfactory and vomeronasal genes is needed to identify possible differences between salamanders and mammals. Although results from Chapter 6 have greatly improved our understanding of what types of receptors are being used for chemoreception, more can still be learned. For example, identification of the two other families of seven transmembrane receptors used in mammalian olfaction, V1Rs and TAARs, has yet to be accomplished in salamanders.

In addition, the technique of double-label *in situ* hybridization could confirm that G proteins are co-expressed with particular receptor families.

Immunocytochemical techniques could be used to identify vomeronasal cells that both respond to pheromones and express certain types of receptors. This identification would be a substantial advance towards identifying the specific receptors that bind courtship pheromones. Co-labeling could be accomplished by first stimulating the vomeronasal organ with a pheromone protein, such as PRF. Recombinant PRF is available from our collaborators and is an ideal tool for this sort of research. Using fluorescent immunocytochemistry, the stimulated cells could be visualized. Then, the same tissue could undergo *in situ* hybridization to determine whether or not the stimulated cells also express V1Rs or V2Rs. This type of work was used in mice to show that male lacrimal peptides stimulated specific receptors in the female vomeronasal organ (Haga et al. 2007).

In conclusion, I have elucidated the types of chemicals signals that male *P. shermani* use during courtship using a comparative framework. By combining the information about neural stimulation and processing with our current knowledge about the evolution of courtship behavior, we better understand how pheromone delivery (and different delivery modes) may have evolved. Most importantly, identifying the family of receptors stimulated by salamander courtship pheromones has taken us one step closer to identifying a specific pheromone receptor, a feat rarely accomplished in chemical ecology.

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