Expression of Vomeronasal Receptors and Related Signaling Molecules in the Nasal Cavity of a Caudate Amphibian (*Plethodon shermani*)

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Abstract

G-protein-coupled receptors are responsible for binding to chemosensory cues and initiating responses in vertebrate olfactory neurons. We investigated the genetic diversity and expression of one family of G-protein-coupled receptors in a terrestrial caudate amphibian (the red-legged salamander, *Plethodon shermani*). We used degenerate RT-PCR to isolate vomeronasal type 2 receptors (V2Rs)—including full-length sequences—and compared them with other vertebrate V2Rs with phylogenetic analyses. We also amplified a salamander G_{olf}, a G-protein usually expressed in the main olfactory epithelium (MOE) of vertebrates, and an ion channel expressed in the rodent vomeronasal organ: trpc2. We then localized mRNA expression of V2Rs, trpc2, and G_{olf} in the olfactory and vomeronasal epithelia with in situ hybridization. The mRNA transcripts of V2Rs and trpc2 were detected solely in the vomeronasal epithelium of *P. shermani*. Furthermore, there were differences in the density of cells that expressed particular subclasses of V2Rs: 2 probes showed sexually dimorphic expression, whereas a third did not. Although G_{olf} mRNA was expressed primarily in the MOE, G_{olf} transcripts also were found in the vomeronasal epithelium. Thus, some aspects of mRNA expression of vomeronasal receptors and related molecules differ between salamanders and frogs, and between salamanders and mice.

Key words: in situ hybridization, pheromone receptors, salamander, trpc2, V2R, vomeronasal organ

Introduction

The organizational complexity of olfactory organs varies across vertebrates. In most fish, chemosensory cues are detected by a single olfactory organ. Recent evidence suggests, however, that the anatomical division of a second olfactory system may have begun to evolve in ancestral sarcopterygians. Lungfish have a specialized set of neurons, located in epithethial crypts in the chemosensory organ, that project to the ventrolateral olfactory bulb, showing that the accessory olfactory system evolved in the aquatic ancestor of lungfish and tetrapods (González et al. 2010). In contrast, most amphibians (and other tetrapods) possess 2 anatomically separate olfactory organs, the main olfactory epithelium (MOE), which is the sensory organ of the main olfactory system, and the vomeronasal organ (VNO), the sensory organ of the accessory olfactory system. Comparative anatomical studies of aquatic amphibians demonstrate that an anatomically separated VNO did not evolve as an adaptation to a ter-restrial lifestyle (Eisthen 2000). Although secondary losses of the VNO have occurred in some vertebrate lineages, functional VNOs are present in many extant taxa and are used for a multitude of diverse sensory functions, from prey detection to social interactions (Halpern 1987; Halpern and Martinez-Marcos 2003).

Molecular signal transduction of chemosensory cues in the MOE and VNO is mediated by several families of G-protein-coupled receptors. The 3 most well-studied families of G-protein-coupled receptors are olfactory receptors (ORs) and vomeronasal type 1 and type 2 receptors (V1Rs and V2Rs). The size of these 3 gene families varies widely across vertebrate taxa; some vertebrate genomes contain over 1000 genes of a particular family, whereas others possess no functional copies (Shi and Zhang 2009). These receptor families are coexpressed with specific G proteins; ORs are coexpressed with G_{olf}, V1Rs with $G_{\alpha i2}$, and V2Rs with $G_{\alpha o}$

(reviewed in Dulac 2000). In addition, an ion channel, transient receptor ion cation channel, subfamily C, member 2 (trpc2) is coexpressed with V1Rs and V2Rs. Trpc2 is present in most vertebrate genomes and functions in pheromone signal transduction in mammals (Liman et al. 1999; Leypold et al. 2002; Stowers et al. 2002). Until now, however, trpc2 expression had not been determined in nonmammalian tetrapods.

With the advent of molecular techniques, attention has expanded from studies limited to VNO anatomy to include studies of the expression of chemosensory receptors. ORs, V1Rs, and V2Rs (as well as trpc2) are expressed in the single olfactory organ of some fish (Cao et al. 1998; Pfister and Rodriguez 2005). In mice and rats, OR expression is generally restricted to the MOE (although a subset of VNO neurons in mice express ORs but do not express G_{olf}, Levai et al. 2006) and V1R/V2R expression is restricted to the VNO (Buck and Axel 1991; Matsunami and Buck 1997). In clawed frogs (*Xenopus laevis* and *Silurana tropicalis*), ORs, V1Rs, and V2Rs are expressed in the MOE, and V2Rs—but not V1Rs—are expressed in the VNO (Hagino-Yamagishi et al. 2004; Kashiwagi et al. 2006; Date-Ito et al. 2008). Goats and humans also express V1Rs in the MOE, a pattern similar to that of anuran V1R expression (Rodriguez et al. 2000; Wakabayashi et al. 2007). Thus, despite the anatomical separation of the MOE and VNO in most tetrapods, V1R/V2R receptor distributions are not restricted to the VNO in all species.

Amphibians, as basal tetrapods, represent a critical group for understanding the evolution of the vertebrate VNO (for review, see Woodley 2010), yet most information on the expression of chemoreceptors in the amphibian VNO has been based on 2 closely-related species of clawed frogs (Hagino-Yamagishi et al. 2004; Date-Ito et al. 2008). Studies of clawed frogs have provided valuable baseline data on vomeronasal receptor diversity and expression (Hagino-Yamagishi et al. 2004; Date-Ito et al. 2008) but may not be representative of amphibians as a whole. First, receptor types and distribution in chemosensory epithelia might differ between aquatic anurans like the clawed frogs and other more terrestrial amphibians. In mammals, V1Rs are known to bind small airborne compounds (e.g., Boschat et al. 2002; Punta et al. 2002), whereas V2Rs mediate detection of large water soluble molecules (e.g., Leinders-Zufall et al. 2009; Haga et al. 2010). Thus, aquatic and terrestrial amphibians may use different families to detect important sensory cues in their environments. Second, most anurans, including clawed frogs (Kelley and Tobias 1999), rely heavily on acoustic signals. Pheromones (generally thought to activate many vomeronasal receptors) are not typically believed to play a prominent role in the social communication of most anurans (Ryan 1988; Gerhardt 1994). Hence, examining additional amphibian species, especially those that are terrestrial and utilize pheromones for communication, can provide valuable comparative data that will elucidate general patterns within amphibians and across vertebrates.

We examined the presence and expression of genes involved in the detection and transduction of chemosensory cues in a fully terrestrial salamander, the red-legged salamander (*Plethodon shermani*). This species is a direct developer; thus, it does not have an aquatic life stage. *Plethodon shermani* is a member of the salamander family Plethodontidae, an amphibian group that has long been recognized to use chemical cues in social and reproductive interactions (Jaeger and Forester 1993; Houck and Arnold 2003). Red-legged salamanders, in particular, have been used for the study of chemical communication during courtship for more than a decade. Males develop specialized glands during the breeding season that produce protein secretions that are delivered to females. Several nonvolatile protein pheromones related to male–female courtship interactions have been characterized in terms of behavioral effects (Rollmann et al. 1999; Houck et al. 2007; Houck et al. 2008), biochemical structure (Rollmann et al. 2000), and molecular evolution (Watts et al. 2004; Palmer et al. 2005, 2007, 2010). Courtship pheromones activate sensory neurons of the VNO (Wirsig-Wiechmann et al. 2002, 2006; Schubert et al. 2006, 2009), stimulate higher brain centers (Laberge et al. 2008), and elicit endocrine changes (Schubert et al. 2009). These studies have documented the key function that chemical signals play in modulating reproductive behavior in this species. In the present study, we focused on the molecular basis for detection of chemical signals in the salamander sensory epithelia, especially the VNO. We first used polymerase chain reaction (PCR) to extensively survey VNO cDNA for V2R sequences and used phylogenetic analyses to evaluate the evolutionary relationships among vertebrate V2Rs. We then examined the expression patterns of particular subfamilies of V2Rs. We also investigated whether males and females differ in their expression of different classes of V2Rs. Finally, for comparison, we also sequenced and localized mRNA expression of G_{olf} and the signal transduction gene, trpc2.

Materials and Methods

Animal collection and RNA extraction

Adult *P. shermani* were collected from Macon County, NC (035°10′48″N 083°33′38″W) during their breeding season and with permits from the NC Wildlife Resources Commission. Animals used for either in situ hybridization or tissue collection were sacrificed by decapitation in accordance with the standards of the Institutional Animal Care and Use Committee at Oregon State University (LAR 3549 to L.D.H.) and Duquesne University (S.K.W.). For RNA extraction, tissue from the MOE and VNO was carefully removed by dissection from the nasal cavity. Tissue from 4 females was pooled and immediately preserved in RNA*later* (Ambion). 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.

Genetic isolation and molecular cloning

Degenerate PCR was used to isolate *P. shermani* orthologs of genes from the MOE and VNO cDNA. Total RNA extracted from the tissues was reverse transcribed into cDNA using the ImProm-II reverse transcription system (Promega) or the FirstChoice RLM-RACE kit (Ambion). To amplify partial V2Rs, we initially designed 4 forward and 2 reverse primers that annealed within the conserved 7-transmembrane domain of the proteins. All combinations (*n* = 8) of these forward and reverse primers were tested and the 2 sets that consistently amplified (Supplemental File 1) were used under a variety of annealing temperatures using GoTaq DNA polymerase (Promega). In order to obtain a diverse set of sequences, we varied the annealing temperature from 53 to 62°C and cloned the PCR products resulting from the 53, 56, and 60 °C reactions.

We also designed degenerate primers to amplify 2 other genes that encode other proteins involved in olfactory signal transduction: trpc2 and G_{olf}. The successful primer pairs used to amplify each is listed for each gene/gene family in Supplemental File 1. All amplicons were cloned using the pGEM-Teasy vector system (Promega) following the manufacturers' protocols. Plasmid DNA was isolated from cultures of unique clones with the Qiaprep spin miniprep kit (Qiagen). The purified DNA was sent for sequencing using T7 or SP6 primers (and/or internal sequencing primers) at the Nevada Genomics Center. 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, 2005).

Phylogenetic analysis of V2Rs

To investigate the evolutionary relationships among vertebrate V2Rs, we compiled a data set containing the deduced amino acid sequences from amphibians (*P. shermani* and *S. tropicalis*), mammals (*Mus musculus*), and fish (*Danio rerio*). The subset of mouse V2Rs included in the phylogenetic analysis included representatives from the A, B, C, and D families. The amino acid sequences of V2Rs for each species were compiled and aligned using the MEGA 5.05 (Tamura et al. 2011) sequence alignment editor. Then, each alignment was trimmed to a uniform length (153 amino acids) that contained a portion of the more conserved 7-transmembrane domain present in the partial *P. shermani* sequences. To reduce the number of sequences included in the analysis, CD-HIT was used on each species alignment with a 90% amino acid identity threshold, with default algorithm and alignment coverage parameters (Li and Godzik 2006). This program reduced the number of sequences in each data set to a subset containing a single representative from each identity group (Table 1). The output from CD-HIT from all species was then compiled into a second larger data set that included 2 taste receptors as outgroups (GenBank accession no.

Table 1 Characteristics of V2R sequences included in the phylogenetic tree

Species	No. of sequences	% Identity among all sequences[a]	Reference
Zebrafish	27	30–90	Shi and Zhang (2007)
Red-legged salamander	15	45–90	This study
Western clawed frog	82	28–90	Shi and Zhang (2007)
Mouse	38	23–90	Young and Trask (2007)

[a]Amino acid identity calculated from alignment in Supplementary File 2.

mouse: AAK39438 and zebrafish: NP_001034614). MUSCLE (Edgar 2004), hosted at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/muscle) was used to generate an alignment of the second data set (Supplemental File 2). A bootstrap consensus tree was generated from the alignment in MEGA 5.05 (Tamura et al. 2011) using the neighbor-joining method with the amino acid model and a Poisson correction. Branch support was tested by performing 1000 bootstrap replicates (Felsenstein 1985).

In situ hybridization

In situ hybridization was performed on tissue sections of the nasal chemosensory epithelia to determine tissue level expression of mRNA using riboprobes targeting fragments of trpc2, G_{olf}, and 3 different partial-length V2Rs. To generate the 3 V2R riboprobes, we used 3 V2R sequences: V2R18 (GenBank accession no. JN805739), V2R26 (JN805743), and V2R29 (JN805760). These sequences were chosen because they were less than 70% identical to each other, which made them unlikely to cross hybridize (Ishii et al. 2004) and thus be more likely to label different subsets of cells.

Preparation of riboprobes for the in situ hybridization analyses followed a protocol previously described by Butler et al. (2001) for generating sense and antisense DIG-labeled riboprobes. An initial PCR was run using the SP6 and T7 primers with 50 ng of plasmid DNA as template. One microgram of each PCR product was then used as a template to synthesize either sense or antisense DIG-labeled riboprobes with SP6 or T7 RNA polymerase using standard procedures (Fisher Scientific, Pittsburgh, PA). PCR products were incubated with the appropriate polymerase, digUTP labeling mix (Roche Diagnostics), DEPC water, RNasin, buffer, and DTT (Takara Bio Inc.) at 37 °C for 2 h. DNA template was then degraded using 1 unit of DNase1 (Fisher Scientific).

To obtain sections of the nasal cavity, animals 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, and submerged in 30% sucrose in PBS overnight. The heads were embedded in OCT embedding

medium (Fisher) and sectioned in the transverse plane using a cryostat. Sections 18 μm thick were mounted onto Superfrost plus slides coated with 10% poly-L-lysine. For each animal, 4 alternate series of slides were obtained such that, in a single series, each section was 72 μm apart from the adjacent section.

Hybridization of the probes to the sections was carried out with reference 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 0.2 N HCL for 15 min, rinsed with SSPE, and incubated with 0.1 M triethanol-amine (pH 8.0) for 5 min. After 2 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/μL cRNA probe, 50% formamide, 1% blocking reagent (Omnipure), 5X SSC, 5 mM EDTA, 0.5 mg/mL Torula RNA (Sigma), 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 3 times with 100 mM Tris (pH 7.5) and 150 mM NaCl. The sections were then incubated for 2 h in 100 mM Tris (pH 7.5), 150 mM NaCl, and blocking reagent (Roche Diagnostics). After incubation, the slides were incubated with alkaline phosphatase–conjugated antiDIG Fab fragment antibody (Roche Diagnostics) for 1 h, washed 3 times with 100 mM Tris (pH 7.5) and 150 mM NaCl, and equilibrated in alkaline phosphatase buffer for 10 min before signals were visualized with the BM purple chromogenic substrate (Roche) for 24–48 h. The reaction was stopped with a solution of MEMFA fixative in DEPC water.

Sex differences in V2R mRNA expression

To assess variation in V2R mRNA expression, we examined V2R expression in both male and female red-legged salamanders using antisense riboprobes made from partial-length V2Rs (18, 26, and 29). Hybridization of adjacent sections with sense probes represented a negative control. Because of the high sequence homology within subfamilies of V2Rs, it is difficult to make gene-specific probes for individual receptor mRNAs. Thus, probes likely bind to closely related receptors (e.g., Silvotti et al. 2007). In order to perform in situ hybridization (ISH) on multiple individuals, 2 batches of ISH were run. Each batch included an equal number of males and females. Slides were examined using an Olympus BX-51 brightfield microscope and the number of cell bodies with clear signal was counted in each slide, including both left and right nasal cavities. The investigator was blind to the sex of the animal. The average diameter of a sensory VNO neuronal cell body in this species is 8 μm (Woodley SK, unpublished data), so by examining every 4th section (i.e., 72 μm apart), we avoided counting the same cell twice.

To determine the density of cells relative to the volume of the VNO, we measured the area of the VNO in both left and right nasal cavities on every 4th section using Image Analysis Pro Plus image analysis software. For each animal, total areas were summed and multiplied by 72 μm (the distance between adjacent sections) to estimate VNO volume (mm³). Cell density was determined by dividing the total number of cells by the volume of the VNO. Data were normally distributed with homogeneous variances and were analyzed with ANOVA. Analysis indicated that there was a significant effect of batch on the number of labeled cells and therefore batch was included as a factor in subsequent analyses.

Digital photography

Slides were photographed using an Olympus DP70 camera. The contrast of each photograph was adjusted in Adobe Photoshop using the automatic contrast adjustment.

Results

Genetic diversity of salamander V2Rs

We screened approximately 180 independent clones of 408–447 bp V2R fragments isolated from the cDNA of female *P. shermani* using 2 combinations of degenerate primers. These *P. shermani* fragments contained 3–4 of the 7 transmembrane regions present in V2Rs because the primers were designed to anneal within 2 of the transmembrane domains. In total, we isolated 89 unique nucleotide sequences. Some of the polymorphism we observed, however, may have been caused by sequencing or cloning error and therefore we only present the unique sequences that were ≤98% similar (at the nucleotide level). This conservative exclusion resulted in the isolation of 34 unique nucleotide sequences from the pooled cDNA from 4 *P. shermani* females (GenBank accession nos. JN805732–JN805765). We also identified multiple sequences that contained frameshift and nonsense mutations; these sequences were not included in our analyses but may have been V2R pseudogenes, which are commonly found in vertebrate genomes (Shi and Zhang 2007).

The *P. shermani* V2R fragments ranged from 45% to 98% similar to each other in nucleotide sequence and the subset used in the phylogenetic tree grouped into 5 divergent subfamilies, although the bootstrap supports on some of the deeper nodes of the tree were not high (Figure 1). Three subfamilies were represented by a single sequence and 2 others contained 5–6 sequences. The majority of *P. shermani* sequences were most similar to the mouse V2Rs that belong to the A, B, and D families (Yang et al. 2005; Young and Trask 2007). Whereas the mouse V2Rs from families A and D grouped together, the mouse family B sequences grouped with some salamander and frog sequences. We did not, however, isolate a salamander sequence that was homologous to the C family of V2Rs. The remaining salamander sequences generally grouped with *S. tropicalis* V2Rs,
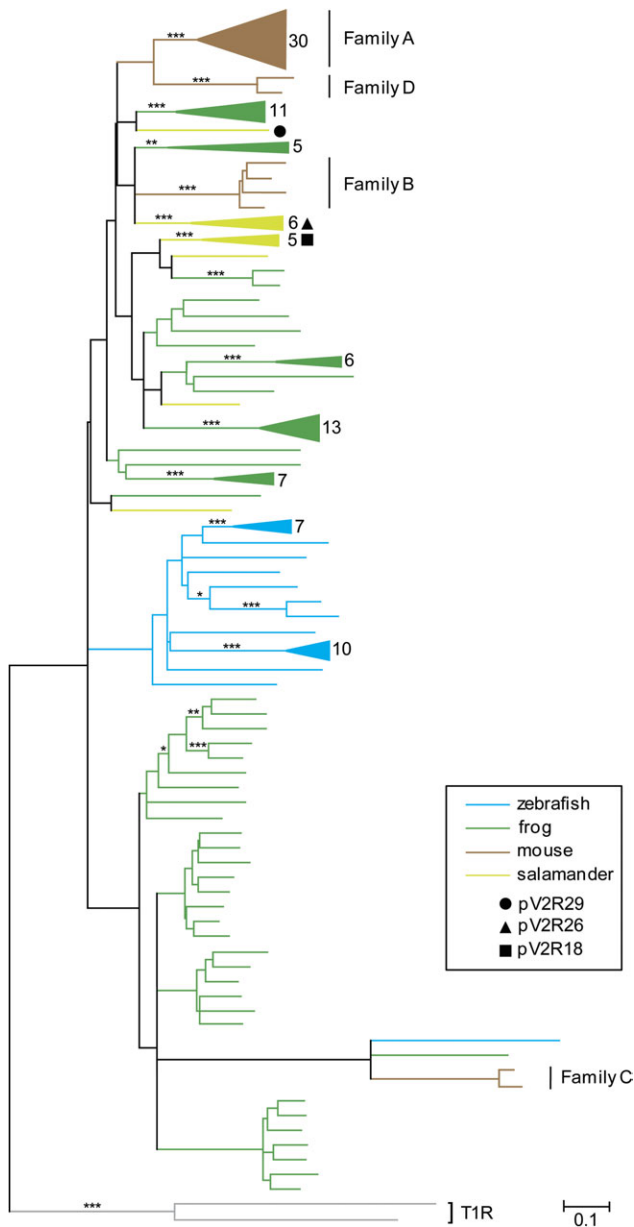
Figure 1 Neighbor-joining tree of vertebrate V2Rs. The tree was constructed with partial-length V2R amino acid sequences from four vertebrates, rooted with two taste receptors. Bootstrap values above 70% are denoted by asterisks above branches (*** = 95–100%; ** = 80–94%; * = 70–79%). Scale bar shows 0.1 amino acid substitutions per site, and symbols designate positions of sequences used to generate riboprobes. Highly supported groups of sequences from a single species have been compressed into wedges (thickness of wedge is proportional to number of sequences in a particular group).

whereas the zebrafish V2Rs formed a separate clade (except for 1 sequence that grouped with the mouse family C).

Using 5′ and 3′ RACE, we were able to obtain the 5′ and 3′ ends of several *P. shermani* V2Rs and used these sequences to design primers (Supplemental File 1) that successfully amplified full-length V2Rs (Figure 2). We isolated 3 unique sequences (JN805766–JN805768). Two of these, however,

were missing an 810-bp region and may have been caused by errors in transcription or posttranscriptional processing (JN805767–JN805768). The 3 sequences were more than 94% similar to each other at the nucleotide level. The transmembrane prediction software (TMHMM) predicted that all 3 sequences contained the 7-transmembrane domain that aligns with those found in *M. musculus* and *X. laevis* V2Rs (Figure 2). The longest *P. shermani* full-length sequence also contained a long extracellular domain, one of the defining features of V2Rs, although it was nevertheless ~80 amino acids shorter than the *X. laevis* or mouse V2Rs. Overall, the *P. shermani* sequence shared 33% amino acid identity with *X. laevis* xV2R1 and 35% with *M. musculus* V2r1. The full-length sequences were most similar to the group of *P. shermani* sequences to which pV2R26 belonged (Figure 1). The *N*-terminal domain was less conserved than was the 7-transmembrane domain between the full-length vertebrate sequences. For example, the amino acid identity between the extracellular domains of the full-length *P. shermani* and *X. laevis* sequences in Figure 2 was 30%, whereas the identity between the 7-transmembrane domains was 49%. Although we tried multiple primer pairs and reaction conditions, we were unable to amplify V1Rs from *P. shermani* VNO or MOE cDNA.

V2R mRNA expression was restricted to the VNO

The *P. shermani* MOE and VNO are located in a single nasal cavity. The MOE covers the majority of the cavity, whereas the VNO is restricted to lateral diverticula (Wirsig-Wiechmann et al. 2002), a common organization in salamanders (Eisthen et al. 1991; Eisthen 1997). For each of 3 V2R fragments (V2R18, 26, and 29), the localization of mRNA expression in the nasal cavity was examined in male and female *P. shermani*. V2R mRNA expression was restricted to the VNO (Figure 3). As expected, there was no labeling in control sections hybridized with sense probes. Each specific V2R probe labeled a small subset of the total numbers of vomeronasal sensory cells, a result consistent to that seen in *X. laevis* (Hagino-Yamagishi et al. 2004). Because V2Rs often vary only slightly in sequence, it was likely that each probe labeled V2Rs that were different but closely related. Expression was distributed throughout the entire rostral–caudal extent of the VNO and was found in the basal half of the neuroepithelium, where *P. shermani* vomeronasal cell bodies are located (Woodley 2007). Out of 23 individuals examined for V2R mRNA expression across the different experiments, we never found labeled cells in the MOE (Figure 3).

Sex differences in expression of some but not all subclasses of V2Rs

In situ hybridization using the 3 different V2R RNA probes revealed sex differences in V2R mRNA expression in the VNO (Figure 4). Females showed significantly higher density
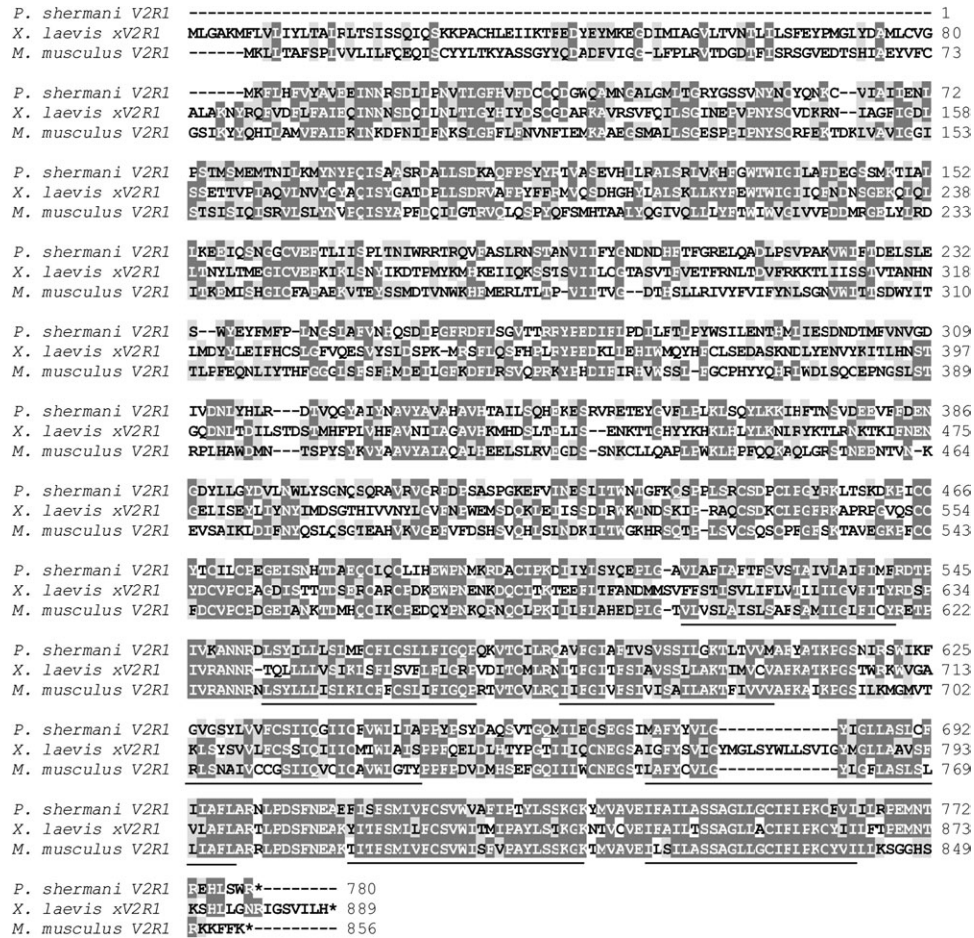
Figure 2 Deduced amino acid sequence of the full-length red-legged salamander V2R1 sequence (JN805766) aligned with *X. laevis* xV2R1 (AB113361) and *M. musculus* V2R1 (AF053985). Putative transmembrane domains are underlined. Dark gray shading indicates identical amino acids and light gray shading indicates conservative substitutions (shading executed in BioEdit; Hall 1999).

of cells labeled with probes targeting V2R18 ($F_{1,8} = 40.3$, $P < 0.001$) and V2R29 ($F_{1,7} = 9.1$, $P = 0.02$) than did males. In contrast, there was no effect of sex on the density of cells labeled with probes targeting V2R26 ($F_{1,7} = 3.2$, $P = 0.12$). Examination of the rostral–caudal distribution of cells' density indicated that the sex differences in the density of cells with RNA for V2R18 and V2R29 were most evident in the rostral VNO (Figure 4).

mRNA expression of trpc2 was restricted to the VNO

Using degenerate primers, we isolated a ~1400 bp fragment of an ion channel, trpc2, from *P. shermani* (JN805769). The predicted amino acid sequence of the salamander trpc2 was similar to the mouse (74% amino acid identity), frog (*S. tropicalis*; 88% identity), and zebrafish (76% identity) sequences. In situ hybridization revealed the ubiquitous expression of trpc2 RNA in the entire extent of the lateral diverticulum of the nasal cavity (VNO; Figure 5), corresponding to delineation of the plethodontid VNO based on morphology established by other investigators (Dawley and Bass 1988; Woodley 2007). Labeling was most intense in the basal half of the chemosensory neuroepithelium, corresponding to the location of the cell bodies. In all individuals, mRNA expression of trpc2 was not seen in the MOE.

mRNA expression of G_{olf} in both the MOE and VNO

G_{olf} was used as a proxy for ORs because work in other species indicated that signal transduction by most ORs requires G_{olf} (Berghard and Buck 1996). To determine tissue expression of G_{olf}, we amplified a fragment of the homolog of the G protein, G_{olf} from *P. shermani* olfactory cDNA (JN805770). The fragment was 954 bps in length and showed strong similarity to other vertebrate G_{olf} proteins (89% amino acid identity with mouse; 93% with *S. tropicalis*; and 83% with zebrafish). The in situ hybridization clearly demonstrated strong and ubiquitous mRNA expression of G_{olf} in *P. shermani* MOE. The staining was distributed throughout the rostral–caudal extent of the MOE. However, there was distinct—but light and sparse—labeling in the lateral diverticulum where the VNO was located (Figure 6).
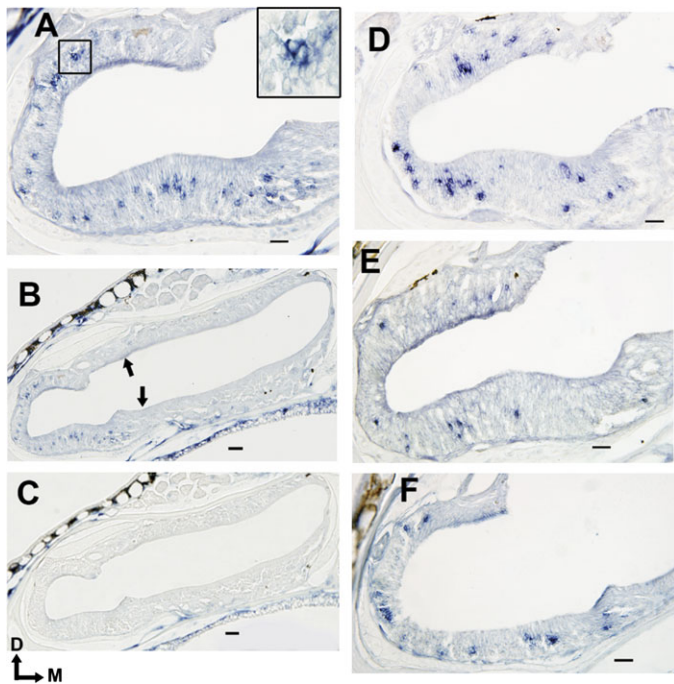
Figure 3 Representative photomicrographs of chemosensory epithelia from red-legged salamanders showing V2R mRNA expression. (**A**) Caudal VNO, showing V2R26 mRNA expression in a male. (**B**) Lower magnification corresponding to Panel A, showing the absence of V2R26 mRNA expression in the medially positioned MOE (the chemosensory epithelium to the right of the arrows). (**C**) Sense control corresponding to Panel C showing no signal in the chemosensory epithelia. (**D**) Caudal VNO, showing V2R29 expression in a male. (**E**) Caudal VNO, showing V2R18 expression in a male. (**F**) Caudal VNO, showing V2R18 expression in a female. Scale bars represent 200 μm (**A,D,E,F**) or 100 μm (**B,C**). Arrows in lower right corner indicate dorsal (D) and medial (M). This figure appears in color in the online version of *Chemical Senses*.

Discussion

Our study represents the first description of the diversity and expression of vomeronasal receptors in a salamander. We show that the terrestrial salamander *P. shermani* possesses a diverse complement of vomeronasal receptors and that some of the receptor subfamilies are expressed by particular subsets of sensory neurons in the VNO but not the MOE. We also found evidence for sex differences in mRNA expression of some but not all V2R subfamilies in *P. shermani*. In addition, we documented the first description of ubiquitous and exclusive expression of trpc2 mRNA transcripts in an amphibian VNO. G_{olf} transcripts were expressed not only throughout the *P. shermani* MOE but also somewhat in the VNO, suggesting that ORs may be expressed in the VNO. These data allow us to make comparisons with other vertebrates, especially the aquatic anurans *X. laevis* and *S. tropicalis* and to gain insight into the evolution of receptor diversity and expression.

One of the objectives of this study was to investigate the evolutionary relationships among the isolated salamander V2Rs and other vertebrate sequences. The phylogenetic analysis revealed that the sequences from a terrestrial salamander were generally more similar to those from an aquatic
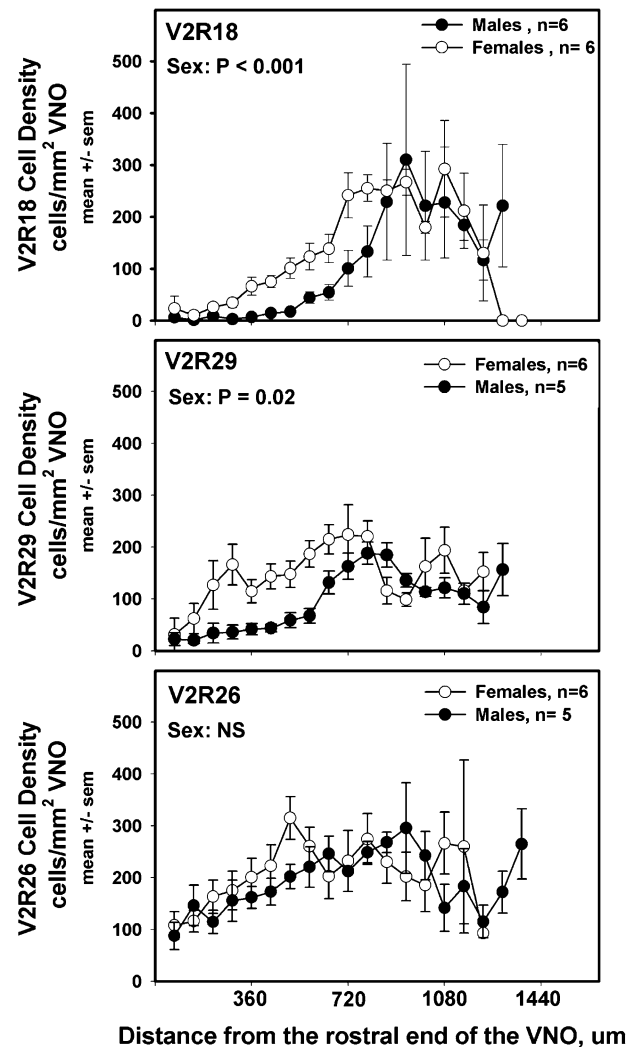

Figure 4 Density of cells with positive hybridization signal for V2R18, V2R29, and V2R26 along the rostral–caudal dimension of the VNO. The densities of labeled cells for V2R18 and V2R29 are greater in *P. shermani* females relative to males. The difference is most evident in the rostral part of the VNO (see text for details).

anuran rather than to fish. This lack of similarity between amphibian and fish sequences suggests that perhaps diversification of amphibian V2Rs occurred in their common tetrapod ancestor during the transition from a completely aquatic to an at least partially terrestrial environment (Shi and Zhang 2007). In addition, both salamanders and frogs possessed V2Rs that appeared to be homologous to the family B in mouse, but there were other groups of salamander and frog sequences that did not show direct homology with mouse sequences. This is a common result when comparing highly divergent vertebrate species because of lineage-specific gene duplication that occurs in particular lineages after speciation. We did not isolate a salamander homolog of the mouse family C, a family that does not show monogenic expression in mice (Martini et al. 2001). There is a single member of this group in the *S. tropicalis* genome (Shi and
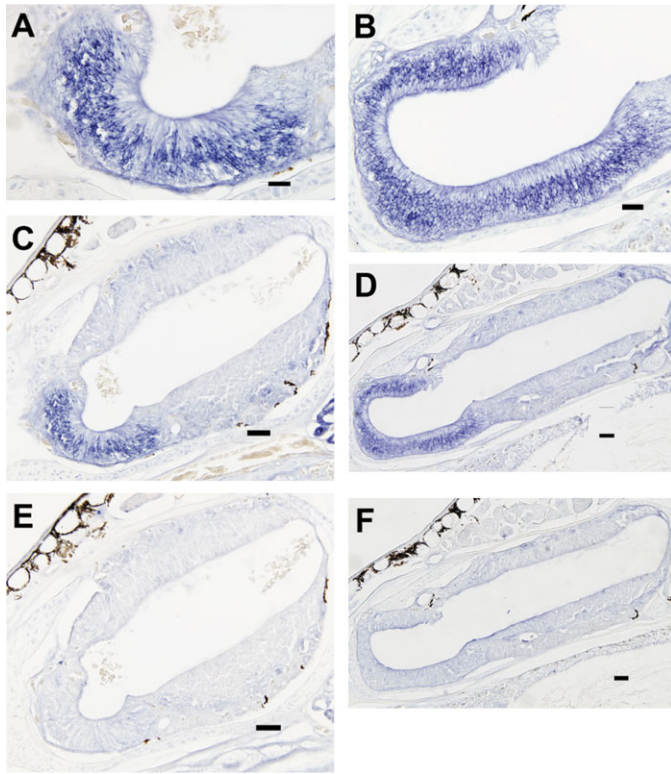
Figure 5 Representative photomicrographs of chemosensory epithelia from male red-legged salamanders showing trpc2 mRNA expression. (**A**) Rostral VNO, showing trpc2 mRNA expression. (**B**) Caudal VNO, showing trpc2 mRNA expression. (**C**) Lower magnification corresponding to Panel A, showing absence of trpc2 mRNA in the medially positioned MOE. (**D**) Lower magnification corresponding to Panel B, showing absence of trpc2 mRNA in the medially positioned MOE. (**E**) Sense control corresponding to Panel C showing no signal in the chemosensory epithelia. (**F**) Sense control corresponding to Panel D showing no signal in the chemosensory epithelia. Scale bars represent 200 μm (**A,B**) or 100 μm (**C–F**). This figure appears in color in the online version of *Chemical Senses*.

Figure 6 Representative photomicrographs of chemosensory epithelia from male red-legged salamanders showing G_{olf} mRNA expression. (**A**) Rostral nasal cavity, showing G_{olf} mRNA expression. Note the strong G_{olf} mRNA expression in the medially positioned MOE (to the right of the arrows). (**B**) Caudal nasal cavity, showing G_{olf} mRNA expression. Note the strong G_{olf} expression in the medially positioned MOE (to the right of the arrows). (**C**) Higher magnification corresponding to Panel A, showing the light mRNA expression in the laterally positioned VNO. (**D**) Higher magnification corresponding to Panel B, showing G_{olf} mRNA expression in the laterally positioned VNO. Inset shows boxed area at higher magnification. (**E**) Sense control corresponding to Panel A showing no signal in the chemosensory epithelia. (**F**) Sense control corresponding to Panel B showing no signal in the chemosensory epithelia. Scale bars represent 200 μm (**C,D**) or 100 μm (**A,B,E,F**). Arrows indicate lateral boundary of the MOE. This figure appears in color in the online version of *Chemical Senses*.

Zhang 2007), but its expression pattern has yet to be determined in amphibians.

We hypothesize that *P. shermani* possesses a large family of V2Rs, like *S. tropicalis*, for several reasons. First, we amplified over 89 unique sequences with 2 sets of primers, although only 34 were retained for analyses. Second, the salamander V2R sequences possess conserved amino acid domains known to be important for functional stability of the molecules, but are also substantially divergent from other vertebrate sequences, and in some cases, from one another. The wide range of sequence similarity among salamander V2R fragments (45–98%) in our sample suggests that these fragments are part of a divergent repertoire because this range is similar to the range of divergence among the mouse A, B, and D families, a group that contains 114 genes (Young and Trask 2007). In addition, the partial sequences isolated in this study span the more conservative 7-transmembrane domain of the receptors. The extracellular domain was less conserved among the 3 full-length V2R sequences we
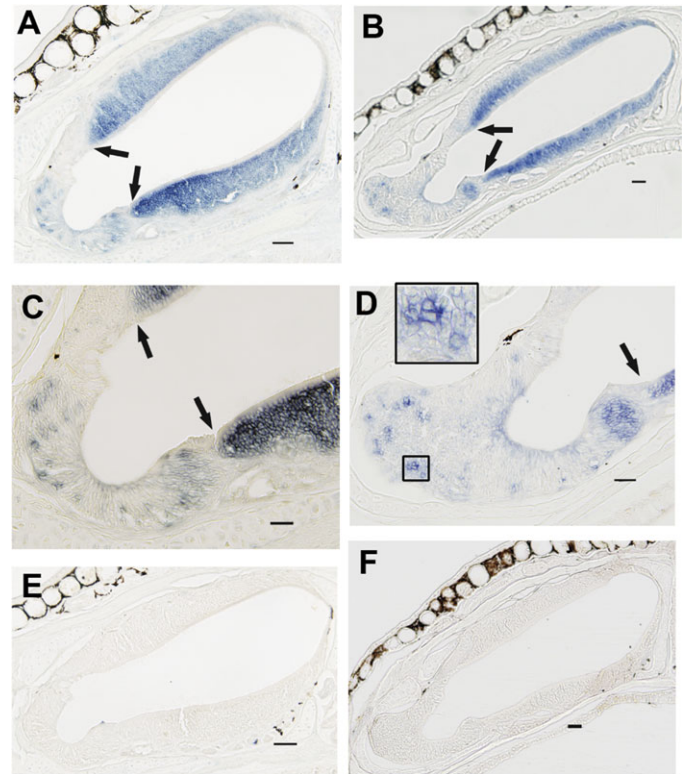
compared. The *N*-termini of V2Rs is generally more variable than the 7-transmembrane domain (Yang et al. 2005), and thus the sequence divergence among the partial *P. shermani* V2Rs may be even greater than that reported here.

In a comparative genomic study, Shi and Zhang (2007) found that the *S. tropicalis* genome contains 249 intact V2R receptors but only 21 V1Rs. In comparison, the mouse genome contains approximately 121 V2Rs (Young and Trask 2007) and 191 V1Rs (Zhang et al. 2007). Overall, the number of V2R genes and V1R genes range from 0–249 genes and 0–270 genes, respectively, in sequenced vertebrate genomes (reviewed in Shi and Zhang 2009). Despite considerable effort, we were unable to amplify any V1Rs from *P. shermani* and thus cannot make any comparisons between V1Rs and V2Rs in this species. Our difficulty in amplifying V1Rs, however, is consistent with the hypothesis

that *P. shermani* may have a fewer V1R than V2R genes, although further study is needed.

Our study provides evidence for divergence in the patterns of V2R mRNA expression among the different groups of salamander V2Rs. For 2 of the probes, the density of cells labeled by the RNA probe was significantly higher in females than in males. A third probe, however, showed no difference in expression between males and females. The results are not due to a sex difference in the rostral–caudal length of the VNO (Woodley 2007); despite the sex difference in rostral–caudal length, the probes differed in whether the V2R cell density was sexually dimorphic.

Because of the high sequence homology within subfamilies of V2Rs, it is difficult to make gene-specific probes for individual receptor mRNAs. Thus, it is important to note that probes likely bound to closely related receptors. However, using a similar strategy, sexually dimorphic expression of V2R mRNA was found in mice, although the expression of particular sequences was biased towards males and was hormone dependent (Alekseyenko et al. 2006). Thus, sex differences in V2R expression may be a common vertebrate pattern, although more studies in additional species are necessary.

The functional significance of the expression of V2Rs in salamanders is unknown, but we hypothesize that V2Rs are involved in signal transduction of pheromones. Even though this species is terrestrial, it responds behaviorally to waterborne chemical cues (Jaeger and Forester 1993; Palmer and Houck 2005). In some plethodontid salamanders, males have an exocrine (mental) gland that produces relatively large, nonvolatile proteins. Several of these proteins activate vomeronasal neurons (Wirsig-Wiechmann et al. 2002, 2006), modulate female receptivity to mating (Rollmann et al. 1999; Houck et al. 2007, 2008) and have been termed courtship pheromones. Current research in mammals indicates that V1R-expressing cells respond primarily to chemical cues that are volatile whereas V2R-expressing cells respond primarily to nonvolatile or water-soluble chemical cues (Boschat et al. 2002; Punta et al. 2002; Leinders-Zufall et al. 2009; Haga et al. 2010). If similar conclusions apply to amphibian chemoreception, then V2Rs may mediate female responses to the proteinaceous pheromones delivered by courting male *P. shermani*.

We present the first description of trpc2 localization in the VNO of a nonmammalian vertebrate. It is likely that VNO neurons coexpress V2R and trpc2 in *P. shermani* because most—if not all—of the VNO cells express trpc2. The strong expression of trpc2 in *P. shermani* VNO mirrors the pattern in mammalian taxa: in rodents, for example, trpc2 is expressed in the VNO and not in the MOE (Liman et al. 1999). This pattern suggests that trpc2 plays a fundamental role in mediating the signal transduction of chemical signals in the VNO of *P. shermani*, as it does in rodents (Leypold et al. 2002; Stowers et al. 2002). Because amphibians are a sister taxon to the rest of the tetrapod lineage, our study suggests that trpc2 may be expressed in the VNO of most

tetrapods that still possess a functional VNO. The trpc2 gene is found in most vertebrate genomes, including sea lampreys and elephant sharks (Grus and Zhang 2009), which lack VNOs. Thus the function of trpc2 in some lineages is not yet clear, although trpc2 is expressed in most microvillous neurons in the zebrafish olfactory rosette (Sato et al. 2005).

Although trpc2 and V2R mRNA were observed in the *P. shermani* VNO, in situ hybridization showed that G_{olf} mRNA is localized to the VNO as well as the MOE, a pattern that has not been seen in anurans. In mammals (Dulac 2000) and *X. laevis* (Date-Ito et al. 2008), ORs are usually coexpressed with G_{olf} in the MOE (although some mice VNO neurons express ORs but do not express G_{olf}; Levai et al. 2006). In the tiger salamander (*Ambystoma tigrinum*), OR mRNA transcripts are found throughout the MOE but not in the VNO (Marchand et al. 2004). We suspect that our use of probes for G_{olf}, rather than specific ORs as used by Marchand et al. (2004), may have revealed an additional pattern of OR expression in the *P. shermani* VNO. Further work on chemosensory receptor expression in amphibians is needed to determine the functional significance underlying the expression localization patterns of specific G proteins and chemoreceptor families.

In both salamanders and anurans, the mRNA expression of vomeronasal and olfactory receptors is not segregated neatly into the VNO versus the MOE as in mice and rats, despite the anatomical separation of the VNO and MOE. In *X. laevis*, limited expression of V2R (and $G_{\alpha o}$) mRNA is found in the posterolateral principal cavity, a part of the MOE that also expresses ORs (and G_{olf}) and V1Rs (and $G_{\alpha i2}$) (Date-Ito et al. 2008). Hagino-Yamagishi et al. (2004) postulated that the *X. laevis* posterolateral principal cavity may be a ''remnant'' of the VNO that was present in ancestral amphibians, and this is the reason V2Rs are expressed in this region. In contrast, we found no evidence of V2R expression in the salamander MOE. The difference in V2R expression between *X. laevis* and *P. shermani* may be because the location of the salamander VNO (in the lateral diverticulum) has been conserved over evolutionary time, unlike the VNO of *Xenopus*. It is also possible that we have not yet identified and characterized the expression of the particular V2Rs genes that are expressed in the *P. shermani* MOE. Investigations of V2R expression in aquatic salamanders and caecilians may be useful for revealing general patterns of chemosensory gene expression in amphibians.

We have not only shown that *P. shermani* and *X. laevis* share similarities in expression but also that there are differences between these 2 taxa that warrant further investigation. Several possibilities may explain the differences in the signaling molecule expression between plethodontid salamanders and clawed frogs. Amphibians originated about 250 million years ago and diverged rapidly such that extant caudates and anurans differ substantially in many ways. For example, ecological and morphological differences between aquatic and terrestrial amphibians may contribute to the

differences in vomeronasal receptor expression in these groups. *Plethodon shermani* is terrestrial, whereas *Xenopus* is aquatic, an important life history difference that may influence the size of V1R and V2R receptor repertoires, and perhaps the distribution of V1R/V2R and OR expression in the chemosensory neuroepithelia (Shi and Zhang 2007). Also, social interactions, especially courtship, are strongly mediated by chemoreception in *P. shermani* (Houck 2009) but largely by acoustic communication in *Xenopus* (Kelley and Tobias 1999). Generally, many plethodontid species respond to both volatile and nonvolatile cues from conspecifics (e.g., Dawley 1986; Dantzer and Jaeger 2007; Palmer and Houck 2005; Schubert et al. 2008). These chemical cues have yet to be characterized but represent additional opportunities for understanding the mechanisms of amphibian chemoreception, especially given the variety of responses that chemical cues elicit in plethodontids. Ultimately, studies of more amphibian species, as well those linking ligands to vomeronasal receptors, will be necessary to determine whether VNO differences between frogs and salamanders reflect differences in phylogenetic history, life history, or use of sensory modalities.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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