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In situ localization of plethodontid courtship pheromone mRNA in formalin-fixed tissue

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Abstract

Male plethodontid salamanders produce courtship pheromones that increase female receptivity. Three protein components of the courtship pheromone cocktail have been characterized in the mental gland of *Plethodon shermani*, the red-legged salamander: plethodontid receptivity factor (PRF), plethodontid modulating factor (PMF), and sodefrin precursor-like factor (SPF). In this study, a streamlined *in situ* hybridization (ISH) protocol, employing a biotinylated oligonucleotide probe, is used to visualize the sites of pheromone expression in formalin-fixed paraffin-embedded *P. shermani* mental gland and post-cloacal tail tissue. Results corroborate previous RT-PCR studies on pheromone expression. PRF and PMF are highly expressed in *P. shermani* mental gland, while SPF expression is more variable. None of the tested pheromones is expressed in dorsal or ventral tail glands. The reported protocol is simple, rapid, and effective, allowing visualization of high-copy mRNA transcript in formalin-fixed tissue.

Keywords: *In situ* hybridization; Pheromone; *Plethodon shermani*; Plethodontidae; PRF; PMF; SPF

1. Introduction

Pheromone delivery is an integral part of courtship in the salamander family Plethodontidae. During the courtship season, males in all four plethodontid lineages develop specialized glandular tissue below the chin, called the mental gland (Houck and Sever, 1994; Sever, 2003). Hypertrophy of the mental gland is androgen dependent (Sever, 1976; Woodley, 1994). During the conserved, stereotyped courtship behavior characteristic of plethodontid salamanders, males deliver courtship pheromones from the mental gland to females through one of several mechanisms (described in Houck and Sever, 1994; Houck and Arnold, 2003; Picard, 2005). Courtship pheromones differ from other reproductive pheromones (e.g., sex attractants, priming pheromones) in that courtship pheromones are transmitted only between potential sexual partners and only during courtship (Arnold and Houck, 1982). Male delivery of plethodontid courtship pheromones increases female receptivity (Houck and Reagan, 1990; Houck et al., 1998). The use of courtship pheromones has been reported in the majority of salamander species studied, making this order unique among vertebrates (Houck, 1986).

Within the complex of *Plethodon* species in the eastern US, the *Plethodon glutinosus* group employs a method of courtship pheromone delivery in which a male rubs or slaps its mental gland on the female's nose. Pheromones enter the nasal cavity via the female's nasolabial grooves, and these aqueous odorans are shunted to the vomeronasal organ (Dawley, 1992). Vomeronasal response (Wirsig-Wiechmann et al., 2002, 2006) is inferred to convey information to the brain (Schmidt et al., 1988; Laberge and Roth, 2004), as in other documented cases of pheromone stimulation to the VNO (Halpern and Martinez-Marcos, 2003). Plethodontid receptivity factor (PRF; isolated by Rollmann et al., 1999)

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is one of the pheromones delivered in this manner. PRF is a 22 kDa protein produced in the mental glands of male *P. shermani* (Stejneger) (formerly *Plethodon jordani*; Highton and Peabody, 2000), a member of the *P. glutinosus* species group. Behavioral tests demonstrate that delivery of this protein to females during courtship significantly decreases time to insemination (Rollmann et al., 1999). RT-PCR surveys have detected PRF mRNA in the mental glands of all eastern *Plethodon* species tested (27 species) (Palmer et al., 2005). Among these species, the PRF protein exhibits wide allelic diversity at the individual (Watts et al., 2004), population (Rollmann et al., 2000), and species levels (Palmer et al., 2005). PRF is not expressed in the mental glands of *Plethodon* species surveyed from the western US, in the sister genus *Aneides*, or in the genera *Batrachoceps*, *Desmognathus*, and *Eurycea*, representing the three other plethodontid lineages (Palmer et al., 2005; for phylogenetic relationships see Mueller et al., 2004). Nor is PRF present in tail skin of *P. shermani* males or females (Palmer et al., 2005).

Two additional protein components of the *P. shermani* courtship pheromone cocktail have been isolated: plethodontid modulating factor (PMF; Feldhoff et al., 1999) and sodefrin precursor-like factor (SPF; Palmer et al., 2007). PMF is a 7 kDa protein that is consistently detected in a 2:1 (PMF:PRF) ratio with PRF in mental gland extract (Rollmann et al., 1999; Feldhoff et al., 1999). PMF is widely expressed among the Plethodontidae; RT-PCR detected PMF expression in species representing all four lineages of the family (Palmer, 2004). When experimentally applied alone to females during courtship, PMF significantly increases time to insemination; however, when applied in conjunction with PRF, courtship time decreases significantly (Houck et al., 2007a). PMF modulates behavior via a population of vomeronasal neurons that are distinct from those stimulated by PRF (Wirsig-Wiechmann et al., 2006).

SPF is reported only at low levels of expression in *P. shermani* mental glands, but is highly expressed in the mental glands of *Desmognathus ocoee* (Palmer et al., 2007). Also, RT-PCR surveys have detected SPF in species of *Aneides*, *Desmognathus*, *Eurycea*, and *Plethodon*, representing three of the four lineages of Plethodontidae (Palmer et al., 2007). Behavioral studies show that SPF significantly decreases time to insemination when applied during courtship in *D. ocoee* (Houck et al., 2007b). The amino acid sequence of the SPF protein shows approximately 22% homology to the precursor of sodefrin, a peptide sex pheromone, found in the aquatic breeding newt *Cynops pyrrhogaster* (described by Kikuyama and Toyoda, 1999).

Except for one study of PMF binding in *P. shermani* tissue (Palmer et al., submitted for publication), expression of these three pheromone proteins has been studied through RT-PCR assays, which are conducted *in vitro*. While RT-PCR is a sensitive technique for detecting low levels of gene expression, it does not specifically locate the sites of gene expression in tissues or identify the cells involved (except in single-cell RT-PCR). *In situ* hybridization (ISH) is an alternate technique for studying gene expression. ISH experiments provide morphological as well as genetic information. This technique can locate gene expression in intact tissues, and can identify the specific cells responsible for expression of a certain gene. ISH is a particularly valuable tool in complex organs, such as skin, that have multiple tissues and cell types. Here, we employ the technique to visualize the location of pheromone expression in mental gland and tail tissue of *P. shermani*. Tail skin is a hypothesized alternative source of pheromone secretions (Sever, 1989; Staub and Paladin, 1997).

ISH can be performed in frozen or fixed tissues, using a variety of probes. Formalin fixation of tissue yields more intact morphology, but fresh-frozen tissue effectively preserves target RNA without the crosslinking that occurs with fixation. Fixed tissue must usually be pretreated with enzymatic or other digests to unmask target sequences. Probes can be short synthesized oligonucleotides, or long strands of DNA or RNA synthesized *in vitro* by transcription, reverse transcription or PCR. Isotopic probes are highly sensitive and allow quantification of signal. However, using radioactive probes raises difficulties in safety and waste disposal. Non-isotopic labels (such as digoxigenin, biotin, or FITC) are easier to use but less effective in detecting expression of genes with low copy numbers. In order to yield a clear signal, factors that must be optimized include: fixation solution and time, protease digest parameters, probe concentration, hybridization time and temperature, and stringency of post-hybridization washes. Kadkol et al. (1999) provide an excellent review of theory and technique that highlights both the strengths and the difficulties of ISH.

One drawback of ISH is the cumbersome protocol required for good results. Long isotopic probes in fresh-frozen tissues yield excellent signal (e.g., see Hollis et al., 2006), but some labs may find this protocol less than ideal for a variety of reasons, including safety concerns about using radioactive materials, background staining due to electrostatic adhesion of very long probes, complicated hybridization buffers, results that take weeks to develop, and the lack of availability of fresh-frozen tissue. Many labs rely on archives of formalin-fixed paraffin-embedded (FFPE) tissues, which store and section well but are problematic for use in mRNA detection due to formalin crosslinking of the mRNA signal. Extensive pretreatment to unmask target sequences increases both time and cost. Here, we report a simplified—but effective—*in situ* hybridization protocol to visualize the sites of pheromone expression in FFPE *P. shermani* tissue, using a short biotinylated oligonucleotide probe.

2. Methods

2.1. Specimen preparation

Twelve preserved male specimens of *P. shermani* were obtained from the collections of SJ Arnold and NL Staub (NLS ID# 1859, 1862, 1863,

1865, 1866, 1868, 1869, 1927, 1929, 1932; SJA ID# 41351, 41352). All specimens were in reproductive condition, as assessed by the presence of well-developed mental glands. Specimens had been sacrificed in MS222 (tricaine methanesulphonate), fixed for 24 h in neutral buffered formalin, rinsed in water for 24 h, and stored in 70% ethanol.

Mental gland, dorsal tail base, and ventral tail (adjacent to the cloaca) tissues were dissected. Excised tissues were embedded and sectioned according to routine histological protocols (Presnell and Schreibman, 1997). Tissues were dehydrated in graded ethanol, cleared in Citrisolv (Fisher, Pittsburgh, PA), embedded in paraffin, sectioned at 12 μm with a rotary microtome, mounted on electrostatically charged Superfrost Plus slides (Fisher) or charged barrier slides (Biogenex, San Ramon, CA), and dried overnight at 50 °C prior to *in situ* hybridization. Mental glands from four specimens, and dorsal and ventral tail skin from four or five specimens (depending on probe), were tested for each pheromone; two additional mental glands were tested for SPF due to high variability of pheromone expression.

2.2. Probe design

The complete mRNA coding sequences of PRF (Accession No: AY499372), PMF (DQ882251–DQ882378), and SPF (DQ097071) were obtained from GenBank. Antisense probes were designed against regions of each coding sequence that are highly conserved both intraspecifically and interspecifically, so that probes would bind to the mRNA of various alleles of each gene. Probe sequences were analyzed for hairpin structures and self-dimerization potential using the IDT OligoAnalyzer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer). The following 5′ biotinylated deoxyribonucleotide probes were purchased from Integrated DNA Technologies (Coralville, IA): PRFPROBE (5′-TAG GGA GGA GGG AGT CAG CAC TGC TCT GAA TGT CCT CAG CAA-3′); PMFPROBE (5′-CTG ACC CTT CTT CAG CTA AAC CAG CTT CCA TAG CGT CAC AGA-3′) and SPFPROBE (5′-GTC TTC CAA GCA GTG CAG ACA CTT TAC CCC ATT CGG TGT GTT-3′). Lyophilized probes were resuspended in RNase-free TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at a concentration of 1 mg/mL and stored at −20 °C. Probe working solutions of 100 μg/mL were made from stock solutions. For *in situ* hybridization experiments, 5 μL working solution was added per mL of hybridization solution to yield a final probe concentration of 500 ng/mL.

2.3. In situ hybridization

Sections were deparaffinized in Citrisolv and hydrated in graded ethanol to DEPC-treated water (DEPC: diethylpyrocarbonate, an RNase inhibitor). A PAP pen (Daido Sangyo Co, Tokyo) was used to draw a hydrophobic barrier around the sections on each Superfrost Plus slide. Sections on barrier slides were already mounted within a Teflon barrier. Sections were overlaid with proteinase K (Ambion, Austin, TX; 50 μg/mL in DEPC–PBS) and incubated in a humid chamber at 37 °C for 30 min. The proteinase digest was quenched for 5 min in glycine buffer (0.2% glycine in DEPC–PBS). Following 5 min washes in DEPC–PBS and DEPC–H_2O, slides were transferred to an incubation tray and allowed to air-dry briefly to remove excess water. Sections were overlaid with hybridization solution (4 mM EDTA, 5× SSC, 50% formamide, 250 μg/mL yeast tRNA [Sigma, St. Louis, MO]; 1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) containing 500 ng/mL oligonucleotide probe. Negative controls were overlaid with hybridization solution alone. Sections were incubated overnight at 42 °C in a humid chamber.

The following day, sections were washed twice for 10 min each in 0.2× SSC pre-warmed to 37 °C, then blocked in pre-warmed *In Situ* Hybridization Blocking Solution (Vector, Burlingame, CA) for 30 min at 37 °C. Blocking solution was tipped off. Sections were overlaid with an alkaline phosphatase–streptavidin conjugate (1:500 in blocking solution; Vector) and incubated 30 min at room temperature. Slides were washed in two changes of 100 mM Tris, pH 9.5, for 3 min each. BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) substrate solution (Vector) was prepared in 5 mL of 100 mM Tris, pH 9.5, according to kit instructions. In an incubation tray, sections were overlaid with substrate solution. The tray was then covered and the slides were incubated 4 h in the dark. Slides were washed in 100 mM Tris, pH 9.5, for 5 min, rinsed briefly in water, and counterstained in 0.1% methyl green for 5 min. After another rinse in deionized water, slides were dehydrated in graded ethanol and cleared in Citrisolv. Coverslips were mounted with Cytoseal 60 (Richard Allan Scientific, Kalamazoo, MI). Slides were viewed with a Leica DME light microscope and photographed using a Canon Powershot G6 camera attached via a Canon G6 adapter tube to a Zeiss Axiolab microscope.

2.4. Controls

Sections of mouse intestine were hybridized with each probe as described above; no binding was detected. Sense probes for PMF and SPF were tested and did not bind in *P. shermani* mental gland tissue. Slides processed without alkaline phosphatase or without the incubation with substrate solution showed no probe binding. Sections of mental gland from one male *Aneides ferreus* (collection of NL Staub, ID# 1679) were hybridized with the PRF probe only; no binding was detected. *A. ferreus* tissue was not used as a negative control for PMF or SPF because RT-PCR results indicate that those proteins are expressed in *Aneides* (Palmer, 2004).

3. Results

The PRF and PMF probes specifically labeled mental glands in all individuals tested, localizing around nuclei on the periphery of each gland (Fig. 1A and B). SPF labeling was more variable; of six individual mental glands tested, two were positive for SPF expression, while four were negative (Fig. 1C). Within each specimen that expressed SPF, labeling intensity varied from gland to gland, so that some glands appeared to be negative for probe binding while others were positive.

No pheromone expression was detected in the granular or mucous glands of tail tissue or adjacent to the mental gland (Fig. 1A and B). Some flask cells were labeled with PMF and SPF, but not PRF, in both mental and tail epidermis, though results were inconsistent. Epidermis in the mental region was negative for PRF and PMF, but showed variable labeling with SPF. Two individuals displayed light to moderate labeling in the cytoplasm of epidermal cells. One individual had a basal layer of darkly labeled epidermal cells. The epidermis did not label at all in three specimens.

4. Discussion

The mental gland binding results reported here are generally consistent with previously published RT-PCR and cDNA library sequencing results (Watts et al., 2004; Palmer et al., 2005; Palmer et al., 2007; Palmer et al., submitted for publication). PMF and PRF are highly and ubiquitously expressed in the mental gland; SPF is present to a lesser extent, with no expression detectable in some individuals. SPF is a rare element in *P. shermani* cDNA libraries (Palmer et al., 2007). It may not be expressed at all in some individuals or, alternately, levels of expression may be too low to be detected by ISH. Given that SPF appears to have been replaced by PRF as the primary courtship pheromone

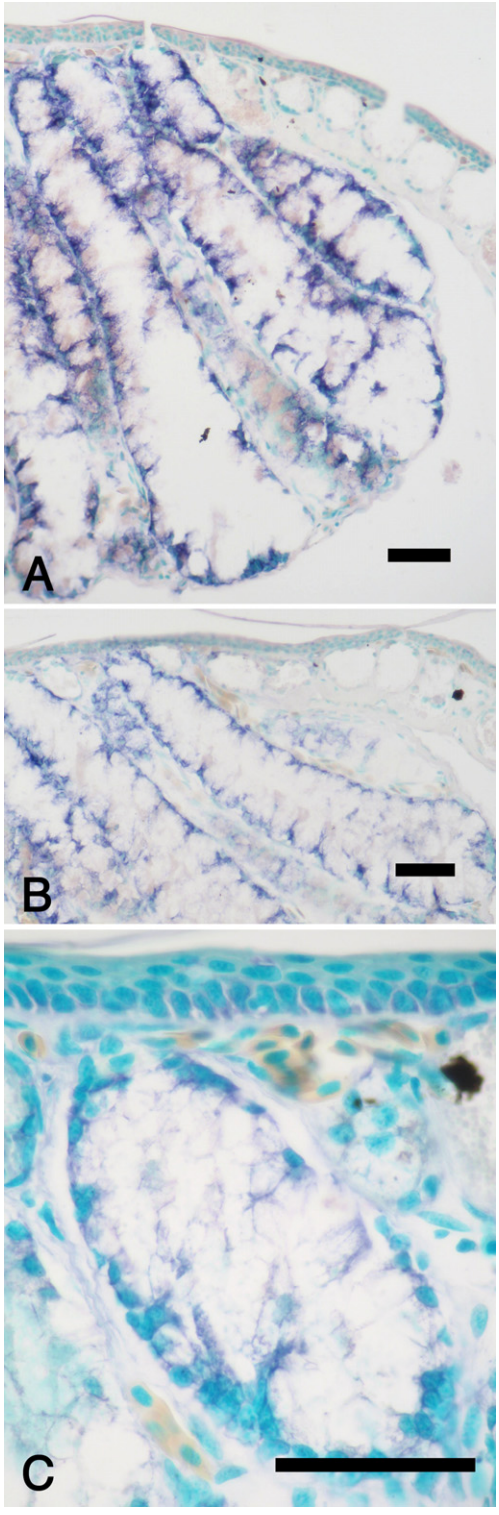

Fig. 1. *Plethodon shermani* mental glands are peripherally labeled with (A) PRF, (B) PMF, and (C) SPF. SPF labeling was variable both within and between individuals. Note unlabeled granular glands to the right of mental glands in A and B. Scale bars 100 μm.

in *P. shermani* (Palmer et al., 2007), it is not surprising that some individuals show little to no expression. The ubiquitous expression of PRF and PMF in *P. shermani* mental gland underscores their importance in this species.

In addition to mental glands, sexually dimorphic tail glands have been suggested as another source of courtship pheromones (Sever, 1989; Staub and Paladin, 1997). In male and female *Aneides lugubris*, Staub and Paladin (1997) describe hypertrophied ventral tail glands (called modified granular glands) that are histochemically similar to those in mental glands. They hypothesize that these glands produce sex- or species-specific pheromones. Sever (1989) suggested that during a behavior termed the tail-straddling walk (when the female's chin rests on the male's dorsal tail base), secreted pheromones could be transferred to the female vomeronasal organ via the nasolabial grooves. Based on these hypotheses, we tested dorsal and ventral *P. shermani* tail skin for the presence of PMF, SPF, and PRF. None of these pheromone proteins was detected in tail glands. Because of the high copy number of mRNA transcripts necessary to produce a protein for secretion, it is unlikely that pheromone RNA was present in amounts too low to be detected. Either these tail glands are not involved in pheromone production in *P. shermani*, or the glands produce different pheromone proteins that have not yet been characterized. Further studies *in situ* are warranted to test for the presence of these courtship pheromones (PMF, SPF, and PRF) in the modified granular glands of male *Aneides*.

Palmer et al. (submitted for publication) report the presence of PMF mRNA transcript in *P. shermani* tail skin, as detected by RT-PCR. However, in the experiments reported here, PMF labeling in tail skin was confined to some flask cells. As noted above, the RT-PCR technique provides no information on the location of mRNA expression. The sensitivity of RT-PCR may have been sufficient for Palmer et al. to detect flask cell mRNA for PMF, even though it makes up only a tiny fraction of the total RNA from sampled skin. Additional work is necessary to determine whether flask cells are a source of pheromones and how they may facilitate pheromone secretion from other glands.

While hyper-expression occurs in the mental gland, PMF expression also is reported at low levels in the intestine, liver, and kidneys of both male and female *P. shermani* (Palmer et al., submitted for publication). It is surprising that a courtship pheromone protein should be expressed in these tissues, particularly in both sexes; the widespread expression might correlate instead to a more generalized function as a secretory carrier protein. However, a protein with a strict carrier function would not be expected to stimulate vomeronasal neurons, as reported by Wirsig-Wiechmann et al. (2006). Further research is needed to characterize the action of this protein.

Levels of SPF expression in tissues other than the mental gland have not been tested, except as reported here. Nor has SPF expression been visualized in the mental gland of *D. ocoee*, the species in which its pheromonal effects have been demonstrated.

The protocol described above is highly effective in detecting abundant mRNA transcripts. The targeted

pheromone proteins are produced en masse for secretion, so there is an expected high copy number of pheromone transcript, resulting in good signal. The protocol may lack sensitivity, however, disparities between RT-PCR results and ISH results may be explained by differences in the sensitivity of these techniques.

Our protocol was designed to include the fewest steps necessary to achieve a good signal. The use of a short oligonucleotide probe minimizes electrostatic interactions that can cause non-specific background staining, eliminating the need for acetylation of the tissue before hybridization. The proteinase digest is necessary and sufficient to rescue probe binding sites from formalin crosslinking. Decrease in proteinase concentration, or length or temperature of digestion, resulted in a dramatic loss of signal (M. Fontana, pers. obs.). Hybridization buffer recipes in many ISH protocols are complicated and time-consuming to make up. The buffer used here, taken from Warner et al. (1997), omits many standard ingredients but retains the typical 50% formamide content and still provides an effective hybridization medium.

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References

Arnold, S.J., Houck, L.D., 1982. Courtship pheromones: evolution by natural and sexual selection. In: Nitecki, M. (Ed.), Biochemical Aspects of Evolutionary Biology. University of Chicago Press, Chicago, IL, pp. 173–211.

Dawley, E.M., 1992. Sexual dimorphism in a chemosensory system: the role of the vomeronasal organ in salamander reproductive behavior. Copeia 1992, 113–120.

Feldhoff, R.C., Rollmann, S.D., Houck, L.D., 1999. Chemical analysis of courtship pheromones in a plethodontid salamander. In: Johnston, R.E., Muller-Schwarze, D., Sorensen, P.W. (Eds.), Advances in Chemical Signals in Vertebrates, vol. 8. Kluwer Academic, Plenum Publishers, NY, pp. 117–125.

Halpern, M., Martinez-Marcos, A., 2003. Structure and function of the vomeronasal system: an update. Prog. Neurobiol. 70, 245–318.

Highton, R., Peabody, R., 2000. Geographic protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the Southern Appalachian Mountains with the description of four new species. In: Bruce, R.C., Jaeger, R.G., Houck, L.D. (Eds.), The Biology of Plethodontid Salamanders. Kluwer Academic, Plenum Press, NY, pp. 31–93.

Hollis, D.M., Coddington, E.J., Moore, F.L., 2006. Neuroanatomical distribution of cannabinoid receptor gene expression in the brain of the rough-skinned newt, *Taricha granulosa*. Brain Behav. Evol. 67, 135–149.

Houck, L.D., 1986. The evolution of salamander courtship pheromones. In: Duvall, D., Muller-Schwarze, D., Silverstein, R.M. (Eds.), Chemical Signals in Vertebrates, Ecology, Evolution, and Comparative Biology, vol. 4. Plenum Press, NY, pp. 173–190.

Houck, L.D., Arnold, S.J., 2003. Courtship and mating behavior. In: Sever, D.M. (Ed.), Reproductive Biology and Phylogeny of Urodela. Science Publishers, Inc., Enfield, NH, pp. 383–424.

Houck, L.D., Bell, A.M., Reagan-Wallin, N.L., Feldhoff, R.C., 1998. Effects of experimental delivery of male courtship pheromones on the timing of courtship in a terrestrial salamander, *Plethodon jordani* (Caudata: Plethodontidae). Copeia 1998, 214–219.

Houck, L.D., Palmer, C.A., Watts, R.A., Arnold, S.J., Feldhoff, P.W., Feldhoff, R.C., 2007a. A new vertebrate courtship pheromone that affects female receptivity in a terrestrial salamander. Anim. Behav.

Houck, L.D., Watts, R.A., Mead, L.M., Palmer, C.A., Arnold, S.J., Feldhoff, P.W., Feldhoff, R.C., 2007b. A new vertebrate pheromone, SPF, increases female receptivity in a salamander. In: Beynon, R., Hurst, J., Muller-Schwarze, D. (Eds.), Chemical Signals in Vertebrates XI. Springer. in press.

Houck, L.D., Reagan, N.L., 1990. Male courtship pheromones increase female receptivity in a plethodontid salamander. Anim. Behav. 39, 729–734.

Houck, L.D., Sever, D.M., 1994. Role of the skin in reproduction and behavior. In: Heatwole, H., Barthalmus, G.T. (Eds.), Amphibian Biology, The Integument, vol. 1. Surrey Beatty and Sons, Australia, pp. 351–381.

Kadkol, S.S., Gage, W.R., Pasternack, G.R., 1999. *In situ* hybridization: theory and practice. Mol. Diag. 4, 169–183.

Kikuyama, S., Toyoda, F., 1999. Sodefrin: a novel sex pheromone in a newt. Rev. Reproduction 4, 1–4.

Laberge, F., Roth, G., 2004. Connectivity and cytoarchitecture of the ventral telencephalon in the salamander *Plethodon shermani*. J. Comp. Neurol. 482, 176–200.

Mueller, R.L., Macey, J.R., Jaekel, M., Wake, D.B., Boore, J.L., 2004. Morphological homoplasy, life history evolution, and historical biogeography of plethodontid salamanders inferred from complete mitochondrial genomes. PNAS 101, 13820–13825.

Palmer, C.A., 2004. Chemical signaling and pheromone evolution in plethodontid salamanders. Ph.D. thesis, Oregon State University, Corvallis OR.

Palmer, C.A., Hollis, D.M., Watts, R.A., Houck, L.D., McCall, M.A., Gregg, R.G., Feldhoff, P.W., Feldhoff, R.C., Arnold, S.J., submitted for publication. Plethodontid Modulating Factor (PMF), a hypervariable salamander courtship pheromone in the snake toxin-like protein superfamily. FEBS J.

Palmer, C.A., Watts, R.A., Gregg, R.G., McCall, M.A., Houck, L.D., Highton, R., Arnold, S.J., 2005. Lineage-specific differences in evolutionary mode in a salamander courtship pheromone. Mol. Biol. Evol. 22, 2243–2256.

Palmer, C.A., Watts, R.A., Houck, L.D., Picard, A.L., Arnold, S.J., 2007. Evolutionary replacement of components in a salamander pheromone signaling complex: more evidence for phenotypic molecular decoupling. Evolution, in press.

Picard, A.L., 2005. Courtship in the zig-zag salamander (*Plethodon dorsalis*): insights into a transition in pheromone-delivery behavior. Ethology 111, 799–809.

Presnell, J.K., Schreibman, M.P. (Eds.), 1997. Humason's Animal Tissue Techniques, 5th ed. Johns Hopkins University Press, Baltimore.

Rollmann, S.M., Houck, L.D., Feldhoff, R.C., 1999. Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. Science 285, 1907–1909.

Rollmann, S.M., Houck, L.D., Feldhoff, R.C., 2000. Population variation in salamander courtship pheromones. J. Chem. Ecol. 26, 2713–2724.

Schmidt, A., Naujoks-Manteuffel, C., Roth, G., 1988. Olfactory and vomeronasal projections and the pathway of the nervus terminalis in ten species of salamanders A whole-mount study employing the horseradish-peroxidase technique. Cell Tissue Res. 251, 45–50.

Sever, D.M., 1976. Induction of secondary sexual characters in *Eurycea quadridigitata*. Copeia 1976, 830–833.

Sever, D.M., 1989. Caudal hedonic glands in salamanders of the *Eurycea bislineata* complex (Amphibia: Plethodontidae). Herpetologica 45, 322–329.

Sever, D.M., 2003. Courtship and mating glands. In: Sever, D.M. (Ed.), Reproductive Biology and Phylogeny of Urodela. Science Publishers, Inc., Enfield, NH, pp. 323–381.

Staub, N.L., Paladin, J., 1997. The presence of modified granular glands in male and female *Aneides lugubris* (Amphibia: Plethodontidae). Herpetologica 53, 339–344.

Warner, C.K., Whitfield, S.G., Fekadu, M., Ho, H., 1997. Procedures for reproducible detection of rabies virus antigen mRNA and genome *in situ* in formalin-fixed tissues. J. Virol. Methods 67, 5–12.

Watts, R.A., Palmer, C.A., Feldhoff, R.C., Feldhoff, P.W., Houck, L.D., Jones, A.G., Pfrender, M.E., Rollmann, S.M., Arnold, S.J., 2004. Stabilizing selection on behavior and morphology masks positive selection on the signal in a salamander pheromone signaling complex. Mol. Biol. Evol. 21, 1032–1041.

Wirsig-Wiechmann, C.R., Houck, L.D., Feldhoff, P.W., Feldhoff, R.C., 2002. Pheromonal activation of vomeronasal neurons in plethodontid salamanders. Brain Res. 952, 335–344.

Wirsig-Wiechmann, C.R., Houck, L.D., Wood, J.M., Feldhoff, P.W., Feldhoff, R.C., 2006. Male pheromone protein components activate female vomeronasal neurons in the salamander *Plethodon shermani*. BMC Neurosci. 7, 26.

Woodley, S.K., 1994. Plasma androgen levels, spermatogenesis, and secondary sexual characteristics in two species of plethodontid salamanders with dissociated reproductive patterns. Gen. Comp. Endocrinol. 96, 206–214.