



Pheromonal communication in urodelan amphibians

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Abstract

Pheromonal communication is an ancient and pervasive sensory modality in urodelan amphibians. One family of salamander pheromones (the sodefrin precursor-like factor (SPF) family) originated 300 million years ago, at the origin of amphibians. Although salamanders are often thought of as relatively simple animals especially when compared to mammals, the pheromonal systems are varied and complex with nuanced effects on behavior. Here, we review the function and evolution of pheromonal signals involved in male–female reproductive interactions. After describing common themes of salamander pheromonal communication, we describe what is known about the rich diversity of pheromonal communication in each salamander family. Several pheromones have been described, ranging from simple, invariant molecules to complex, variable blends of pheromones. While some pheromones elicit overt behavioral responses, others have more nuanced effects. Pheromonal signals have diversified within salamander lineages and have experienced rapid evolution. Once receptors have been matched to pheromonal ligands, rapid advance can be made to better understand the olfactory detection and processing of salamander pheromones. In particular, a large number of salamander species deliver pheromones across the skin of females, perhaps reflecting a novel mode of pheromonal communication. At the end of our review, we list some of the many intriguing unanswered questions. We hope that this review will inspire a new generation of scientists to pursue work in this rewarding field.

Keywords Olfactory · Reproduction · Salamander · Signaling · Vomeronasal

Introduction

Chemical communication is widespread across vertebrates and is particularly prevalent in salamanders. Salamander social behavior is strongly influenced by chemical signals, unlike frogs and toads, where acoustic communication prevails. Salamanders, while primarily restricted to moist and relatively cool habitats, have a diverse array of lifestyles. Many species are biphasic, whereby aquatic larvae metamorphose into a terrestrial life stage. However, some species are fully aquatic and others are fully terrestrial, having direct development that bypasses the aquatic larval form. Finally, adults can alternate between terrestrial (nonbreeding) and aquatic (breeding) lifestyles annually.

This variety of lifestyles makes salamanders an excellent model to understand the impact of terrestrial versus aquatic habitats on chemical communication.

As are all extant lineages, amphibians are a mix of derived and ancestral characters. They originated about 315 million years ago (mya), and thus, the view that they are transitional or intermediate between fish and reptiles is incorrect. In addition, salamanders diverged from the other amphibian orders about 290 mya (San Mauro 2010). The long evolutionary history of pheromonal signaling within salamanders makes them an ideal nonmammalian model to understand the function and evolution of pheromones.

We define pheromones as chemical signals used in social interactions between individuals of the same species (Wyatt 2017). Our definition encompasses simple molecules that trigger stereotypical responses as well as molecular mixtures with more subtle effects on behavior and physiology. Although pheromones are used in many contexts in salamanders, they are most studied in courtship and mating. Several salamander pheromones have been biochemically, molecularly, physiologically, behaviorally,

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and phylogenetically characterized. The first vertebrate peptide pheromone discovered was from a salamander and was involved in courtship behavior (Kikuyama et al. 1995); many more have been discovered subsequently (Table 1). Because many salamander pheromones are peptides or proteins, there is a relatively direct relationship between the genetic sequence and the molecular structure, facilitating study of the functional properties and molecular evolution of pheromonal signals.

Salamanders have a simple nasal cavity lined with sensory neuroepithelia consisting of both a main olfactory epithelium and an anatomically separate accessory (vomeronasal) epithelium. Surprisingly, many salamander pheromones do not appear to be delivered to, and detected by, the olfactory system. In fact, in the lineage of plethodontid salamanders, the ancestral form of pheromone delivery is through the skin (transdermal delivery), with olfactory delivery evolving later in a small subset of species.

With this review, we first provide a general overview that highlights common themes of salamander pheromonal communication. Then, we describe the rich diversity of salamander pheromonal communication according to taxonomic family. We direct readers to previous reviews of amphibian olfaction and chemical communication for more details (Dawley 1998; Eisthen and Polese 2006; Houck 1998; Kikuyama et al. 2005; Reiss and Eisthen 2008; Woodley 2015, 2010, 2014). Our goals with this review are to provide an update on this growing field, place the

work in a phylogenetic perspective, and inspire new research by highlighting many intriguing questions that remain unanswered.

General themes of salamander pheromonal communication

Context

Chemical communication is the basis of much social communication in salamanders: they rarely vocalize and are primarily nocturnal when visual cues are more difficult to detect. Salamander social behavior is rich and complex, including courtship, sexual behavior, parental care, aggression, territoriality, dominance, and submission (Houck and Arnold 2003; Jaeger et al. 2016; Woodley 2018). For our review, we focus on female–male reproductive interactions related to courtship and mating, for which most is known. At a proximate level, reproductive pheromones typically modulate behavioral motivation; at an ultimate level, they increase reproductive success by bringing together suitable mating partners, contributing to mate choice, and synchronizing male and female interactions.

Salamander male–female reproductive interactions typically consist of the following: (1) searching for, or attempting to attract suitable mating partners; (2) mate choice, where a female or male chooses a mating partner;

Table 1 Salamander pheromones that function in male–female interactions related to mating

| Pheromone | Chemical nature | Sex | Source | Invariant within species? | Species in which first described | Origin (mya) | Citation |
|----------------------|-----------------|--------|---------------|---------------------------|--|--------------------|------------------------|
| 15(epi)-PF2 α | Prostaglandin | Female | Oviduct | Yes | Korean salamander <i>Hynobius leechii</i> | ? | (Eom et al. 2009) |
| Sodefrin | Peptide | Male | Cloacal gland | Yes | Japanese firebelly newt <i>Cynops pyrrhogaster</i> | 34–26 ^a | (Kikuyama et al. 1995) |
| Silefrin | Peptide | Male | Cloacal gland | Yes | Sword-tail newt <i>Cynops ensicauda</i> | 34–26 ^a | (Yamamoto et al. 2000) |
| Imorin | Peptide | Female | Oviduct | Yes | Japanese firebelly newt <i>Cynops pyrrhogaster</i> | ? | (Nakada et al. 2017) |
| Persuasins | Protein | Male | Cloacal gland | Yes | Palmate newt <i>Lissotriton helveticus</i> | 51–45 ^b | (Maex et al. 2018) |
| PRF | Protein | Male | Mental gland | No | Red-legged salamander <i>Plethodon shermani</i> | 17 ^c | (Rollmann et al. 1999) |
| PMF | Protein | Male | Mental gland | No | Red-legged salamander <i>Plethodon shermani</i> | 66 ^c | (Houck et al. 2007) |
| SPF | Protein | Male | Mental gland | No | Ocoee salamander <i>Desmognathus ocoee</i> | 300 ^d | (Houck et al. 2008b) |

^aJanssenswillen et al. (2015a)

^bMaex et al. (2018)

^cWilburn et al. (2017a)

^dVan Bocxlaer et al. (2015)

and (3) mating behavior. Pheromones may play a role in all these interactions. While some species shed gametes into the water and fertilization is external, the vast majority of species have internal fertilization (Fig. 1). In species with internal fertilization, sperm are passed to the female via a spermatophore, which is a gelatinous base with a sperm cap. For insemination, a female positions her cloacal vent over the spermatophore and transports the sperm cap into her cloaca. The sperm are stored in a cloacal storage organ until the female ovulates, at which point the sperm and egg fuse, and the fertilized eggs are deposited externally. Pheromones play a key role in the synchronization of female and male behavior in species with internal fertilization.

Historically, female salamanders were viewed as passive participants in mating, needing to be persuaded via male pheromones to mate. However, it is clear that female–male interactions are bi-directional, with both males and females exchanging physical or sensory cues including pheromones (Staub et al. 2020). The interactions likely reflect the outcome between natural and sexual selection pressures for each sex.

Salamander courtship and mating behaviors have been studied in a diversity of species, facilitating phylogenetic comparisons (Arntzen and Sparreboom 1989; Halliday 1990; Houck and Arnold 2003). Many aspects of salamander courtship and mating behaviors have been evolutionarily conserved over millions of years, while other components

are evolutionarily labile. Pheromonal communication likely arose approximately 290 million years ago, close to the origin of salamanders (Van Bocxlaer et al. 2015). As such, salamander courtship and mating has been the focus of studies in the evolution and function of signaling systems (Arnold et al. 2017; Janssenswillen et al. 2015a; Wilburn et al. 2017a).

Sources

Amphibians have highly glandular skin with a subset of these glands producing pheromones (Houck and Sever 1994; Sever 2003). Glands that produce pheromones can be clustered or scattered diffusely across the body. While many glands are hypothesized to secrete pheromones based on their secretory cycle or behavioral evidence (e.g., Jaeger and Gabor 1993; Largen and Woodley 2008; Rupp and Sever 2018), only a few glands are confirmed sources of pheromones. By confirmed, we mean that the pheromone itself has been identified either via extraction, antibody labeling, or mRNA labeling, and the response of the receiver has been demonstrated.

Confirmed sources of pheromone production include the cloacal glands and the mental gland. The ancestral function of cloacal glands is hypothesized to be pheromone production; they are called dorsal cloacal glands in salamandrids and vent glands in other families (Sever 2003).

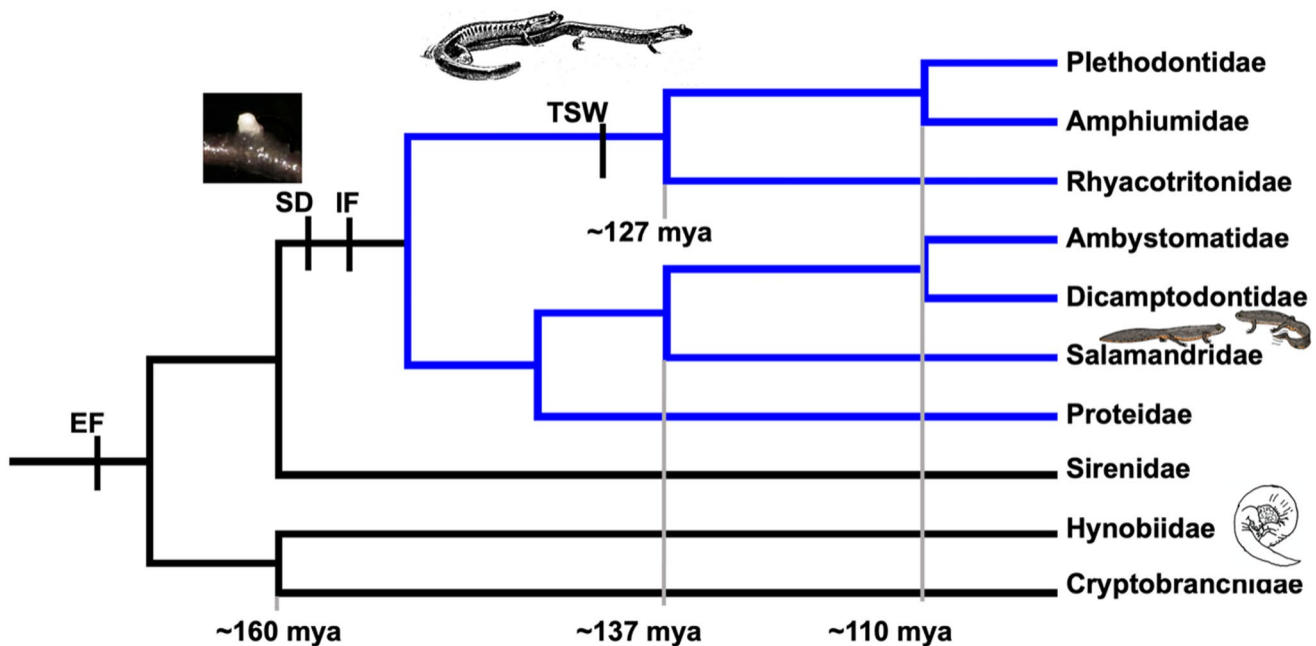


Fig. 1 Phylogeny of salamander families with key evolutionary transitions (dates from Doten et al. 2017; Shen et al. 2015). EF=external fertilization, black horizontal lines; IF=internal fertilization, blue horizontal lines; SD=spermatophore deposition (with picture of a spermatophore from a spotted salamander (*Ambystoma maculatum*), deposited on a twig in the water); TSW=tail-straddling walk. Note

that while TSW is present in Plethodontidae and Rhyacotritonidae, mating behavior of Amphiumidae has not been observed (images adapted from Park et al. 1996 [*Hynobius* male with egg clutch]; Treer et al. 2013 [salamandrid courtship]; photo of tail-straddling walk used with permission from SJ Arnold)

Mental glands are submandibular glands found in almost all plethodontids. They are thought to have evolved from salivary glands (Wilburn et al. 2014).

Dorsal cloacal glands and mental glands are sexually dimorphic and hypertrophy during the breeding season in parallel with androgen levels (Woodley 1994). For example, the hypertrophied cloacal gland of the Smooth Newt, *Triturus vulgaris*, represents 10% of a male's mass and has been compared to a peacock's tail (Verrell et al. 1986). The seasonal hypertrophy of mental glands of plethodontid salamanders is so striking, it has been likened to tumor development, with massive proliferation of specialized tissue dedicated to pheromone and protein synthesis (Wilburn and Feldhoff 2019). Additional glands are hypothesized to produce pheromones involved in courtship and mating, such as the modified granular glands on the dorsal tail base of plethodontid salamanders (Staub and Paladin 1997). Pheromones in females are much less studied, but the ciliary cells of the oviduct are a source of pheromones in salamandrid salamanders (Nakada et al. 2017).

An intriguing possibility is that microbiota could contribute to pheromone production. Although most studies of salamander skin microbiota focus on antimicrobial activity (e.g., Pereira et al. 2018), studies in frogs show that volatile odors produced by skin microbiota are sex-specific, suggesting involvement in signaling (Brunetti et al. 2019). Also, a large body of work shows that salamanders respond to scents in feces (reviewed in Jaeger et al. 2016). Although speculative, chemical cues from the feces could provide information about the gut microbiome for use in mate assessment.

Chemical structures

In animals, many types of molecules possess signaling properties including steroids, steroid metabolites, alcohols, ketones, amino and bile acids, peptides, and proteins (Wyatt 2017). Differences in molecular structure contribute to the different properties of pheromones. Pheromones that function in aquatic environments are soluble in water; those in terrestrial environments are nonvolatile or volatile. Nonvolatile pheromones are used in short-range communication while volatile pheromones have a longer range.

Most salamander pheromones are proteins and peptides (Table 1). The first amphibian pheromone discovered was sodefrin, which is found in the cloacal gland of male Japanese firebelly newts (*Cynops pyrrhogaster*) and attracts females (Kikuyama et al. 1995). Sodefrin is a water-soluble decapeptide, cleaved from a precursor protein, that is invariant within males of a population and signals species and sex (Kikuyama et al. 1995). Females produce a pheromone called imorin, a tripeptide from oviductal glands, which attracts males (Nakada et al. 2017). Pheromones like sodefrin and imorin fit the classic definition of a pheromone as an invariant molecule

that conveys information on species and sex, and triggers a simple reflexive behavioral response in recipients.

Subsequent study of salamander pheromones revealed a more complicated story (detailed in Woodley 2010, 2014). In plethodontid salamanders, the male mental (submandibular) gland produces a multicomponent mixture of proteins. During courtship, after species and sex recognition have occurred, mental gland secretions are directly applied to females during courtship. Mental gland secretions are most studied in two species of plethodontid salamanders (red-legged salamander [*Plethodon shermani*] and Ocoee salamander [*Desmognathus ocoee*]), where the secretions were shown to increase female receptivity to mating. Transcriptomic and protein biochemistry studies revealed that the mental gland contains dozens of pheromonal isoforms from diverse gene families, which are variable both within and among species (detailed in Woodley 2010, 2014).

Using the tools of molecular biology and bioinformatics, study of dozens of additional species of salamanders revealed an array of variable proteinaceous molecules with sequence similarity to known pheromones from red-legged and Ocoee salamanders. One group of proteins, called the sodefrin precursor-like factor (SPF) family, is present in several salamander families and even in some frogs, indicating an ancient origin in amphibian evolution (Bossuyt et al. 2019; Janssenswillen et al. 2015a). As the name suggests, sodefrin is a member of the SPF family of proteins, but being a decapeptide, is much smaller than the other members of the SPF family of proteins (Iwata et al. 1999; Van Bocxlaer et al. 2016). In species in which behavioral tests have been done, SPF-family proteins influence female interest in males and mating (Houck et al. 2008b; Van Bocxlaer et al. 2015).

Pheromonal signaling is ancient and there has been much lineage-specific divergence, often through gene duplication followed by mutation and neofunctionalization. As one example, SPF proteins originated 300 mya and diversified through multiple gene duplications. Some of the duplications corresponded to speciation events, but others did not. As a result, there are two SPF subfamilies (alpha and beta) of orthologous proteins (Janssenswillen et al. 2015a; Van Bocxlaer et al. 2015), many of which have pheromonal properties.

Detection

Sensory systems. Pheromones are usually detected by sensory neurons housed in the nasal cavity (Wyatt 2017). Like many other tetrapod vertebrates, salamanders have a main olfactory system (MOS) and an anatomically distinct accessory olfactory system (AOS). The AOS is also called the vomeronasal system and includes a vomeronasal sensory epithelium called the vomeronasal organ (VNO) (Fig. 2). Historically, the vertebrate MOS was believed to detect

general odorants while the AOS was thought to be specialized to detect socially-relevant chemical signals such as pheromones. However, this dichotomy is false as both the MOS or AOS can detect pheromones (Baum and Cherry 2015). Likewise, the vertebrate AOS is not specialized to detect only pheromones, but is also involved in the detection of nonpheromonal stimuli like those from predators and prey (Baxi et al. 2006; Placyk and Graves 2002).

An anatomically separate accessory olfactory system was once thought to be an adaptation to a terrestrial lifestyle. However, studies in salamanders refute this idea: the VNO is present in most aquatic species of salamanders, in aquatic larvae of species that are terrestrial as adults, and is an ancestral character for the amphibian and amniote lineage (Baxi et al. 2006; Eisthen 2000). Furthermore, evidence is growing that lungfish have a VNO homolog (González et al. 2010; Nakamuta et al. 2012; Wittmer and Nowack 2017).

An unusual and remarkable aspect of salamander pheromonal communication is that many pheromones do not enter the nasal cavity via the nares to activate sensory neurons. In the vast majority of plethodontid salamander species, courtship pheromones are delivered through the skin and not directly to the nasal cavity (Fig. 3). Transdermal delivery of courtship pheromones is the ancestral condition in the family, with olfactory (through the nares) delivery of courtship pheromones evolving in only a few groups of salamanders (~ 30 species out of 488 species of plethodontid salamanders, AmphibiaWeb 2020). Although the behaviors

and pheromones associated with transdermal delivery have been well studied, the mechanism whereby pheromones can pass through the skin to affect behavior and physiology is completely unknown.

Anatomy of sensory systems. In salamanders, both larval and adult forms, the anatomy of the nasal cavity is simple, consisting of a dorsoventrally flattened tube extending from the external naris to the internal naris which empties into the roof of the mouth (Fig. 2). The nasal cavity has a lateral recess or diverticulum (Dawley 2017; Reiss and Eisthen 2008). Sensory neurons of the main olfactory system (main olfactory epithelium, MOE) line the main chamber while those of the accessory olfactory system (vomeronasal epithelium or organ, VNO) line the recess or diverticulum. This simple anatomy made salamanders an early model for research on olfaction (Kauer 2002). In contrast, the nasal cavity of the anuran amphibians has multiple interconnected chambers that are remodeled during metamorphosis (Reiss and Eisthen 2008).

The salamander MOE contains both ciliated and microvillar sensory neurons while the VNO contains only microvillar sensory neurons (Eisthen and Polese 2006; Różański and Żuwała 2019). Additional types of sensory neurons have been reported in coastal giant salamanders (*Dicamptodon tenebrosus*) and fire salamanders (*Salamandra salamandra*) (Rozanski and Zuwała 2020; Stuelpnagel and Reiss 2005). In some species of salamanders, the sensory neurons of the MOE are

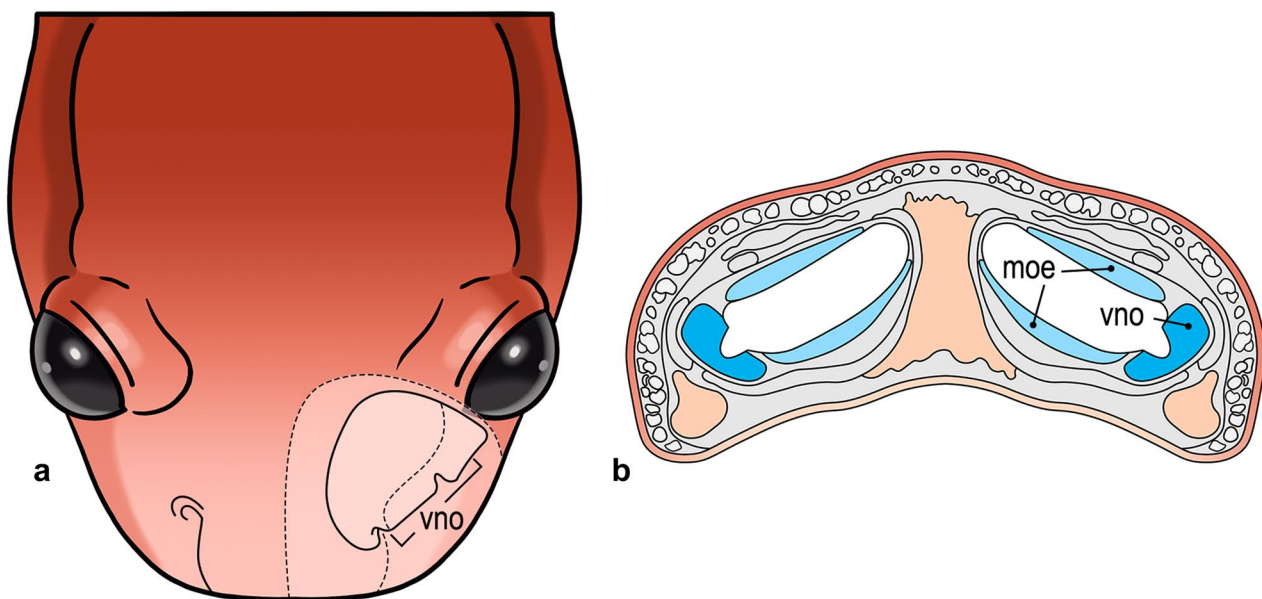


Fig. 2 **a** Dorsal view of the head of a generalized plethodontid salamander. On the right, the cut-away shows the outline of the nasal cavity. The VNO, starting anteriorly at the external naris and lining the lateral portion of the nasal cavity, is indicated (adapted from Dawley 1992). **b** Transverse section of an upper jaw from

a generalized plethodontid salamander. The dorsal surface is the epidermis (dark orange); the ventral surface is the roof of the mouth (pale orange). The main olfactory organ (MOE) and VNO are indicated. Large glands are shown in orange. Illustrations by Angel Hawari

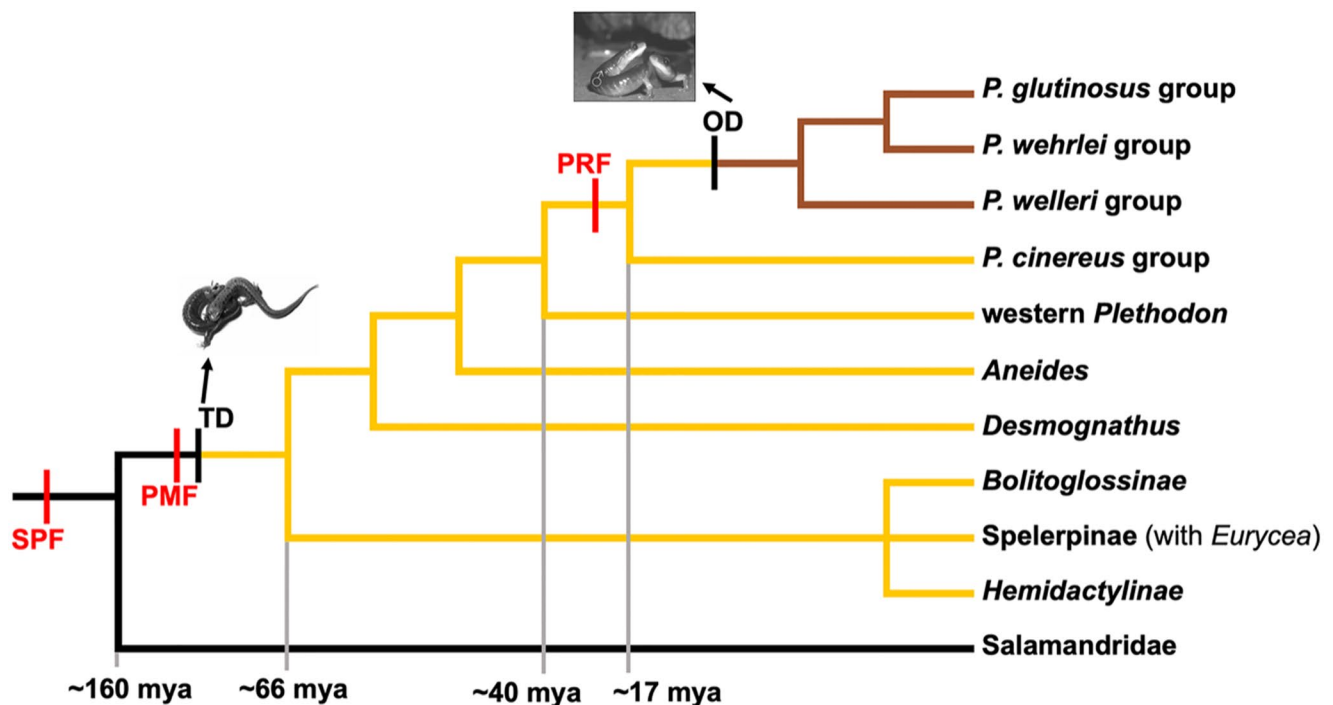


Fig. 3 Phylogeny of plethodontid pheromones (SPF, PMF, and PRF) showing the distribution of the ancestral transdermal mode and the derived olfactory mode of mental gland pheromones (dates from Shen et al. 2015). OD=olfactory delivery, horizontal brown lines; TD=transdermal delivery, horizontal yellow lines. Note that tran-

scripts for PMF were found in axolotls (Hall et al. 2016). If the transcripts are shown to correspond to proteinaceous pheromones, the origin of PMF may be earlier than shown here (images used with permission from SJ Arnold)

arranged in strips separated by nonsensory ridges while other species have continuous sheets of olfactory sensory neurons. Strips separated by nonsensory ridges are found in more aquatic species but it is unclear if this pattern is related to aquatic lifestyles (Eisthen 2000; Rózański and Żuwała 2019).

In some species of salamanders, the volume of the VNO is larger in males than females, even after correcting for differences in body size (Dawley 1992; Woodley 2007). There is also evidence for plasticity in the size of the VNO, which is largest during the summer foraging season compared to the mating season in late fall/early spring (eastern red-backed salamanders, *Plethodon cinereus*) (Dawley and Crowder 1995). This result is consistent with a potential role of the VNO in foraging (Placyk and Graves 2002), but more work is needed to understand VNO plasticity.

Salamander sensory neurons of the MOE and VNO express many of the same signaling molecules as other vertebrates, including olfactory receptors (ORs), vomeronasal receptors in the V2R family, and associated G-proteins and ion channels (Fig. 4) (Kiemnec-Tyburczy et al. 2012; Marchand et al. 2004; Mohrhardt et al. 2018; Nakada et al. 2014; Zhou et al. 1997). The distribution

of these molecules contrasts somewhat with patterns in rodents and varies among salamanders (Table 2).

One important difference between salamanders and rodents is that G α olf is found in the VNO in salamanders (Table 2). Because G α olf is associated with ORs, this suggests that ORs are expressed in the salamander VNO. Another important difference is the lack of evidence for V1Rs in salamanders. Kiemnec-Tyburczy et al. (2012) were unable to detect V1Rs in red-legged salamanders, and Nakada et al. (2014) found only a few cells with G α i2 in the MOE (not the VNO) in Japanese firebelly newts, suggesting a reduced role for V1Rs in salamanders. Although the VNO is no longer accepted as an adaptation to life on land, the V1R to V2R ratio was proposed to be higher in terrestrial species (Silva and Antunes 2017). This hypothesis is not supported by data in red-legged salamanders (*P. shermani*), which are terrestrial, yet apparently lack V1Rs. Another difference is that salamanders lack the clear apical and basal distribution of VRs found in mice (Kiemnec-Tyburczy et al. 2012; Nakada et al. 2014) (Table 2).

The neuroanatomy of the salamander olfactory systems has been well studied and homologous areas to mammalian olfactory processing areas have been identified (Kokoros and Northcutt 1977; Laberge et al. 2006; Laberge and Roth

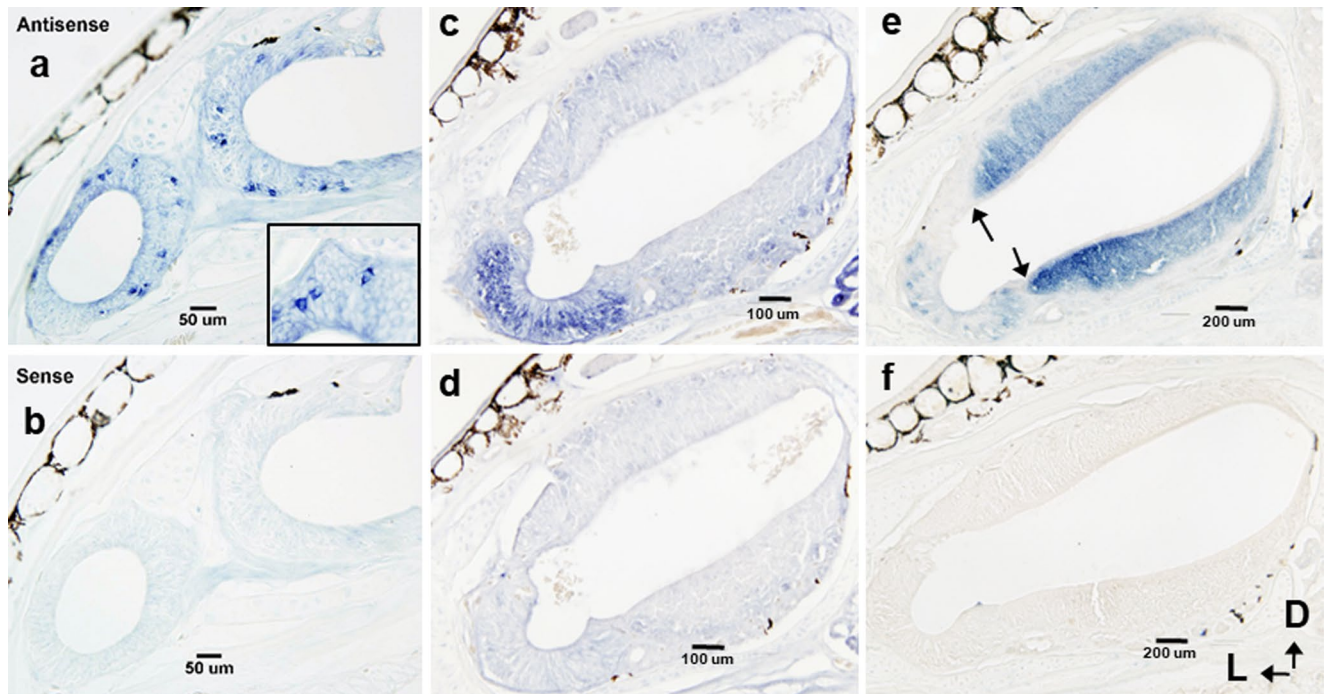


Fig. 4 Representative photomicrographs of the main olfactory epithelium and the vomeronasal epithelium from red-legged salamanders (*P. shermani*) using in situ hybridization to show mRNA expression of V2R (a, b) and TRPC2 (c, d) in the VNO, and Gαolf (e, f)

in the VNO and MOE. The top row is antisense and the bottom row is sense. Inset shows cells in higher magnification. Arrows show the boundary between the VNO (lateral) and MOE (medial). D=dorsal, L=lateral

2005). Briefly, in amphibians, the sensory neurons of the chemosensory epithelia of the nasal cavity send axons to three main sites: (1) the main olfactory bulb (MOB), located on the rostral telencephalon; (2) the accessory olfactory bulb (AOB), a bulge on the lateral telencephalon caudal to the MOB; and (3) and extrabulbar pathways, where sensory neurons of the MOE skip the olfactory bulb and project directly to areas of the hypothalamus (Eisthen and Polese 2006; Schmidt et al. 1988).

The sensory neurons of the salamander MOE project to the MOB. Surprisingly, in Japanese firebelly newts, some sensory neurons from the ventral part of the MOE projected to the AOB as well (Nakada et al. 2014; Schmidt and Roth 1990). More studies are necessary to determine if this projection pattern is found in other salamander species. Interestingly, in Axolotl salamanders (*Ambystoma mexicanum*) as well as frogs, the axon of a single olfactory sensory neuron

Table 2 Location of signaling molecules in rodents and salamanders

| Signaling molecule | Mice | Red-legged salamander ^a | Japanese firebelly newt ^b | Tiger salamander ^c , mudpuppy ^d |
|--------------------|--------------|------------------------------------|--------------------------------------|---|
| OR | MOE | | | MOE |
| Gαolf | MOE | MOE, VNO ^e | MOE (apical), VNO ^e | |
| V1R | VNO (apical) | None found | | |
| Gαi2 | VNO (apical) | | MOE ^c | |
| V2R | VNO (basal) | VNO | | |
| Gαo | VNO (basal) | VNO | MOE (basal), VNO | |
| TRPC2 | VNO | VNO | | |

^aKiemnec-Tyburczy et al. (2012)

^bNakada et al. (2014)

^cMarchand et al. (2004)

^dZhou et al. (1997)

^ePresent in a relatively small number of cells

bifurcates and innervates several different MOB glomeruli (Weiss et al. 2020). This wiring pattern appears to be unique to amphibians, suggesting that it originated early in amphibian evolution. Its significance is unclear.

The sensory neurons of the salamander VNO project axons to the AOB (Nakada et al. 2014; Schmidt and Roth 1990). While the morphology of the MOB was uniform across 10 species of salamanders, the morphology of the AOB was variable in the shape and number of terminal fields of the sensory neuron projections (Schmidt and Roth 1990). Those species with direct development (i.e., terrestrial throughout life) had fewer terminal fields compared to species possessing an aquatic larval stage.

From the olfactory bulbs, information is conveyed to more central brain areas (reviewed in Eisthen and Polese, 2006). Briefly, projection neurons of the MOB travel through the medial and lateral olfactory tracts to areas of the pallium, striatum, and amygdala. Projection neurons of the AOB travel via the accessory olfactory tract to reach the amygdala.

Areas of the telencephalon receiving olfactory and vomeronasal input were studied in red-legged salamanders (*P. shermani*) (Laberge et al. 2006; Laberge and Roth 2005; Roth and Laberge 2011). Stimulation of the olfactory nerve (ON) resulted in evoked potentials throughout the entire telencephalon. Stimulation of the vomeronasal nerve (VN) evoked potentials in the lateral telencephalon and amygdala. Recording from 254 telencephalic neurons found that 13% exclusively responded to stimulation of the VN. These areas were typically located in the “extended vomeronasal amygdala” that included the striatopallial transition area and vomeronasal amygdala, as described previously (Laberge et al. 2006). Twenty-two percent of the recorded neurons responded to stimulation of the ON, mostly in the caudal pole of the telencephalon. Finally, almost 60% of recorded neurons showed excitation in response to stimulation of either the ON or the VN. These were usually in the lateral pallium, the striatopallial transition area and amygdala. Many of these neurons responded very quickly to nerve stimulation, consistent with direct synaptic input from the olfactory bulbs. Thus, there is a high amount of direct convergence of olfactory and vomeronasal input in the salamander telencephalon. This lends further credence to the notion that the vomeronasal and olfactory sensory systems perform complementary and overlapping functions.

Pheromonal receptors. Despite the evidence that the salamander MOE and VNO express ORs and VRs (reviewed above), the exact receptors that bind to particular pheromones are unknown, which means deciphering olfactory coding of salamander pheromones is challenging. However, pheromones that enter the nasal cavity through the nares (olfactory delivery) activated sensory neurons in the VNO (Kikuyama et al. 1995; Wirsig-Wiechmann et al. 2006). A

relatively small number of VNO neurons responded to stimulation with pheromones, suggesting that individual sensory neurons express only one or a few of the larger group of VRs, similar to mammals (Mohrhardt et al. 2018). As discussed more below, there is also some evidence that pheromones may activate sensory neurons of the MOE. For those pheromones that are delivered transdermally, the mechanism involved in sensory transduction has not been identified.

Although the receptors recognizing the known salamander pheromones are unknown, we can infer some of the evolutionary dynamics between receptor and ligand by considering the evolution of the ligand (reviewed in Wilburn et al. 2017a). The courtship pheromones in plethodontid salamanders have undergone incessant and rapid molecular evolution over millions of years. They have elevated rates of nonsynonymous-to-synonymous substitutions in their protein coding sequences, which are consistent with positive natural selection. While both a male pheromone and a female receptor are shaped by natural selection, modelling of ligand-receptor dynamics suggest that the male ligand is relatively more constrained by sexual selection to match a female receptor, compared to the female receptor. As a consequence, the male ligand moves in evolutionary space to track the female receptor, which is itself, moving in space shaped by natural selection. These co-evolutionary dynamics between the male pheromones and female receptors are akin to a tango, a dance where partners form a tight embrace around an axis (Wilburn et al. 2017a). It would be interesting to know if similar dynamics are at play with female pheromones and male receptors, but data on female pheromones are sparse.

Hormonal regulation

As with other vertebrates, amphibian pheromonal communication related to reproduction is highly modulated by hormones (Woodley 2015). Hormonal regulation of pheromone production and courtship behavior in red-bellied newts is well studied and has recently been reviewed (Kikuyama et al. 2019). Here, androgens and prolactin together mediate hypertrophy of the cloacal gland and the production of pheromones during the breeding season (Toyoda et al. 1993, 1994). These hormones also increase expression of male tail fanning, a courtship behavior that delivers pheromones towards females, via actions on brainstem cells involved in tail fanning, as well as brain areas linked to reproduction (Toyoda et al. 2005, 1993). Arginine vasotocin also plays a role (Haraguchi et al. 2010; Toyoda et al. 2003). While less is known about female pheromone production, males are most attracted to

female-derived water if females have elevated estradiol and prolactin levels (Toyoda et al. 1994).

Hormones also regulate investigation of pheromones. In red-legged salamanders, testosterone increased the length of cirri, structures that facilitate the ability to sample the substrate for nonvolatile chemical cues (Schubert et al. 2006). Testosterone also increased chemo-investigation and behavioral preferences for female-derived chemical cues (Schubert et al. 2006). Levels of chemo-investigation were sexually dimorphic and differed depending on female reproductive condition, suggesting that estradiol may modulate female interest in chemical cues (Schubert et al. 2008).

Finally, hormones regulate sensitivity to pheromones. In red-bellied newts, the female VNO was more responsive (measured via electric field potentials) to sodefrin if females had elevated estradiol and prolactin levels (Iwata et al. 2013; Kikuyama and Toyoda 1999; Toyoda and Kikuyama 2000). In contrast, in red-legged salamanders, there was no evidence that female reproductive condition modulated the sensitivity of the female VNO to male mental gland pheromones (Schubert et al. 2008). Thus in red-legged salamanders, differential responses to male pheromones may be due to differences in chemo-investigation rather than sensitivity of the VNO.

In mammals, pheromones can exert priming effects by increasing levels of hormones. In red-legged salamanders, exposure to mental gland pheromones elevated plasma corticosterone (Schubert et al. 2009). However, this result was not replicated, so it is unclear whether priming effects of salamanders pheromones are common (Wack et al. 2012).

Pheromonal communication by family

There are currently 755 species of salamanders across 10 families, inhabiting Holarctic and Neotropical regions. Evidence for pheromonal communication related to courtship and mating is found in all salamander families. Red-legged salamanders (*P. shermani*) and Japanese firebelly newts (*C. pyrrhogaster*) have received focused attention, and pheromonal communication has been demonstrated at genetic, biochemical, molecular, physiological, and behavioral levels in these species. Indirect evidence for pheromonal communication exists in many additional species. Below we illustrate the fascinating diversity of salamander pheromonal communication by reviewing representatives in each family of salamanders (Fig. 1). We begin with families with internal fertilization where sperm transfer occurs using a spermatophore. In these groups, complex communication between males and females helps to coordinate pick up by the female of the sperm-cap from the spermatophore. We end with families with external fertilization (Fig. 1).

Salamandridae

Salamandrid salamanders, which include the newts, are found in temperate areas of Europe, North Africa, Asia, and North America. Adults are typically terrestrial except during the breeding season, when they become secondarily aquatic for breeding. As the breeding season approaches, the tail fin of the male often enlarges and changes color, and the dorsal cloacal gland hypertrophies (Halliday 1990; Sever et al. 1990). Pheromones are important for finding and assessing appropriate mates and for coordinating male–female sexual interactions. In addition, male interference is common, where rival males may attempt to interfere with the mating of other males (e.g., Verrell 1989).

Courtship and mating behaviors are elaborate and highly variable among salamandrid species (Kieren et al. 2018). Sources of variation are as follows: (1) whether males exhibit tail fanning, where the male beats, undulates, waves, flicks, or vibrates its tail during courtship, presumably to direct cloacal gland pheromones towards females, and (2) whether males and females clasp together in a behavior called amplexus. Below, we describe salamandrid chemical communication according to genus.

Lissotriton, *Ichthyosaura*, *Triturus*. The elaborate courtship of these three genera of European newts has been extensively studied. Tail morphology is dramatically sexually dimorphic, with large and colorful crests in males of many species. There is no amplexus or prolonged physical contact with females, but males vigorously move their tails in a lateral fanning motion prior to spermatophore deposition (Halliday 1990). Tail fanning is energetically demanding and males that tail fan more have higher mating success (Green 1991; Teyssedre and Halliday 1986). Early behavioral work with great crested newts (*Triturus cristatus*) suggested that females were responding to a male-derived chemosignal (Malacarne and Vellano 1982, 1987).

More recent work has concentrated on palmate newts (*Lissotriton helveticus*). Female behavior is dramatically modulated by water that previously contained courting males, especially when males exhibited tail fanning behavior. That is, when two females were put in water that had previously held a male exhibiting tail fanning behavior (courtship behavior), the females attempted to court each other by following each other, touching the other female's tail with their snouts, and waving their tails (Treer et al. 2013). These behaviors were seen only when placed in water from a conspecific male, not a heterospecific male, indicating species recognition (Treer et al. 2013; Van Bocxlaer et al. 2015). Examination of the male water found proteins that were absent in water from noncourting males. Tests of females with different fractions of the water revealed a 20 kDa glycosylated protein that

itself induced female mating behavior (Van Bocxlaer et al. 2015). Sequencing revealed that it was a protein in the SPF-family of proteins, which were originally discovered in plethodontid salamanders. Examination of the male dorsal cloacal gland revealed that about 8% of the transcripts coded for 31 unique SPF proteins, of which 5 isoforms were most abundant (Van Bocxlaer et al. 2015). The function of the remaining 92% of reads was not explored. Similar proteomic and transcriptomic studies were done in other species, and orthologues to the SPF family of proteins were found in the dorsal cloacal gland of male alpine newts (*Ichthyosaura alpestris*), smooth newts (*Lissotriton vulgaris*), and Montadon's newt (*L. montandoni*) (Osikowski et al. 2008; Treer et al. 2018).

In addition to SPF family pheromones, a novel pheromone called Persuasin was discovered in palmate (*L. helveticus*) and alpine newts (*I. alpestris*). In both species, a 15 kDa protein was released from the male cloacal gland during courtship. The protein alone increased the expression of female mating behavior, although effects were strongest when combined with proteins from the SPF family (Maex et al. 2018). Persuasin evolved about 51–45 mya in the *Lissotriton* and *Ichthyosaura* lineages and has no homology with other vertebrate protein families.

Some of the earliest work on the olfactory detection of salamander pheromones was done in the great crested newt (*T. cristatus*). Here, perfusion of the nasal cavity of females with crude extracts of the dorsal cloacal gland increased the frequency and amplitude of spike potentials in the ventral olfactory bulb (Cedrini and Fasolo 1971). The response did not show adaptation to continuous perfusion over several minutes. In contrast, only weak responses were seen in response to tap water or female-derived aquarium water (Cedrini and Fasolo 1971). These experiments attest to the potency of pheromones from the male cloacal gland and are consistent with behavioral observations that males fan their tails for an extended period of time, presumably directing pheromones towards females.

Female pheromone production in European newts is understudied, but some evidence suggests that males detect the reproductive state of female via chemical cues. Female palmate newts (*L. helveticus*) tail fan, perhaps to direct pheromones to males (Treer et al. 2013). The olfactory cues of large, fecund, female smooth newts (*L. vulgaris*), were preferred by males compared to smaller females (Verrell 1986). Finally, plugging the nares (nostrils) of male great crested newts (*T. cristatus*) reduced male sexual behavior (Malacarne and Vellano 1982).

Cynops. The first amphibian pheromone discovered, sodefrin, was from the male dorsal cloacal gland (sometimes called the abdominal cloacal gland) of the Japanese firebelly newt, *Cynops pyrrhogaster* (Kikuyama et al. 1995). Similar to European newts, courtship involves vigorous tail

movements by the male and culminates in spermatophore deposition. Sodefrin, a decapeptide produced by the male dorsal cloacal gland, strongly attracted reproductively active females (Kikuyama et al. 2009, 1995; Toyoda et al. 2004). Related peptides with pheromonal properties were found in additional species and populations of *Cynops* (Nakada et al. 2007; Yamamoto et al. 2000). They differed from sodefrin by 1 to 2 amino acids and were species- or population-specific. That is, females were only attracted to water with cues from males from their population, not water from heterospecific males or males from a different population (Nakada et al. 2007; Yamamoto et al. 2000).

Although sodefrin by itself is a potent modulator of female behavior, it is in the SPF family of salamander pheromones and may be part of a multi-component pheromonal system (Van Bocxlaer et al. 2016). Sodefrin is much smaller than SPF-like proteins because of differences in post-translational processing due to a gene insertion (Iwata et al. 1999; Van Bocxlaer et al. 2016). The dorsal cloacal gland of *Cynops* also contained proteins with sequence similarity to SPF (Van Bocxlaer et al. 2016). In fact, 85% of the transcripts in the male cloacal gland were in the SPF family. It is unclear whether SPF-like proteins contribute to pheromonal signaling in *Cynops*, because earlier studies showed that water lacking sodefrin was ineffective (Kikuyama et al. 1995). However, the co-occurrence of sodefrin and SPF family proteins in *C. pyrrhogaster* is reminiscent of the co-occurrence of Persuasin and SPF family pheromones in palmate newts (*L. helveticus*).

Pheromones involved in male–female reproductive interactions are also produced by female Japanese redbelly newts (Nakada et al. 2017). A tripeptide, called imorin, attracted reproductively active males but not females or nonreproductive males. Males preferred water derived from reproductively active females, and removal of oviducts attenuated the attractive properties of the female water (Toyoda et al. 1994). Imorin is produced in the oviduct and is the first peptide pheromone to be discovered in a female vertebrate.

Sodefrin and imorin are detected by sensory neurons located in the VNO. Sodefrin activated VNO cells of reproductively active females in a dose-responsive manner (Iwata et al. 2013; Toyoda and Kikuyama 2000; Toyoda et al. 2004). The VNO of males and nonreproductive females did not respond to sodefrin. Similarly, imorin activated VNO cells of reproductively active males but not nonreproductive males or females (Nakada et al. 2017). Consistent with findings in the VNO of mammals, only a few cells responded to sodefrin and imorin, suggesting that VNO cells express receptors for only one or a few of the potential array of ligands (Dulac and Torello 2003). Interestingly, a few areas of the main olfactory epithelium also responded to stimulation of sodefrin (Toyoda and Kikuyama 2000). As with mammals, it is very difficult to identify the receptors that

bind to the pheromonal ligands because of the low number of cells expressing receptors for them. This is an impediment to understanding the code that shapes neural responses to pheromonal ligands.

Notophthalmus. In eastern red-spotted newts, *Notophthalmus viridescens*, mating includes tail fanning as well as dorsal amplexus prior to spermatophore deposition (Verrell 1982). While in amplexus, the male rubs glands from its cheek (genial glands) over the nares of the female. However, amplexus is facultative and may be skipped if the female is highly receptive and rival males are absent. Tail height and the size of the cloacal gland and cheek glands enlarge in the male during the breeding season under the influence of hormones (Pool and Dent 1977). Analysis of mRNA transcripts of the cheek and cloacal glands found transcripts with sequence similarity to pheromones in the SPF family. There was a similar amount and diversity of SPF isoforms in both cheek and cloacal glands (Janssenswillen et al. 2015b). SPF transcripts were also found in female cheek glands, but at reduced levels. More work is needed to determine whether proteins corresponding to the cloacal gland mRNA are released by courting males and whether they affect female behavior. Studies should also test responses of other males, since a previous paper suggested that male cloacal glands were the source of a 33 kDa protein that repelled males, perhaps related to the intense male–male competition for access to mates (Park and Propper 2001; Rohr et al. 2004). Study of cheek glands in males and females is also warranted.

In addition to a role in courtship and mating behavior, pheromones contribute to mate attraction and mate choice. The attraction of males to water containing conspecific males and females varied depending on the relative numbers of males and females (Dawley 1984; Rohr et al. 2002). For example, males were repelled by olfactory cues from large groups of males, perhaps to avoid male–male competition (Park and Propper 2002, 2001). In addition, males preferred olfactory cues from larger females (possessing more eggs) versus smaller females, suggesting that female olfactory cues contribute to male mate choice for more fecund females (Verrell 1985). Compared to males, there was less evidence that females used olfactory cues in locating males or in mate choice (Dawley 1984; Gabor et al. 2000).

Taricha. Salamanders in the genus *Taricha* are endemic to the Western USA. During courtship, the male clasps the female in a dorsal amplexus behavior that lasts for hours, prior to dismounting and depositing a spermatophore in front of the female (Propper 1991). After insemination, a male re-clasps the female in an amplexus that can last days. The post-insemination amplexus may function in mate guarding because multiple males will gather around a male–female pair in a mating ball. Tail fanning is not part of the courtship and mating ritual.

During the pre-insemination amplexus, the male rubs its chin on the female’s head, presumably transferring pheromones from a specialized submandibular gland onto the female’s nares (Hippe et al. 2014; Propper 1991). Putative male pheromones from submandibular glands have not been examined. However, the male cloacal gland contains mRNA transcripts with sequence similarity to the SPF family of proteins used as pheromones in other species of salamanders (Janssenswillen et al. 2015a). The number of transcripts in the glands of rough-skinned newts (*T. granulosa*) is much less than in those species that exhibit tail fanning behavior but lack the tactile stimuli of amplexus, suggesting that cloacal gland pheromones are less important in *Taricha*, perhaps because nonchemical cues like visual or tactile cues stimulate females.

While the evidence for male pheromones in *Taricha* is circumstantial, there is strong evidence that females emit potent pheromones. In red-bellied newts (*Taricha rivularis*), males were attracted to the chemical cues of females, often swimming upstream many meters seeking females (Tewit 1955). In rough-skinned newts (*T. granulosa*), rubber models of females that were coated with female chemical cues were clasped by males (Thompson et al. 1999). The female chemical cues enhanced and prolonged male clasping behavior via the arginine vasotocin somatosensory processing circuit (Thompson et al. 2008). The source and nature of the putative female pheromones is unclear, but appears to be secreted from the female skin and is not proteinaceous (Thompson et al. 2008).

Pleurodeles. Pheromonal communication is believed to be important in Iberian ribbed newts (*Pleurodeles waltl*), but has been little studied. Courtship includes amplexic clasping during which the male may place its protruded cloaca onto the nares of the female (cloacal imposition, Janssenswillen and Bossuyt 2016). This behavior likely transfers chemical signals to the female. After cloacal imposition by the male, the female opens its cloacal vent (cloacal gaping), before proceeding with additional courtship behaviors and sperm transfer. Female cloacal gaping can be induced by exposure to water that had previously contained a courting male–female pair (Janssenswillen and Bossuyt 2016). As with other salamanders, the male dorsal cloacal glands contain mRNA transcripts corresponding to the SPF-family of salamander pheromones (Janssenswillen et al. 2015a).

Plethodontidae

The family Plethodontidae is the most speciose, with 488 species in 28 genera (AmphibiaWeb 2020). Plethodontid salamanders are distributed predominantly in North, Central, and (partially) South America. They have internal fertilization and most have direct development, in which the embryo hatches with a miniature adult body form. In

most species, courtship occurs on land. Because this group has internal fertilization and a spermatophore is required to transfer sperm from the male to the female, courtship and mating behavior function to coordinate males and females for successful sperm transfer (Arnold et al. 2017). In particular, all plethodontid salamanders exhibit tail-straddling behavior, where the female straddles the tail of the male and the couple walks together in tandem. Tail-straddling behavior is a required prelude to spermatophore deposition and insemination (Houck and Arnold 2003).

Surprisingly, most species employ transdermal pheromonal delivery during courtship, with the male rubbing its mental gland (a confirmed pheromone source on the male's chin) on the female's head, back, and tail (Fig. 3). Transdermal pheromone delivery is associated with a scratching behavior, where the male scratches the skin of the female with specialized sexually dimorphic premaxillary teeth during pheromone application. It is hypothesized that the abrasion of the female's skin facilitates diffusion of pheromones and other molecules to the circulatory system (Arnold et al. 2017). As an extreme example, two species of *Desmognathus* use a "bite and seize" technique of delivery, in which the pheromone cocktail is delivered from the mental gland that empties inside the mouth at the base of the teeth on the dentary (Arnold et al. 2017). It is not known how transdermal pheromone delivery works in terms of how the signal is recognized by the female. Regardless of the mechanism, experimental application of mental gland extract to the dorsum of females increased female receptivity, as shown by reduced the time required to reach tail-straddling walk and spermatophore deposition (Houck and Reagan 1990; Houck et al. 2008b).

Olfactory delivery of mental gland pheromones is found in a handful of species (Fig. 3). Olfactory delivery evolved within one genus of plethodontids; in the eastern large *Plethodon* clade of species, the male slaps its mental gland on the nares on the female during courtship (Arnold et al. 2017). Experimental application of mental gland extract to the nares of females increased female receptivity, as shown by a reduction in the time required to reach tail-straddling walk and spermatophore deposition (Houck et al. 1998; Rollmann et al. 1999). The pheromones travel up the nasolabial grooves and into the vomeronasal organ, where receptors are then activated (Kiemnec-Tyburczy et al. 2011; Schubert et al. 2006, 2008; Wilburn et al. 2017b; Wirsig-Wiechmann et al. 2002, 2006).

The effects of the mental gland pheromones are relatively subtle. They consist of complex blends that are transferred to females at a relatively late stage of courtship and are not involved in species identification or mate attraction (Rollmann et al. 2003). Because these pheromone blends serve only to accelerate the pace of mating, pairs will mate even if the mental gland of the

male has been removed (Houck et al. 1998; Houck and Reagan 1990). This contrasts with the newt pheromones such as sodefrin and imorin, which are simple, invariant molecules that have a relatively straightforward effect on species recognition and mate attraction (Kikuyama et al. 1995; Nakada et al. 2017).

Three families of pheromones have been identified in the mental gland of plethodontids: plethodontid modulating factor (PMF), plethodontid receptivity factor (PRF), and SPF (Table 1). These pheromones are rapidly evolving reproductive proteins (Wilburn et al. 2017a; Wilburn and Swanson 2016); they occur as complex mixtures in the mental gland, and different lineages use different pheromone cocktails (Rollmann et al. 2000). They are also variable within a species (Chouinard et al. 2013). Interestingly, European common frogs (*Rana temporaria*) secrete proteins during amplexus that are structurally similar to PMF, an example of convergent evolution. This group of proteins, called amplexins, are produced from glands under the nuptial pads (Willaert et al. 2013). The function of amplexins, and the response to them, remains to be tested.

The three pheromones identified in plethodontids show intriguing differences. For example, while SPF and PMF are relatively old pheromones, PRF has more recently replaced SPF in eastern species in the *Plethodon* clade (Fig. 3) (Palmer et al. 2005; Wilburn et al. 2014). In addition, the pheromones differ in the number and bioactivity of isoforms. For example, Ocoee salamanders (*D. ocoee*) have a single PMF isoform, eastern red-back salamanders (*P. cinereus*) have six, and red-legged salamanders (*P. shermani*) have more than 30 (Wilburn et al. 2012). In red-legged salamanders, one isoform of the pheromone PRF increased female receptivity (Houck et al. 2008a). In contrast, for PMF, a mixture of at least three isoforms increased female receptivity (Wilburn et al. 2015) while the predominant isoform (PMF-G) reduced receptivity when applied singly (Houck et al. 2007). PMF, a hypervariable protein because of this high number of isoforms, activated more neurons in the vomeronasal organ when delivered as an isoform mixture rather than when administered as a single isoform (Wilburn et al. 2017b). The specific mechanisms of neuronal activation due to this synergy among isoforms remains unknown. Wilburn et al. (2017b) raise the possibility that some of the isoforms are acting like signature mixtures by relaying identity information rather than eliciting a specific response. There are six classes of SPF isoforms in Ocoee salamanders (*D. ocoee*), and the mixture varied between individuals, though all males studied had the most abundant SPF form (SPF-I-01) (Doty et al. 2016).

Structural analyses give us insight into molecular function of different components of the pheromones. PMF and SPF are types of three-finger proteins (TFPs) (Doty et al. 2016; Wilburn et al. 2014). This class of proteins has evolved a diverse array of functions in different

animal lineages while maintaining a highly conserved TFP structure. PMF however is different from other TFPs because the third finger has unique structural flexibility, which is hypothesized to allow continued receptor binding despite receptor changes (Wilburn et al. 2014). SPF also has altered disulfide linkages, which may change the 3D conformation (Doty et al. 2016).

The mental gland itself, the only confirmed source of pheromones in plethodontids, is starting to benefit from molecular scrutiny. The mental gland transforms seasonally into a pheromone-producing organ. At the height of the breeding season, PRF and PMF made up 85% of the secretory product in mental glands of red-legged salamanders (*P. shermani*) (Feldhoff et al. 1999; Houck et al. 2007). In Ocoee salamanders (*D. ocoee*), SPF made up more than 30% of the mental gland protein (Doty et al. 2016; Houck et al. 2007). The function of additional components of the mental gland is also being investigated. Transcriptome analysis identified that cold-inducible RNA-binding protein (CIRBP) binds to the highly conserved 3' untranslated-region of the PMF mRNA to regulate its translation (Wilburn and Feldhoff 2019).

Putative pheromones encoded by other genes which have been co-opted for signaling is an emerging field as well (Wilburn et al. 2014). For example, a cysteine-rich secretory protein (CRISP) was found in male eastern red-back salamander (*P. cinereus*) mental gland extracts, but its function here is not known (it is involved in sperm mechanics in mammals). In addition, there is evidence for peptide pheromones with sequence similarity to glucagon, vasoactive intestinal peptide, relaxin, and others (Doty et al. 2016). These hormone-like peptides are suggested to modulate female behavior, and if they have hormonal properties, may be involved in the signal transduction mechanism involved in transdermal delivery.

Pheromones are often hypothesized to contribute to the divergence of populations over time. In a direct test of this hypothesis, the composition of pheromone mixtures was examined in different clades of eastern red-back salamanders (*P. cinereus*), but there was no effect of genetic relatedness or of geography on pheromone mixtures (Kunkel et al. 2019). This result is surprising due to the limited dispersal ability of eastern red-back salamanders (Kunkel et al. 2019). Testing more divergent populations and testing this hypothesis with additional species can begin to address how pheromonal divergence might correlate with population divergence.

Other glands are hypothesized to secrete pheromones in males and females, such as the ventral and dorsal cloacal glands, and glands on the dorsal tail base (Rollins and Staub 2017; Sever 1989, 1988b). Pheromone production,

via mRNA transcripts, has been confirmed in the cloacal glands and dorsal tail base glands in male red-legged salamanders (*P. shermani*) (Chouinard 2016).

Ambystomatidae

Salamanders in the family Ambystomatidae are endemic to North America. In most species, adults are terrestrial, but adopt an aquatic lifestyle when they return to the pond to breed. Other species are fully aquatic (axolotl, *Ambystoma mexicanum*) and or have fully terrestrial adults that mate on land (marbled salamander, *A. opacum*). The courtship of many ambystomatid species includes behaviors suggestive of chemical communication. In aquatically breeding species, both females and males undulate their bodies and tails, perhaps to waft pheromones towards their partners, and also nudge the opposite-sex cloacal area with their snouts (Houck and Arnold 2003).

Study of chemical communication in this group has focused on the axolotl (*A. mexicanum*). In females, exposure to body rinses from males resulted in increased mating behavior and activated sensory neurons of both the MOE and VNO as measured by electro-olfactograms (EOGs) (Park et al. 2004). Similarly, male axolotls were behaviorally responsive to female-derived water, and rinses from females activated MOE and VNO sensory neurons as measured by EOG (Park et al. 2004). Males may be able to gauge the reproductive state females via chemical cues, as exposure to water from females of different reproductive conditions resulted in differential EOG responses in males.

Tail fanning by males likely directs cloacal pheromones towards females. Proteinaceous compounds were found in the water from sexually active axolotl males. The proteins were orthologues to SPF, a family of pheromones found in salamandrid and plethodontid salamanders (Maex et al. 2016). Examination of mRNA transcripts from the cloacal gland of male axolotls revealed multiple SPF sequences as well as a sequence similar to PMF, which is a pheromone in some plethodontid salamanders (Hall et al. 2016; Maex et al. 2016). Future studies should confirm the pheromonal function of the cloacal gland secretions by examining behavioral and olfactory responses. It would be interesting to examine cloacal gland secretions of the fully aquatic axolotl with those of Marbled Salamanders (*A. opacum*), which mates on land, and Spotted Salamanders (*A. maculatum*), a species which is normally terrestrial but becomes secondarily aquatic for breeding, to determine if there are differences related pheromonal communication in water versus land.

There is strong evidence that female axolotls produce pheromones. Males often nudge the female cloacal area, perhaps responding to signals from the cloacal glands or emanating

from the oviducts (Houck and Arnold 2003). Furthermore, there is behavioral and EOG evidence that males can detect and distinguish among chemical signals from females of different reproductive conditions (Park et al. 2004).

Dicamptodontidae

This small family (one genus, four species) of salamanders is found in the western USA and includes both terrestrial and aquatic species. Very little is known about pheromonal signaling in this group. Cloacal glands have been examined, and a vent gland that secretes at the cloacal opening is hypothesized to produce pheromones (Sever 1992).

Rhyacotritonidae

This small family (one genus, four species) of semi-aquatic salamanders is found exclusively in the Pacific NW of the USA. They have internal fertilization and aquatic larvae (Petranka 2010), and females and males participate in a tail-straddling walk during courtship, considered homologous to the tail-straddling walk of plethodontids (Doten et al. 2017). Species of *Rhyacotriton* have distinctive square lobes around the cloacal vent that house glands (Sever 1988a). Pheromones are hypothesized to be released from these glands during the tail waving display during courtship, but this has not been confirmed (Sever 1988a).

Proteidae

Proteids (two genera, eight species) are aquatic and paedomorphic (retaining larval features as adults). Cloacal glands in male and female proteids are well described, but their function in producing pheromones is unknown (Sever 2013). Proteids are unusual for salamanders because they lack a distinguishable VNO (Eisthen 2000), suggesting that pheromones are not the main mode of social communication, or that the main olfactory system detects them. Because pheromonal production is ancestral for salamanders, how proteids detect them or whether they produce them, is an intriguing question.

Amphiumidae

Members of this family (three species) have reduced limbs and paedomorphic features. Breeding is aquatic and fertilization is internal but little is known about mating behavior (Houck and Arnold 2003; Petranka 2010). Females have cloacal vent glands, typically only found in males in the other salamander families. Whether these vent glands produce pheromones is not known, although some of these glands

do secrete onto the epidermis just posterior to the cloacal opening (Sever 1992). Some granular glands are grouped on the dorsal tail surface, an important site of courtship contact between male and female plethodontids but their function has not been investigated (Pereira et al. 2018).

Cryptobranchidae, Hynobiidae, and Sirenidae

Hynobiids, Cryptobranchids, and Sirenids breed in the water and have external fertilization (Fig. 1). With external fertilization, complex communication between males and females to coordinate sperm-cap pick up from the spermatophore is unnecessary. However, pheromones are involved in other aspects of reproductive success. For example, male Korean salamanders (*Hynobius leechii*) were attracted to females who recently ovulated or were ovipositing. The olfactory cues did not derive from female cloacal glands (Park and Sung 2006). Analysis of water containing reproductive females revealed the presence of a prostaglandin (15(epi)-PF₂α) that itself affected male behavior and activated sensory neurons of the MOE of males (Eom et al. 2009). Responses of the VNO were not measured because of its small size in this species. The ability to identify females about to release ova is advantageous because the sex ratio is male-biased and multiple males will fight to fertilize female ova (Park and Park 2000). Prostaglandins serve as sex pheromones in many fish (Sorenson and Stacey 2006), and it would be interesting to determine if prostaglandin pheromones are common in salamanders with external fertilization.

Like many salamanders with internal fertilization, male Korean salamanders exhibit a body and tail undulation behavior that might function in disseminating pheromones from cloacal glands towards females (Kim et al. 2009). Both males and females display a smelling behavior in courtship, but what exactly is being detected is still under investigation (Park et al. 1996).

Sexual behavior and chemical communication between the sexes is under-studied in the aquatic salamanders in the cryptobranchid and sirenid families (Sever 2013). In cryptobranchids, both males and females have anterior ventral cloacal glands, but a pheromonal function has not been established. In sirens, both male and female sirens lack cloacal glands, so if pheromones are secreted, they would be from an alternate source.

Conclusions and future directions

As we are highly visual creatures, it is difficult for humans to fully understand a perceptual world shaped by chemical cues. Yet, salamanders rely on complex chemical signaling for social communication and have done

so for hundreds of millions of years. The natural history background available for salamanders provides a rich context for understanding pheromonal communication. At the same time, the field has greatly benefited from new molecular techniques that have increased understanding of pheromonal variation through evolutionary time, between populations, and within individuals. Although a large number of pheromones have been identified, receptors have not yet been identified, limiting our ability to explore neural detection and processing. Surprisingly, variation in pheromone type does not seem to correlate with aquatic or terrestrial habitat or sensory modality. For example, SPF is important in both aquatic and terrestrial environments, and is a component of both olfactory and transdermal delivery. Below we list some of the many unanswered intriguing questions.

- What is the function of individual variation in pheromonal signals? Do pheromones affect male mating success and are they shaped by sexual selection?
- How can the SPF family of proteins function in such diverse contexts, including both terrestrial and aquatic environments, and with both olfactory and transdermal transmission?
- Why have salamander pheromones experienced such rapid and incessant evolution? Is this restricted to pheromones that function in female-male interactions, perhaps due to co-evolution with female receptors? Is this pattern of change unique to salamanders or is it widespread in reproductive proteins?
- What receptors are recognizing salamander pheromones? Does the variation in receptors match that of the pheromones? How are the multi-component pheromonal mixtures coded in the olfactory system? Do multi-component signals act in an additive, synergistic, or antagonistic fashion?
- How are transdermally delivered pheromones processed? Are they detected by olfactory and vomeronasal receptors or do they represent a novel mode of communication?
- How are chemical cues and other sensory cues (visual and tactile) processed by the nervous system to enable multi-modal signaling?
- Most studies have focused on male pheromones acting on female recipients. What can be learned by examining female pheromones more thoroughly?
- Ample behavioral and morphological evidence indicates that pheromones are important for marking territories. What is the nature of pheromonal communication in non-reproductive contexts?
- How do skin microbiota influence pheromone production? Do microbiota and skin glands together participate in pheromone production?

- A true phylogenetic comparison of pheromone signaling requires more data on individual lineages. Will study of the relatively neglected species identify new types of pheromones and new hypotheses of protein evolution?
- With their reliance on chemical communication, are salamanders particularly vulnerable to environmental pollutants?

Given how much we have learned since the last review (Woodley 2015), we predict many more pheromones and signaling molecules will be identified in the next 10 years. Only a handful of salamander species have been examined, and further study, especially in understudied groups, should provide additional insight on the role of pheromones. We hope that this review inspires a new generation of scientists to pursue work in this rewarding field.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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