Chem. Senses 33: 623–631, 2008

doi:10.1093/chemse/bjn027
Advance Access publication June 5, 2008

A Recombinant Courtship Pheromone Affects Sexual Receptivity in a Plethodontid Salamander

Lynne D. Houck[1], Richard A. Watts[1], Stevan J. Arnold[1], Kathleen E. Bowen[2], Karen M. Kiemnec[1], Hilary A. Godwin[3], Pamela W. Feldhoff[2] and Richard C. Feldhoff[2]

[1]Department of Zoology, 3029 Cordley Hall, Oregon State University, Corvallis, OR 97331, USA, [2]Department of Biochemistry, University of Louisville, Louisville, KY 40292, USA and [3]Department of Environmental Health Sciences, University of California–Los Angeles, Los Angeles, CA 90095, USA

Correspondence to be sent to: Lynne D. Houck, Department of Zoology, 3029 Cordley Hall, Oregon State University, Corvallis, OR 97331, USA. e-mail: houckl@science.oregonstate.edu

Abstract

Pheromones are important chemical signals for many vertebrates, particularly during reproductive interactions. In the terrestrial salamander *Plethodon shermani*, a male delivers proteinaceous pheromones to the female as part of their ritualistic courtship behavior. These pheromones increase the female's receptivity to mating, as shown by a reduction in courtship duration. One pheromone component in particular is plethodontid receptivity factor (PRF), a 22-kDa protein with multiple isoforms. This protein alone can act as a courtship pheromone that causes the female to be more receptive. We used a bacterial expression system to synthesize a single recombinant isoform of PRF. The recombinant protein was identical to the native PRF, based on mass spectrometry, circular dichroism spectra, and a behavioral bioassay that tested the effects of recombinant PRF (rPRF) on female receptivity (21% reduction in courtship duration). The rPRF appears to mimic the activity of a mixture of PRF isoforms, as well as a mixture of multiple different proteins that comprise the male courtship gland extract. Pheromones that are peptides have been characterized for some vertebrates; to date, however, rPRF is one of only 2 synthesized vertebrate proteins to retain full biological activity.

Key words: *Plethodon shermani*, plethodontid receptivity factor, recombinant protein pheromone, red-legged salamander, vertebrate pheromones

Introduction

Pheromones are chemical signals that are released from an individual and elicit a specific response in a member of the same species (Karlson and Lüscher 1959). In vertebrates, pheromone signals can elicit a variety of responses in both females and males, including mate attraction, maternal behavior, and reproductive cycle modulation (Meredith 1983; Jemiolo et al. 1986; Kikuyama et al. 1995; Novotny, Jemiolo, et al. 1999; Novotny, Ma, et al. 1999; Wabnitz et al. 1999; Nakada et al. 2007). A behavioral response to pheromone signals often can be observed and quantified, but actually isolating and identifying the specific pheromone compounds that elicit the response is a continuing challenge in chemical ecology. Much of the research on vertebrate pheromones has focused on mammalian communication (Brennan and Zufall 2006). To date, however, only 7 mammalian pheromones have been chemically synthesized and then validated with an experiment that reveals full behavioral response to the synthetic pheromone. The number of characterized pheromones is even lower in amphibians. Prior to this study, pheromones were characterized for one anuran (the magnificent tree frog, *Litoria splendida*; Wabnitz et al. 1999) and 2 congeneric species of newts (*Cynops* spp., family Salamandridae; Kikuyama and Toyoda 1999; Yamamoto et al. 2000; Nakada et al. 2007). These amphibian pheromones are peptides, and each has been synthetically produced and the synthetic product behaviorally validated (Table 1).

We now report the characterization and validation of a male pheromone present in a multispecies group of lungless salamanders within the genus *Plethodon* (family Plethodontidae). Plethodontid salamanders are nocturnal animals that rely heavily on chemical communication to locate prey items, as well as to identify potential mates. The pheromone we characterized is not used for mate location, however, but is only delivered after courtship interactions between

Table 1 Vertebrate pheromones that have been synthesized and that retain biological response

	Pheromone	Type	Function	Species	Common name	Production	References	Class
1	7a,12a,24-Trihydroxy-5acholan-3-one 24-sulfate	NV organic	Causes searching behavior and preference in females	*Petromyzon marinus*	Sea lamprey	Glandular cells in male gills	(1)	Agnatha
2	17α,20β-Dihydroxy-4-pregnen-3-one or 17α,20β-dihydroxy-4-pregnen-3-one sulfate	NV organic	Attracts males	*Carassius auratus*	Goldfish and carp	Ovaries	(2), (3)	Actinopterygii
3	15-Keto-prostaglandin F2α or prostaglandin F2α	NV organic	Increases male GnRH release and sperm production	*C. auratus*	Goldfish	Ovaries	(4)	Actinopterygii
4	Mixture of (*Z*)-24-tritriaconten-2-one, (*Z*)-26-pentatriaconten-2-one, 2-tritriacontanone, and 2-pentatriacontanone	NV organic	Attracts females	*Clarias gariepinus*	African catfish	Seminal vesicles	(5)	Actinopterygii
5	Etiocholanolone glucuronide	NV organic	Attracts gravid females	*Gobius jozo*	Black goby	Leydig cells	(6)	Actinopterygii
6	L-kynurenine	Amino acid	Attracts males	*Oncorhynchus masou*	Masu salmon	Ovulated female urine	(7)	Actinopterygii
7	Silefrin	Peptide	Attracts females	*Cynops ensicauda*	Sword-tailed newt	Male abdominal gland	(8)	Amphibia
8	Sodefrin (and variant aonirin)	Peptide	Attracts females	*Cynops pyrrhogaster*	Red-bellied newt	Male abdominal gland	(9), (10)	Amphibia
9	Splendipherin	Peptide	Attracts females	*Litoria splendida*	Magnificent tree frog	Male rostral and parotoid glands	(11)	Amphibia
10	PRF	Protein	Increases female receptivity to mating	*Plethodon shermani*	Red-legged salamander	Male mental gland	(12)	Amphibia
11	Mixture of (*Z*)-24-tritriaconten-2-one and (*Z*)-26-pentatriaconten-2-one	NV organic	Induces males to court females	*Thamnophis sirtalis parietalis*	Canadian red-sided garter snake	Female skin	(13)	Reptilia
12	Mixture of *cis*-4-decenal and octanal	V organic	Attracts other conspecifics	*Aethia cristatella*	Crested auklet	Nape feathers	(14)	Aves
13	Mixture of 2-heptanone, *trans*-5-hepten-2-one, *trans*-4-hepten-2-one, *n*-pentyl acetate, *cis*-2-penten-1-yl-acetate, and 2,5-dimethylpyrazine	V organic	Delays onset of puberty in females	*Mus musculus*	Mouse	Female urine	(15)	Mammalia
14	Mixture of 2-(*sec*-butyl)-4,5-dihydrothiazole and 2,3-dehydro-exo-brevicomin	V organic	Accelerates puberty in females, attracts females, and causes intermale aggression	*M. musculus*	Mouse	Male urine	(16), (17), (18)	Mammalia
15	6-Hydroxy-6-methyl-3-heptanone	V organic	Accelerates puberty in females	*M. musculus*	Mouse	Male urine	(19)	Mammalia
16	Mixture of *E*,*E*-α- and *E*-β-farnesenes	V organic	Induces estrus and causes intermale aggression	*M. musculus*	Mouse	Male urine	(18), (20), (21)	Mammalia
17	MUPs (mixture of 4)	Protein	Causes intermale aggression	*M. musculus*	Mouse	Male urine	(22)	Mammalia
18	(Methylthio) methanethiol	V organic	Attracts females	*M. musculus*	Mouse	Male urine	(23)	Mammalia

Table 1 Continued

	Pheromone	Type	Function	Species	Common name	Production	References	Class
19	Dodecyl propionate	V organic	Stimulates maternal anogenital licking of pups	*Rattus norvegicus*	Rat	Pup preputial gland	(24)	Mammalia
20	2-methylbut-2-enal	V organic	Causes infant rabbits to search and grasp nipple	*Oryctolagus cuniculus*	Rabbit	Milk	(25)	Mammalia
21	Phenylacetic acid	V organic	Provokes male investigation	*Meriones unguiculatus*	Mongolian gerbil	Male and female sebaceous abdominal gland secretions	(26)	Mammalia
22	(*Z*)-7-dodecen-1-yl acetate	V organic	Increases frequency of male arousal behaviors	*Elephas maximus*	Asian elephant	Female urine during estrus	(27)	Mammalia
23	5α-Androst-16-en-3-one or 5α-androst-16-en-3α-ol	NV organic	Causes an estrous female to assume a receptive stance during mating	*Sus scrofa*	Pig	Male submaxillary gland	(28)	Mammalia

Synthesis can be either by recombinant peptide expression or by chemical synthesis. The compound (or mix of compounds) must elicit the same conspecific response to the native compound. The synthesized version must be active in an inert solvent. NV, nonvolatile; V, volatile. Numbers in References column refer to the citations: (1) Li et al. (2002), (2) Dulka et al. (1987), (3) Sorensen et al. (1995), (4) Sorensen et al. (1988), (5) Resink et al. (1989), (6) Colombo et al. (1980), (7) Yambe et al. (2006), (8) Yamamoto et al. (2000), (9) Kikuyama et al. (1995), (10) Nakada et al. (2007), (11) Wabnitz et al. (1999), (12) present study, (13) Mason et al. (1989), (14) Hagelin et al. (2003), (15) Novotny et al. (1986), (16) Jemiolo et al. (1986), (17) Novotny et al. (1985), (18) Novotny, Ma, et al. (1999), (19) Novotny, Jemiolo, et al. (1999), (20) Ma et al. (1999), (21) Novotny et al. (1990), (22) Chamero et al. (2007), (23) Lin et al. (2005), (24) Brouette–Lahlou et al. (1991), (25) Schaal et al. (2003), (26) Thiessen et al. (1974), (27) Rasmussen et al. (1997), and (28) Melrose et al. (1971).

a male–female pair already have begun. A plethodontid pair will engage in a stereotyped sequence of courtship interactions that culminate in sperm transfer via a spermatophore (a gelatinous base supporting an apical sperm cap). One of the courtship actions that precedes sperm transfer is the "tail-straddling walk," during which the female follows behind the male while straddling his tail and with her chin on his tail (a behavior that is characteristic of all plethodontid salamanders). This "walk" requires both the male and the female to modulate their courtship behaviors in response to the other partner (Arnold 1976). After the pair has been in the tail-straddling walk, the pair halts and the male lowers his cloaca to the substrate and begins to deposit a spermatophore. Deposition takes approximately 5–7 min, and if sperm transfer is to occur, the female must be sufficiently receptive to remain in contact with the male during that time. Thus, it is critical for the male to use the female's behavior to judge the level of her receptivity and then time his spermatophore deposition accordingly. If a female does not appear to be sufficiently receptive, the male has the option of delivering pheromones to the female during the courtship. These courtship pheromones increase the female's receptivity (judged by courtships that lead to spermatophore deposition more quickly), as compared with courtships in which the female only received a saline control (Rollmann et al. 1999, 2003). Thus, the duration of courtship (from the female entering into tail-straddling walk to spermatophore deposition) is an indirect measure of female receptivity, and receptivity can be influenced by male courtship pheromones.

Plethodontid male courtship pheromones are secreted from specialized mental (submandibular) glands that hypertrophy seasonally (Sever 1976). In our 2 main study species (*Plethodon shermani* and *Desmognathus ocoee*), we have extracted the pheromones expressed in the male mental glands and experimentally delivered these protein pheromones to female salamanders. In each of these species, experimental delivery of conspecific courtship pheromones decreased courtship time (Houck and Reagan 1990; Rollmann et al. 1999; Houck et al. 2007). Pheromone delivery and specialized mental glands occur in the majority of the >380 species within the family Plethodontidae. In fact, courtship pheromone delivery in plethodontids apparently has occurred for over 100 million years (see Houck and Arnold 2003).

In *P. shermani*, the delivery of courtship pheromones typically occurs during tail-straddling walk. The male temporarily halts and turns back toward the female (while the female continues to straddle his tail) and "slaps" his gland on her nares (Arnold 1976). This slapping behavior typically is repeated many times during tail-straddling walk. The pheromones enter the female's nasal cavity (Dawley and Bass 1989) and stimulate cells in the vomeronasal organ (Wirsig-Wiechmann et al. 2002, 2006). Biochemical characterization of the gland extract of male *P. shermani* revealed that a 22-kDa protein, termed plethodontid receptivity factor (PRF), was highly expressed in male mental glands (Rollmann

et al. 1999; Fontana et al. 2007). A purified solution containing multiple PRF isoforms increased female receptivity when experimentally delivered to her nares (Rollmann et al. 1999). Multiple isoforms of PRF are expressed in an individual male's gland, and PRF shows significant amino acid variation at multiple levels: individual, population, and species (Rollmann et al. 2000; Watts et al. 2004; Palmer et al. 2005).

We hypothesized that the recombinant expression of PRF would verify that a single isoform can influence female receptivity. In this study, we report 1) the development of an expression system to produce a functionally active recombinant pheromone protein and 2) the results of a behavioral experiment that tested whether the expressed protein alone could modify female behavior.

Materials and methods

Recombinant protein preparation

Recombinant PRF (termed rPRF) was expressed as a precursor protein with a cleavable N-terminal 6xHis affinity tag. We chose the *P. shermani* PRF isoform 3 (GenBank accession number AF181482) for expression because this isoform is abundantly expressed in the mental glands of males in our study population from Macon County, North Carolina. Isoform 3 was amplified by polymerase chain reaction with primers designed to attach a *Kpn*I restriction site and the sequence for an enterokinase cleavage site (DDDDK) immediately before the initial Glu^{1} residue. Downstream, we attached a stop codon immediately after the final codon and then a *Hind*III restriction site. This construct was inserted into the *Kpn*I/*Hind*III sites of pET32(a) (Novagen, San Diego, CA), and then this expression plasmid was transformed into an *Escherichia coli* strain CodonPlus-(DE3)-RIPL (Stratagene, La Jolla, CA) for protein expression.

Bacterial expression of soluble rPRF was performed at 25 °C with high efficiency (~100 mg/l). Cultures (Terrific Broth supplemented with 100 μg/l ampicillin) were grown using standard methods and with overnight protein expression induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside. The bacteria were harvested by centrifuging and stored at –80 °C.

Initial purification of the soluble rPRF fusion protein was conducted using His-Bind Resin (Novagen) at 4 °C and with buffers and methods described in the supplied protocol. Briefly, the bacteria were resuspended in binding buffer (5 mM imidazole) supplemented with DNase (10 μg/ml) and lysozyme (10 mg/ml) and then lysed in an Emulsiflex/C5 High Pressure Homogenizer (Avestin, Inc., Ottawa, Canada). The lysate was sonicated and clarified by centrifuging, and the 20–90% ammonium sulfate saturation fraction was then obtained using standard procedures. The protein was resuspended in binding buffer and applied to the His-Bind Resin (50 ml bed volume). The column was washed with 6 successive column volumes of binding buffer, 6 of wash buffer (60 mM imidazole), and 3 of elution buffer (1 M imidazole). Final purity was estimated at >95%, and the yield was measured at over 100 mg/l of bacterial culture.

Recombinant enterokinase (EMD Biosciences, Madison, WI) was used to cleave the His-tag. Cleavage products were separated from rPRF by HLPC (~99% purity). After cleavage, the final rPRF protein was shown by mass spectral analyses to have a mass (21,641 Da) identical to the native isoform (isoform 3 in Rollmann et al. 1999). The protein concentration of the rPRF solution was adjusted to the final concentration of 0.7 μg/μl in 0.5× phosphate-buffered saline (PBS) using ultra filtration.

To characterize the secondary structures of rPRF and the native PRF isoform 3, circular dichroism (CD) analyses were performed using a 0.1-cm path length cell at 0.2-nm intervals with 5 scans (182–260 nm) averaged at 20 °C (Jasco J-810 Spectropolarimeter). Far-UV CD spectra of high-performance liquid chromatography–purified rPRF and PRF isoform 3, in 20 mM potassium phosphate buffer (pH 7.4), were taken at a protein concentration of 0.11 and 0.04 μg/μl, respectively. The resultant spectra were corrected for the buffer signal and interpreted with Olis GlobalWorks algorithms (www.olisweb.com).

Courtship trials

Male and female *P. shermani* in breeding condition were collected from Macon County, North Carolina (035°10′48″N, 083°33′38″W) during August 2005. Animals were collected with the appropriate permits from North Carolina Department of Wildlife. The animals were housed at Oregon State University for the duration of the behavioral observations, which were staged over 8 nights between 7 and 19 September 2005. Methods and animal care were approved by Oregon State University's Institutional Animal Care and Use Committee (LAR 3007 to L.D.H.). Animal care followed Houck et al. (1998). Briefly, animals were individually housed and kept on a natural photoperiod. Each animal was fed waxworms (*Galleria mellonella*) weekly.

Before courtship trials were staged, animals were randomly assigned to pairs and prescreened (to determine their propensity to court in a laboratory environment) by allowing each pair to remain together in the same box overnight. The following morning, the animals were put back in their maintenance boxes. Each courtship box was checked for the presence of a spermatophore base, which was taken as an indication of a successful courtship. Most animals were given 3 courtship encounters, and animals that mated at least once were selected to be used in the courtship trials. This selection process favored females that had a higher likelihood of mating in the laboratory.

To ensure that females only received pheromones delivered by the researchers, we anesthetized each male used in the courtship trials and surgically ablated its mental gland. Deglanded males included in the experiment were given at least 2 weeks to recover before they were used in behavioral

trials. Males fully recover and court normally after this procedure (Houck LD, unpublished data).

Once reproductively active animals were selected, they were randomly reassigned to different male–female pairs before the onset of the courtship trials. During a trial, each male–female pair was placed in a clear plastic box (9 × 17 × 30 cm) under low light conditions. Trials were conducted during the time of night when the animals normally would be found courting in the field (2200 to 0100 h).

The delivery of either rPRF or a control saline solution to each female followed the protocol of Houck et al. (2007). To summarize, the onset of experimental pheromone delivery was designed to coincide with a male's natural pheromone delivery behavior to control for tactile stimulation. Once a pair was engaged in tail-straddling walk and the deglanded male had attempted pheromone delivery by touching the female's nares, either rPRF (0.7 μg/μl in 0.5× PBS, a concentration used in prior behavioral experiments) or 0.5× PBS was delivered to the female using a micropipette. On a given observation night (=trial), the first type of treatment (rPRF or 0.5 × PBS) was randomly assigned to the first female to engage in tail-straddling walk. Subsequent treatments alternated between rPRF and 0.5× PBS such that both treatments were given to multiple females, but an individual female received only one type of treatment. Overall, each treated female received a total of 15 μl of solution onto her nares (5 μl every 10 min). Due to temporal limitations (each observer can only keep track of a limited number of courting pairs at the same time, and trials need to be staged during the peak courtship season), we were unable to test multiple stimuli (e.g., rPRF and native PRF) during the same courtship season.

During each trial night, observers recorded observations using focal animal sampling (Altmann 1974). As a measurement of courtship duration, we recorded the time from when a female entered tail-straddling walk to the termination of spermatophore deposition because these behaviors were unambiguous and easily scored by all observers. For all pairs (pheromone and saline treated), we also recorded the number of slaps that a male administered during courtship and whether the female was inseminated.

Data analysis

Data on the mean duration of courtship were analyzed using a 1-tailed *t*-test with alpha = 0.05. We used a 1-tailed test based on results from prior behavioral experiments showing that PRF pheromones significantly increased female receptivity and thus resulted in a reduced courtship duration (e.g., Rollmann et al. 1999). Data on the average number of slaps (males attempted pheromone delivery even though they were deglanded) were analyzed using a 2-tailed *t*-test with alpha = 0.05. Data on insemination success for pheromone-treated and saline-treated females were analyzed using a 2 × 2 contingency table with alpha = 0.05.

Results

The bacterial expression system and subsequent purification produced a single isoform of rPRF. Due to sequence similarity with the interleukin (IL)-6 family of cytokines (4 α-helix bundle structures; Rollmann et al. 1999), rPRF was expected to have a high α-helical content similar to that of the native isoforms. The CD spectrum shown in Figure 1 indicated a predominately α-helical structure, particularly the trough between 208 and 222 nm, which is similar to the documented structure of known secondary structures of the IL-6 cytokines (e.g., Somers et al. 1997). The helical contents of the native PRF (isoform 3) and rPRF were identical (Figure 2) within typical experimental conditions (Table 2).

rPRF decreased the mean courtship time by approximately 14 min (21%): mean of saline treatment = 66 min and mean of rPRF treatment = 52 min. Thus, the behavioral assay confirmed that rPRF increased female receptivity (t_{26} = 3.04, $P \leq 0.01$, 1-tailed *t*-test) in *P. shermani* (Figure 2).

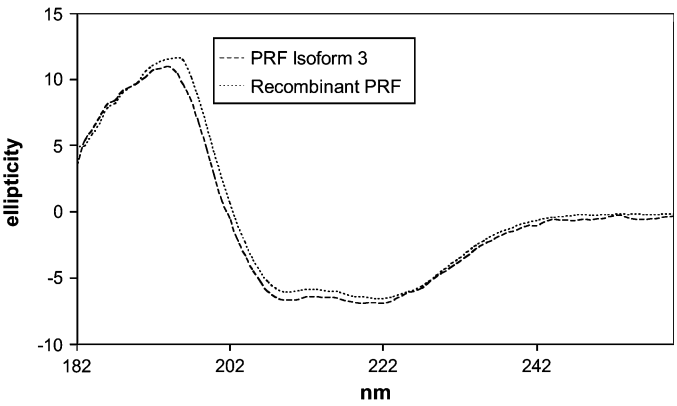

Figure 1 The CD spectrum of high-performance liquid chromatography–purified rPRF (0.11 mg/ml) and purified PRF isoform 3.

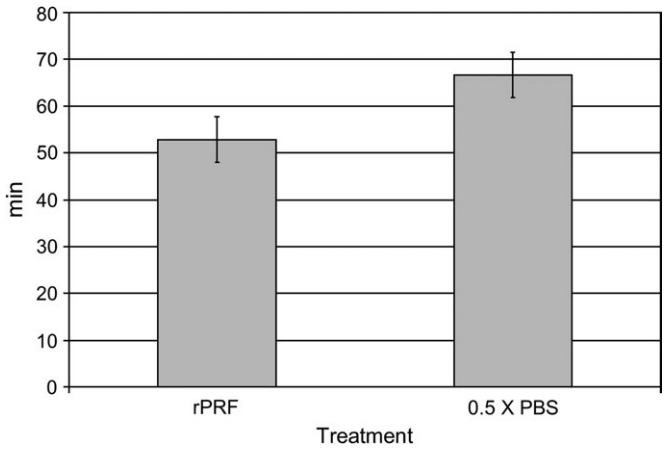

Figure 2 Differences in courtship duration between females treated with a solution of rPRF (pheromone treatment) or 0.5× PBS (saline control) during courtship.

Table 2 Secondary structures calculated from the CD data for the natural PRF isoform 3 and the rPRF

Protein sample	Helical content	Sheet structure	Other[a]	SD[b]
PRF isoform 3	57%	5%	38%	0.06
rPRF	57%	9%	34%	0.04

[a]Turns and disordered.
[b]Typical standard deviation (SD) is ±0.07 and represents the difference between the calculated spectrum and original data.

The only effect of rPRF on a specific behavior pattern was the duration of tail-straddling walk (i.e., time from commencement of tail-straddling walk to spermatophore deposition). There was no difference between the treatment and the control groups in 1) the mean number of times males attempted to deliver pheromones during tail-straddling walk ($t_{26} = 0.112$, $P < 0.911$, 2-tailed t-test) or b) the relative number of females in each treatment group that were inseminated ($\chi^2_1 = 0.022$, $P > 0.882$).

Discussion

In *P. shermani* salamanders, the average courtship duration of male–female pairs was significantly reduced by the experimental delivery of a single recombinant isoform of the male courtship pheromone PRF. This behavioral response to pheromone delivery represents one of the first demonstrations in vertebrates that a nonpeptide protein can be synthesized and still retain the full effects of the native pheromone protein (for a major urinary protein [MUP] example, see Chamero et al. 2007).

The behavioral effect of rPRF (21% reduction in average courtship duration) is similar to effects documented in 2 behavioral tests previously conducted with *P. shermani* courtship pheromones. In comparison with the current results, rPRF mimicked the activity of 1) a purified blend of multiple native PRF isoforms: 15% reduction in courtship time (Rollmann et al. 1999) and 2) the combined proteins contained in pooled secretions obtained from multiple male mental glands: 22% reduction in courtship time (Rollmann et al. 2003). The difference in average courtship durations among these 3 experiments (21%, 15%, and 22%) most likely is due to experimental variation in different years (e.g., minor differences in the relative timing of prescreening animals for courtship propensity and of staging courtship trials). In short, the results of behavioral tests over multiple years confirm that PRF consistently had significant effects on female receptivity.

We interpret the effects of PRF primarily in terms of sexual selection, as the courtship pheromone functions to influence the female's mate choice. In fact, the male's repeated delivery of PRF apparently acts physiologically to focus the female's attention, in effect "persuading" her to continue mating with him. When the male judges that the female is sufficiently receptive (presumably from some aspects of the female's behavior; Arnold 1977), only then will he begin the process of spermatophore deposition. Thus, as this chemical persuasion is successful, the male is more likely to inseminate the female. The significance of pheromone delivery is realized when one observes the actions of a female that already is receptive (prior to her interactions with a male). In this situation, the female actually will approach the male and immediately initiate the tail-straddling walk. In these rare cases, the male completely eliminates courtship pheromone delivery and moves rapidly into spermatophore deposition (Houck, personal observations; and see Halliday 1975). Thus, a pheromone that increases the female's interest in the male can be critical to the male's mating success. In addition, a mating pair that spends less time in tail-straddling walk is more likely to avoid disruption of the courtship (e.g., by rival males) or (reflecting natural selection here) more likely to avoid becoming the target of local predators (Houck 1986).

These effects of PRF on mate choice and mating success are important but still do not reflect the full story of the PRF signal and the female response to this signal. More light is shed on the nature of the PRF pheromone by comparing changes in the amino acid sequences of PRFs not only within and between populations but also across a multispecies *Plethodon* lineage that spans at least 15 million years of divergence. These molecular comparisons reveal that PRF has changed substantially (in terms of amino acid sequences) from species to species, resulting in highly significant variation in the male signal (Rollmann et al. 2000; Watts et al. 2004; Palmer et al. 2005). This kind of extraordinary variation also has been documented in certain other vertebrate chemical signals (Emes et al. 2004). High numbers of pheromone polymorphisms, for example, are found in the MUPs of mice and other rodents: over 2000 different MUP sequences have been deposited in GenBank (Beynon and Hurst 2004). The complex mixture of MUPs apparently confers information about individual identity (Hurst et al. 2001), rather than eliciting a specific behavioral response (but see Novotny, Ma, et al. 1999; Chamero et al. 2007). Another highly variable pheromone signal has been described in male mice: a 7-kDa peptide (transcribed from a multigene family that contains at least 23 genes) is secreted from the extraorbital lacrimal gland to convey information to females (Kimoto et al. 2005). These examples have in common the likelihood that sexual selection is acting on the male signal through the powerful agent of female mate choice (for further details, see Watts et al. 2004). In contrast, variation in the courtship pheromone signal of certain male newts more likely is a result of natural selection, not sexual selection. In these *Cynops* species (see Table 1), variation has been documented for the decapeptide pheromone sodefrin (Nakada et al. 2007). Males in 2 separate populations express different variants of this decapeptide, and females are more stimulated by the pheromone produced by males from their own population.

Whether or not these pheromone signals are highly variable, peptides and proteins appear to be important nonvolatile chemical signals, particularly in amphibians—the first vertebrate group to possess a discrete vomeronasal organ. Kikuyama et al. (2002) noted that amphibian pheromones are predominantly amino acid based, and this trend is supported by the recent research (Palmer et al. 2005; Houck et al. 2007). In studying these peptides and proteins, synthesis presents unique challenges: expression in many cell lines is not difficult but signal effectiveness also is determined by the correct posttranslational modifications and 3-dimensional conformation. Some proteins that act as pheromones in the native form have lost partial or full activity when recombinantly expressed. Purified MUPs had a puberty-accelerating effect on female mice, but recombinant MUPs did not (Novotny, Ma, et al. 1999). In hamsters, a cloned version of the aphrodisin pheromone did not elicit mating behavior from males (Jang et al. 2001), although the native molecule had behavioral effects (Singer et al. 1986). Also, Singer et al. (1976) claimed that male hamsters still showed some response to synthesized dimethyl disulfide, but reactions were only 20–40% of the response to the native vaginal secretions and the absence of any smaller, bound molecules was not demonstrated. Together, these results suggest that context dependence is important, and thus, the increasing complexity of social behaviors in some mammalian groups may have necessitated more complex cues to aid social communication (for review, see Johnston 2003).

The expression system we have developed for PRF isoforms will allow us to test the effectiveness of individual isoforms, as well as isoform combinations. We also will be able to test whether females have different isoform preferences, as suggested by the extreme isoform variation already documented for *P. shermani*. In addition, this system can help determine which sites in the protein are important for biological activity, using techniques such as site-directed mutagenesis. Finally, manipulating PRF isoforms may help us gain an understanding of the evolutionary processes that produce and maintain protein polymorphisms.

Funding

National Science Foundation Grants (IOB-0110666, IOB-0416724 to L.D.H., IOB 0416834 to R.C.F.).

Acknowledgements

We thank Elyse Vaccaro and Stacey Smith for assistance in collecting animals and with behavioral observations and Audrey Thompson for assistance with protein purification. We also thank the Directors and Foundation of the Highlands Biological Station for providing facilities and support for our fieldwork and research.

References

Altman J. 1974. Observational study of behavior: sampling methods. Behaviour. 48:1–41.

Arnold SJ. 1976. Sexual behavior, sexual interference and sexual defense in the salamanders *Ambystoma maculatum*, *Ambystoma tigrinum*, and *Plethodon jordani*. Z Tierpsychol. 42:247–300.

Arnold SJ. 1977. The evolution of courtship behavior in new world salamanders with some comments on old world salamandrids. In: Taylor DH, Guttman SI, editors. The reproductive biology of amphibians. New York: Plenum Press. p. 141–180.

Beynon RJ, Hurst JL. 2004. Urinary proteins and the modulation of chemical scents in mice and rats. Peptides. 25:1553–1563.

Brennan PA, Zufall F. 2006. Pheromonal communication in vertebrates. Nature. 444:308–315.

Brouette-Lahlou I, Amouroux R, Chastrette F, Cosnier J, Stoffelsma J, Vernet-Maury E. 1991. Dodecyl propionate, attractant from rat pup preputial gland: characterization and identification. J Chem Ecol. 17: 1343–1354.

Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, Cravatt BF, Stowers L. 2007. Identification of protein pheromones that promote aggressive behaviour. Nature. 450:899–903.

Colombo L, Marconato A, Belvedere PC, Frisco C. 1980. Endocrinology of teleost reproduction: a testicular steroid pheromone in the black goby, *Gobius jozo* L. Boll Zool. 47:355–364.

Dawley EM, Bass AH. 1989. Chemical access to the vomeronasal organs of a plethodontid salamander. J Morphol. 200:163–174.

Dulka JG, Stacey NE, Sorensen PW, Van Der Kraak GJ. 1987. A sex steroid pheromone synchronizes male-female spawning readiness in goldfish. Nature. 325:251–253.

Emes RD, Beatson SA, Ponting CP, Goodstadt L. 2004. Evolution and comparative genomics of odorant- and pheromone-associated genes in rodents. Genome Res. 14:591–602.

Fontana MF, Houck LD, Staub NL. 2007. *In situ* localization of plethodontid courtship pheromone mRNA in formalin-fixed tissue. Gen Comp Endocrinol. 150:480–485.

Hagelin JC, Jones IL, Rasmussen LEL. 2003. A tangerine-scented social odour in a monogamous seabird. Proc R Soc Lond B Biol Sci. 270: 1323–1329.

Halliday TR. 1975. An observational and experimental study of sexual behaviour in the smooth newt, *Triturus vulgaris* (Amphibia: Salamandridae). Anim Behav. 23:291–322.

Houck LD. 1986. The evolution of salamander courtship pheromones. In: Duvall D, Muller-Schwarze D, Silverstein RM, editors. Chemical signals in vertebrates. Vol. 4. New York: Plenum Press. p. 173–190.

Houck LD, Arnold SJ. 2003. Courtship and mating behavior. In: Sever DM, editor. Phylogeny and reproductive biology of urodela (Amphibia). Enfield, (NH): Science Publishers. p. 383–424.

Houck LD, Bell AM, Reagan-Wallin NL, Feldhoff RC. 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 LD, Palmer CA, Watts RA, Arnold SJ, Feldhoff PW, Feldhoff RC. 2007. A new vertebrate courtship pheromone, PMF, affects female receptivity in a terrestrial salamander. Anim Behav. 73:315–320.

Houck LD, Reagan NL. 1990. Male courtship pheromones increase female receptivity in a plethodontid salamander. Anim Behav. 39:729–734.

Hurst JL, Payne CE, Nevison CM, Marie AD, Humphries RE, Robertson DH, Cavaggioni A, Beynon RJ. 2001. Individual recognition in mice mediated by major urinary proteins. Nature. 414:631–634.

Jang T, Singer AG, O'Connell RJ. 2001. Induction of c-fos in hamster accessory olfactory bulbs by natural and cloned aphrodisin. Neuroreport. 12:449–452.

Jemiolo B, Harvey S, Novotny M. 1986. Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. Proc Natl Acad Sci USA. 83:4576–4579.

Johnston RE. 2003. Chemical communication in rodents: from pheromones to individual recognition. J Mammal. 84:1141–1162.

Karlson P, Lüscher M. 1959. 'Pheromones': a new term for a class of biologically active substances. Nature. 183:55–56.

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

Kikuyama S, Toyoda F, Ohmiya Y, Matsuda K, Tanaka S, Hayashi H. 1995. Sodefrin: a female-attracting peptide pheromone in newt cloacal glands. Science. 267:1643–1645.

Kikuyama S, Yamamoto K, Iwata T, Toyoda F. 2002. Peptide and protein pheromones in amphibians. Comp Biochem Physiol B. 132: 69–74.

Kimoto H, Sachiko H, Sato K, Touhara K. 2005. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature. 437:898–901.

Li W, Scott AP, Siefkes MJ, Yan H, Liu Q, Yun SS, Gage DA. 2002. Bile acid secreted by male sea lamprey that acts as a sex pheromone. Science. 296:138–141.

Lin DY, Zhang S-Z, Block E, Katz LC. 2005. Encoding social signals in the mouse main olfactory bulb. Nature. 434:470–477.

Ma W, Miao Z, Novotny MV. 1999. Induction of estrus in grouped female mice (*Mus domesticus*) by synthetic analogues of preputial gland constituents. Chem Senses. 24:289–293.

Mason RT, Fales HM, Jones TH, Pannell LK, Chinn JW. 1989. Sex pheromones in snakes. Science. 245:290–293.

Melrose DR, Reed HCB, Patterson RLS. 1971. Androgen steroids associated with boar odour as an aid to the detection of oestrus in pig artificial insemination. Br Vet J. 127:497–501.

Meredith M. 1983. Sensory physiology of pheromone communication. In: Vandenberg JG, editor. Pheromones and reproduction in mammals. New York: Academic Press. p. 199–252.

Nakada T, Toyoda F, Iwata T, Yamamoto K, Conlon JM, Kato T, Kikuyama S. 2007. Isolation, characterization and bioactivity of a region-specific pheromone, [Val8]sodefrin from the newt *Cynops pyrrhogaster*. Peptides. 28:774–780.

Novotny M, Harvey S, Jemiolo B. 1990. Chemistry of male dominance in the house mouse, *Mus domesticus*. Experientia. 46:109–113.

Novotny M, Harvey S, Jemiolo B, Alberts J. 1985. Synthetic pheromones that promote inter-male aggression in mice. Proc Natl Acad Sci USA. 82: 2059–2061.

Novotny M, Jemiolo B, Harvey S, Wiesler D, Marchlewska-Koj A. 1986. Adrenal-mediated endogenous metabolites inhibit puberty in female mice. Science. 231:722–725.

Novotny M, Jemiolo B, Wiesler D, Ma W, Xu SHF, Tian-Min Xie T-M, Carmack M. 1999. A unique urinary constituent, 6-hydroxy-6-methyl-3-heptanone, is a pheromone that accelerates puberty in female mice. Chem Biol. 6:377–383.

Novotny MV, Ma W, Wiesler D, Zidek L. 1999. Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. Proc R Soc Lond B Biol Sci. 266:2017–2022.

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

Rasmussen LEL, Lee TD, Zhang A, Roelofs WL, Daves GD Jr. 1997. Purification, identification, concentration and bioactivity of (z)-7-dodecen-1-yl acetate: sex pheromone of the female Asian elephant, *Elphas maximus*. Chem Senses. 22:417–437.

Resink JW, Schoonen WGEJ, Alpers PC, File DM, Notenbloom CD, Van Den Hurk R, Van Oordt PGWJ. 1989. The chemical nature of sex attracting pheromones from the seminal vesicle of the African catfish, *Clarias gariepinus*. Aquaculture. 83:137–151.

Rollmann SM, Houck LD, Feldhoff RC. 1999. Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. Science. 285: 1907–1909.

Rollmann SM, Houck LD, Feldhoff RC. 2000. Population variation in salamander courtship pheromones. J Chem Ecol. 26:2713–2723.

Rollmann SM, Houck LD, Feldhoff RC. 2003. Conspecific and heterospecific pheromone effects on female receptivity. Anim Behav. 66: 857–861.

Schaal B, Coureaud G, Langlois D, Giniès C, Sémon E, Perrier G. 2003. Chemical and behavioural characterization of the rabbit mammary pheromone. Nature. 424:68–72.

Sever DM. 1976. Morphology of the mental hedonic gland clusters of plethodontid salamanders. J Herpetol. 10:227–239.

Singer AG, Agosta WC, O' Connell RJ, Pfaffmann C, Bowen DV, Field FH. 1976. Dimethyl sulfide: an attractant pheromone in the hamster vaginal secretion. Science. 191:948–950.

Singer AG, Macrides F, Clancy AN, Agosta WC. 1986. Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge. J Biol Chem. 261:13323–13326.

Somers W, Stahl M, Seehra JS. 1997. 1.9 Å crystal structure of interleukin 6: implications for a novel mode of receptor dimerization and signaling. EMBO J. 16:989–997.

Sorensen PW, Hara TJ, Stacey NE, Goetz FW. 1988. F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. Biol Reprod. 39:1039–1050.

Sorensen PW, Scott AP, Stacey NE, Bowdin L. 1995. Sulfated 17,20ß-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. Gen Comp Endocrinol. 100:128–142.

Thiessen DD, Regnier FE, Rice M, Goodwin M, Isaacks N, Lawson N. 1974. Identification of a ventral scent marking pheromone in the male Mongolian gerbil (*Meriones unguiculatus*). Science. 184:83–85.

Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP. 1999. Aquatic sex pheromone from a male tree frog. Nature. 401:444–445.

Watts RA, Palmer CA, Feldhoff RC, Feldhoff PW, Houck LD, Jones AG, Pfrender ME, Rollmann SM, Arnold SJ. 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 CR, Houck LD, Feldhoff PW, Feldhoff RC. 2002. Pheromonal activation of vomeronasal neurons in plethodontid salamanders. Brain Res. 952:335–344.

Wirsig-Wiechmann CR, Houck LD, Wood LM, Feldhoff PW, Feldhoff RC. 2006. Male pheromone protein components activate female

vomeronasal neurons in the salamander *Plethodon shermani*. BMC Neuroscience. 2006:26.

Yamamoto K, Kawai Y, Hayashi T, Ohe Y, Hayashi H, Toyoda F, Kawahara G, Iwata T, Kikuyama S. 2000. Silefrin, a sodefrin-like pheromone in the abdominal gland of the sword-tailed newt, *Cynops ensicauda*. FEBS Lett. 472:267–270.

Yambe H, Kitamura S, Kamio M, Yamada M, Matsunaga S, Fusetani N, Yamazaki F. 2006. L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. Proc Natl Acad Sci USA. 103:15370–15374.

Accepted April 24, 2008