Proteinaceous Pheromone Affecting Female Receptivity in a Terrestrial Salamander

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A 22-kilodalton protein was isolated from the submandibular (mental) gland of the male terrestrial salamander, *Plethodon jordani* (family: Plethodontidae). This proteinaceous pheromone, termed plethodontid receptivity factor (PRF), was experimentally delivered to the female during courtship and shown to increase female receptivity. In most plethodontid salamanders, ovulation occurs weeks or months after insemination, so the pheromone-induced change in receptivity is the only known function of PRF. The messenger RNAs corresponding to isoforms of PRF were transcribed into complementary DNA, cloned, sequenced, and shown to have homology with cytokines of the interleukin-6 family. Pheromone activity would represent a previously unrecognized function for cytokines.

Pheromones are chemical signals that can provide information on species identity, gender, and reproductive condition to other individuals within a species. Pheromones may modulate reproductive interactions between potential mates, affecting species recognition as well as mate persuasion. Behavioral effects of pheromones have been documented for a wide variety of species, but very few vertebrate pheromones have been chemically identified [see, for example, (*1, 2*)]. We report here the biochemical identification of a male courtship pheromone in the terrestrial salamander *Plethodon jordani* (Plethodontidae). Courtship pheromones are delivered only after a mate has been located and after initial courtship interactions have commenced. In plethodontid salamanders, courtship pheromones function to increase female receptivity, thus increasing the probability that the female will be inseminated (*3*). We present evidence that a single protein component of the male courtship pheromone is sufficient to affect female behavioral response. Furthermore, we identified a gene that codes for this pheromone component.

During the courtship season, male *P. jordani* develop a specialized (mental) gland underneath the chin that produces courtship pheromones. The female typically receives these nonvolatile courtship pheromones only during one particular stage of courtship, tail-straddling walk (*4*). A pair is in tail-straddling walk when the female walks forward with the male while straddling his tail and resting her chin on his tail base. While in this tail-straddling position, the male regularly turns back and actively applies his gland to her nares during courtship, thereby delivering courtship pheromones (*5*). This highly restricted delivery mode prevents rival conspecifics or predators from detecting the pheromone. In contrast, male aquatic-breeding salamanders (ambystomatids and salamandrids) typically broadcast pheromones into the water. These pheromones may attract females, but these females already are highly receptive, and usually will ovulate within a few hours or days (*2, 6*). Maximal receptivity, insemination, and ovulation are not closely linked in plethodontid salamanders because oviposition typically occurs weeks or even months after pheromone delivery (*6, 7*). Also, a female does not receive nutritional or other materials from male pheromone delivery, and clutch size is unaffected. Thus, the only known function of plethodontid courtship pheromones is to influence female receptivity.

We isolated and characterized the courtship pheromone for *P. jordani* to identify the specific pheromone component that alters female receptivity. Biochemical analyses of mental gland extracts revealed that proteins of ~10 to 25 kD were the primary pheromone components (*8*). Using a combination of Mono-Q anion-exchange high-performance liquid chromatography (HPLC) and gel filtration chromatography, we isolated and purified the primary pheromone component, a ~22-kD protein that we term plethodontid receptivity factor (PRF) (*9*). Separations based on Mono-Q anion-exchange HPLC revealed several PRF peaks (Fig. 1). SDS-PAGE gels and amino acid sequencing confirmed that multiple isoforms of PRF exist. These isoforms show 90% homology in their NH_2-terminal amino acid sequence and in molecular weight (*8*).

The effects of purified isoforms of PRF on female receptivity were then tested through the experimental delivery of treatment solutions directly to the female's nares (*10*). The males had their mental glands excised so that pheromone delivery could be experimentally controlled (*9*). After the mental gland was ablated, the male still behaved as though the gland were present. In staged courtship encounters, 11 male-female pairs each were scored when the female received the pheromone treatment (purified PRF) and also (on a different night) when the female received the saline control treatment. Purified PRF significantly decreased the time a pair spent in tail-straddling walk relative to the control (one-tailed t test; $P < 0.013$; Table 1). The 11 male-female pairs varied in the time spent in tail-straddling walk, yet PRF reduced courtship time for 9 out of the 11 pairs (the times for one pair were identical for both treatments).

The reduction in courtship time is significant in that courtship pheromones apparently alter female behavior in a way that induces the male to deposit a spermatophore sooner. The male responds to cues from female behavior and is more likely to deposit a spermatophore if a female coordinates her forward movements with those of the male (*5*). The physiological mechanism underlying female behavioral response in *P. jordani* is not known. The response to courtship pheromones is a robust phenomenon, however, as demonstrated by eliciting such a response in females that had been preselected on the basis of an already high propensity to court under laboratory conditions (*10*).

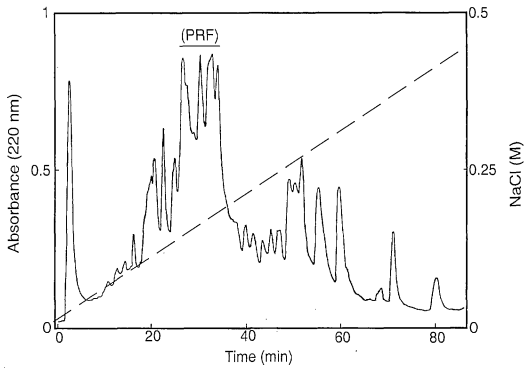

Fig. 1. Anion-exchange HPLC chromatogram of *P. jordani* pheromone extract. Samples of the mental gland extract (1.2 to 1.7 ml) were separated as described (9). Multiple isoforms of PRF exist. These isoforms were pooled and further purified by a second round of anion-exchange HPLC followed by gel filtration chromatography.

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The DNA sequences for four PRF isoforms were determined (*11*). Every salamander synthesizes multiple isoforms. The derived amino acid sequences are shown in Fig. 2. Searches of protein databases revealed sequence homology to members of the interleukin-6 (IL-6) cytokine family (*12*). IL-6 cytokines function in a variety of regulatory activities that can be pleiotropic or cell type specific. Members of this family have similar molecular masses to PRF and often show low homology (15 to 20%) in their amino acid sequences (*13*). It is noteworthy, therefore, that PRF shows 22% identity with rat cardiotrophin and 15% with rat ciliary neurotrophic factor (Fig. 2). Many additional sites show conservative amino acid substitutions. Sections of PRF also have greater than 60% identity with segments of the human genome (Fig. 2) on chromosome 7 where other members of the IL-6 family have been located (*14*). Together, these observations suggest that PRF may be an IL-6–type cytokine acting in a specific manner on the neurological system of female salamanders to increase female receptivity.

Table 1. Differences in the duration of courtship behavior (tail-straddling walk) for pairs in which the female was treated once with a saline control and once with PRF on separate nights. PRF significantly reduced courtship duration ($P < 0.013$, one tailed t test).

Male-female pair	Saline (in min)	PRF (in min)	Duration is shorter for:
1	47	44	PRF
2	60	30	PRF
3	61	55	PRF
4	30	22	PRF
5	47	39	PRF
6	42	39	PRF
7	40	28	PRF
8	67	59	PRF
9	35	27	PRF
10	53	53	Same
11	35	42	Saline

Our characterization of *P. jordani* courtship pheromones is only the second amphibian pheromone that has been biochemically identified (*2*). In earlier work on an aquatic-breeding salamander (*Cynops pyrrogaster*; Salamandridae), a decapeptide produced by a breeding male was identified and shown to be attractive to females (*2*). Unlike in terrestrial plethodontid salamanders, the demonstrated action of this decapeptide is as a sex attractant: females that are already highly receptive apparently use this male-produced pheromone to locate a potential mate. In contrast, most terrestrial plethodontid salamanders have a relatively lengthy mating season, so insemination can occur several months before oviposition. Plethodontid courtship pheromones, therefore, differ from other vertebrate chemical signals in that these specialized signals are produced and transmitted during courtship interactions with a female that is not ready to ovulate. In some mammals, male odors also may elicit a female reproductive response [for example, the "boar effect" (*15*)], but in these cases, the effective male odor typically is not produced by a specialized gland and only estrous females respond. Thus, the demonstrated responses of female plethodontid salamanders, reported here and in (*16*), represent the only known vertebrate examples of pheromone-induced changes in receptivity for females that will be inseminated long before oviposition occurs. Plethodontid salamanders, therefore, may serve as a model system to study changes in female receptivity that are independent from ovulatory events.

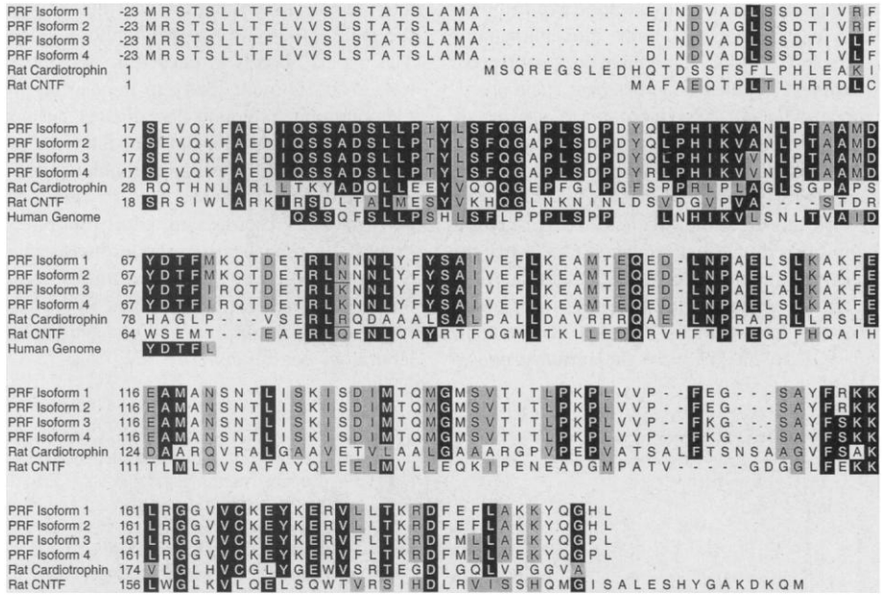

Fig. 2. Comparison of PRF isoforms with two members of the IL-6 cytokine family, rat cardiotrophin and rat ciliary neurotrophic factor (CNTF), and with segments of the human genome (line 7; GenBank accession numbers AC003085 and AC004946). Residues of PRF identical to the IL-6 members and the human genome segments are shaded in black. Conservative substitutions are shaded in gray. Purified isoforms of PRF have an identical 23-residue signal sequence preceding the unblocked NH_2-terminus (−1 to −23). Differences among PRF isoforms (relative to isoform 1) are found at positions 1, 7, 15, 51, 58, 71, 72, 80, 109, 152, 158, 175, 182, 183, 186, and 191.

References and Notes

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9. Each male was anesthetized in a mixture of 4% ether in water. The mental gland was excised and placed into a 0.8 mM acetylcholine chloride solution for 30 min. Extracted pheromone components were then centrifuged (10 min; 14,000*g*) and the supernatant frozen at −80°C. Pooled gland extracts were later thawed and filtered through a 0.2-μm filter (non–protein binding) and applied to a Mono-Q column (FPLC HR 5/5; Pharmacia, Piscataway, NJ) at 50 mM tris-HCl, pH 8.0. The column was eluted with the same buffer, at 1 ml/min, with a NaCl gradient (5.0 mM NaCl/min). To further purify PRF, we ran enriched fractions of PRF isoforms separately on the Mono-Q column again and eluted the column with a NaCl gradient of 3.3 mM NaCl/min. The PRF fractions were further purified on a Sephadex Superfine G-75 column (1.6 cm by 15.5 cm; Pharmacia, Piscataway, NJ) previously equilibrated with one-half strength Dulbecco's phosphate-buffered saline at a flow rate of 7.9 ml/hour. A pool of the highly purified PRF isoforms was adjusted to 0.7 mg/ml, the approximate concentration of PRF present in the whole extract used in previous experiments (*3*), and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on 15% tris-tricine gels. No contaminants were detected on SDS-PAGE gels when 5 μg was stained with Coomassie Brilliant Blue R-250, suggesting a purity of ≥ 98%. Reversed-phase HPLC analysis of similarly prepared PRF isoforms suggests that typical purity is ~99%. We did not use isoforms prepared from cloned genes in this bioassay system because it is unknown if the activity of individual isoforms will depend only on the primary amino acid sequence or will include posttranslational modifications.
10. About 400 adult males and 400 gravid females were collected from a single population in North Carolina (83°33′38″N longitude and 35°10′49″W latitude). The appropriate collecting permits (North Carolina permit number 0120 to S.M.R.) and animal care protocols (University of Chicago animal care and use protocol number 70511; Oregon State University number 1997) were obtained. After collection, courtship encounters were staged between male-female pairs to assess individual tendency to mate under laboratory conditions. Every salamander received multiple courtship opportunities. Males and females were then ranked according to courtship propensity. For the experiments, we selected males and females

that had the highest courtship propensities, in order to maximize the probability of obtaining animals willing to court under experimental conditions. We are aware that such prescreening may bias the results by increasing overall courtship rates. Any bias, however, would tend to minimize the probability of demonstrating courtship pheromone effects and thus render significant results more reliable. The experiment comprised eight trial nights, with three "days off" between each trial night. Courtship encounters were staged by placing male-female pairs into individual courtship boxes at dusk. Delivery of male courtship pheromones was controlled experimentally because each male lacked a mental gland. The time a pair spent in tail-straddling walk was recorded. An increase in female receptivity was operationally defined as a decrease in the time a pair spent in tail-straddling walk. A female that engaged in tail-straddling walk became a candidate for treatment delivery when the courting male turned back and physically contacted the female's snout with his chin. At this time, the first of three 5-μl drops of the appropriate treatment solution was delivered to the nares of the female. Typically, the male delivers courtship pheromones multiple times during tail-straddling walk. The experimental delivery of three drops (10 min apart) mimics natural male delivery. Females received either a pheromone (purified PRF pool from the G-75 column) solution or saline (control) solution. If a female received a treatment solution on the first trial night, the female received the opposite treatment on the following trial night. The saline (control) solution was the same vehicle used in the purification of PRF.

11. A third of the amino acid sequence of the 22-kD PRF protein was determined by direct NH_2-terminal sequencing of PRF or tryptic peptides on an Applied Biosystems 470A Gas Phase Peptide/Protein Sequencer coupled to a 120A HPLC [R. C. Feldhoff unpublished material]. We isolated mRNA from frozen mental glands, then used reverse transcriptase polymerase chain reaction (RT-PCR) for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA). The 5′ and 3′ ends of the PRF transcript were isolated with degenerate oligonucleotides (designed from NH_2-terminal amino acid sequence) and RACE-PCR techniques [M. A. Frohman, *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis Ed. (Academic Press, San Diego, 1990), chap. 4]. PRF cDNA–specific primers were then constructed to amplify a 648–base pair coding region from single-stranded cDNA. PCR products were ligated into a derivative of pZerO-2.1 (Invitrogen, Carlsbad, CA) and chemically transformed. Sequencing templates were prepared by PCR directly from five colonies with standard primers. Templates were sequenced with a dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and run on an ABI377XL Automated Sequencer. The translated cDNA sequences showed homology (see GenBank accession numbers AF181480–181483) with independent amino acid sequencing.

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17. We thank S. J. Arnold, E. Adams, P. Andolfatto, P. Feldhoff, M. Kreitman, T. Morton, C. Palmer, E. Stahl, R. Steinberg, and J.-B. Warne-Reese for technical advice, help in the field, or manuscript review. We also wish to thank the Highlands Biological Station (HBS) and R. Bruce for the use of their facilities. Supported by NSF grants IBN-9709355 (R.C.F.) and IBN-9615473 (S.J.A. and L.D.H.), NSF dissertation improvement grant 9801210 (S.M.R.), Hinds Fund University of Chicago (S.M.R.), and HBS Grants in Aid (L.D.H. and S.M.R.).

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Water Vapor Absorption in Arthropods by Accumulation of Myoinositol and Glucose

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Hydrophilic soil arthropods have been thought to respond to soil desiccation exclusively by migrating to deeper soil layers. Numerous studies have shown that their survival below 90 percent relative humidity dry weight, is limited to hours. However, little attention has been paid to physiological adaptations to more realistic desiccation regimes, such as at the permanent wilting point of plants (98.9 percent relative humidity). A water vapor absorption mechanism is described that allows a common soil collembolan, *Folsomia candida*, to remain active down to below the permanent wilting point. A reevaluation of the water physiology of this widespread and diverse animal group is required.

Soil-dwelling arthropods have several characteristics that distinguish them from surface-living forms, in particular with respect to water balance. These characteristics include small size, epidermal respiration, and high integumental permeability to water (*1*). In fact, soil-dwelling Collembola are reported as having no physiological or metabolic means of regulating water loss and generally have high integumental permeability compared with other terrestrial arthropods (*2*, *3*). These characters seem to match the soil environment, where the pore humidity is normally very close to 100% relative humidity (RH). However, during dry periods, evapotranspirative removal of water throughout the root zone may reduce pore humidity. At about 98.9% RH, at which the water potential is at −15 bars, plants normally wilt (*4*). The hemolymph osmolality of soil arthropods, such as Collembola, is usually about 300 mosm/kg (*5*), roughly corresponding to an osmotic pressure of −8 bars and 99.4% RH. Thus, at soil pore relative humidities below 99.4%, which occur frequently, Collembola and other soil arthropods must tolerate, or in some way avoid, a net efflux of water.

Folsomia candida (Willem) is able to survive more than a week at 98.2% RH (*6*). This RH corresponds to a water potential deficit of about 17 bars between the environment and the normal body fluid osmotic pressure of these animals. We investigated this phenomenon by monitoring the water content, the hemolymph osmotic pressure, and the sugar and polyol (SP) contents of *F. candida* over a 7-day period of dehydration in an atmosphere maintained at a constant 98.2% RH. The response of these animals was compared with that of animals kept in an atmosphere at 99.6% RH without free water and with animals kept at 100% RH with access to free water.

As was expected, animals held in an atmosphere at 98.2% RH dehydrated rapidly during the initial 24 hours. They lost a third of their initial total water content, equivalent to a loss of half of their osmotically active body water (OAW) (Fig. 1), and became wrinkled in appearance and inactive, responding only to strong tactile stimulation. Controls in 99.6 and 100% RH remained unchanged in the same period. This pattern was repeated in the osmotic pressure of the body fluids, where the dehydrated animals approached water potential equilibrium with their environment at −24 bars (Fig. 2). Net water efflux was therefore close to zero at this time. The initial osmotic pressure of the animals was −7.5 bars. A loss of half of their OAW should result in an osmotic pressure of about −15 bars, so these animals should still be suffering from a water potential deficit with their environment. However, the animals actively increased their osmotic pressure to halt water efflux. Part of the explanation for this active increase in osmolality can be found in the synthesis of myoinositol (Fig. 3). This polyol, undetectable in control animals, constituted 1.4% of the dry weight of animals after the first 24 hours. Under the assumption that all myoinositol was dissolved in OAW, the contribution to the osmotic pressure would be about 5 bars, leaving less than 4 bars to be explained by other osmolytes.

The animals did not become iso-osmotic but within 48 hours of desiccation reestablished their hyperosmoticity to their sur-

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