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Olfactory Responses of Male and Female Red-spotted Newts to Sex Pheromones from the Opposite Sex

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Functional characterization of sex pheromones in olfactory responses is essential for the study of chemical communications in amphibians. Using Y-maze olfactory preference tests, we have investigated the olfactory responses of male and female red-spotted newts, *Notophthalmus viridescens*, to the crude extracts of the opposite sex's genial and cloacal pheromones. Both male and female cloacal pheromone extracts caused the opposite sex to initiate olfactory responses by leaving the starting area in the Y-maze, but only subject males exposed to female cloacal pheromone extracts completed olfactory choice by entering the side arm of the Y-maze which received the pheromones. For genial pheromone extracts, only female genial pheromone extracts induced initial olfactory responses from test males. Neither male nor female genial pheromone extracts made the opposite sex complete olfactory choice. Pre-exposure of test females to male pheromone extracts increased the likelihood of initial olfactory responses. The latency for initial olfactory responses of test females that were previously exposed to male genial extracts was significantly shorter than that of control females.

To maximize reproductive fitness, successful mating is crucial (Clutton-Brock and Vincent, 1991). During mating, to detect, locate, and select potential mates, animals generally combine information from several different sensory systems such as visual, auditory, and olfactory. Behavioral functions of chemical cues in mating have received relatively little attention (Houck, 1998), although olfactory cues play an important role in the mating of amphibians (Halliday, 1990), where long-distance visual cues are unreliable, for instance in aquatic habitats (Dodson et al., 1994).

The mating behavior of the red-spotted newt has been intensively studied in the laboratory as well as in the field (Arnold, 1977; Verrell, 1982; Massey, 1988). When male newts encounter females, they exhibit either 'hula' or 'amplexus' courtship displays. When a male encounters a sexually response female that usually stays close to the male, he will attempt to directly transfer a spermatophore via 'hula' display (Arnold, 1977), a series of lateral undulations of the body, which begin in the pectoral region and pass down to the end of the tail. The display lasts about four min. When a female shows a low level of sexual responses by moving away from a male, the male will try to amplexus the female by clasping the female's neck with his hind limbs. Males also use this amplexus courtship mode when male-male competition is relatively high in the courting area (Verrell, 1983). Amplexus courtship lasts for one to three hours and is likely to reduce the possibility of losing females to rival males. During the amplexus, males rub openings of the genial pheromone glands on their cheeks against the female's nostrils to increase female sexual responses. Upon the completion of the courtship display, males deposit a spermatophore (sperm package) on the substrate while leading females ahead to the spermatophore. A successful mating is achieved when a female picks up the spermatophore with the lips of her cloaca. In laboratory experiments, amplexus courtship mode has a higher probability of successful mating (60%) than 'hula' mode (30%; Verrell, 1982). In the field, hula courtship is rare (Massey, 1988).

To find potential mates and to increase sexual responses, red-spotted newts use chemical cues such as sex pheromones. Males can detect and locate females using female sex pheromones (Dawley, 1984). Males discriminate between small and large females also by using female sex pheromones (Verrell, 1985). Both male and female newts each have two pheromone-releasing glands, the cloacal and genial glands. Genial pheromone glands have openings on each cheek behind the eyes. Females have about a third as

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many openings as do males (Pool and Dent, 1977a). Cloacal glands are a part of the accessory glands of the urogenital apparatus and anatomical and histological features have been shown in detail (Dent, 1970). Pheromones from male genial glands have been known to increase female sexual responses (Arnold, 1977; Verrell, 1982; 1988). Females exposed to male genial glands during amplexus become quiescent and more readily follow a courting male during the deposition of spermatophores (Rogoff, 1927). Such increased female sexual responses could result in increased mating success of the courting male (Verrell, 1988). Detailed functions of each pheromone in olfactory responses is still lacking although those could further elucidate our understanding about the function of sex pheromones in amphibian reproduction.

In this study, we have investigated olfactory responses of male and female red-spotted newts, *Notophthalmus viridescens*, to the crude pheromone extracts from cloacal and genial glands of the opposite sex using Y-maze olfactory preference tests. We also studied whether pre-exposure to male cloacal and genial pheromone extracts changes the likelihood of initial olfactory responses of test females to male odors. We found that different pheromones may induce different olfactory responses.

Materials and Methods

Animal husbandry

Red-spotted newts in breeding condition were purchased from a local supplier (Sullivan Co., Nashville, TN) and kept as previously reported (Park and Propper, 2001). Upon arrival in the laboratory, male and female newts were kept in separate aquaria (80 × 40 × 50 cm) containing approximately 40 gallons of aged tap water at a density of no more than 20 individuals per tank. We placed pieces of pottery at the bottom of the aquarium and submerged several paper towels to provide hiding places. They were kept on a photoperiod schedule of 16 h light: 8 h dark (lights on at 0600 h), to mimic the spring breeding season. The water temperature ranged 10-12℃. We fed live black worms and half of the water, approximately 20 gallons, was regularly changed by siphoning once a week.

Methods of Y-maze olfactory preference test

To investigate olfactory responses of male and female newts to sex pheromone extracts from the opposite sex, we executed olfactory preference tests using a standard Y-maze olfactometer (Park and Propper, 2001). Two arms of the Y-maze (4.5 × 22 × 5 cm) continuously received aged tap water at a flow rate of 60 mL/min from a single reservoir (55 × 25 × 50 cm) containing 500 mL of aged tap water. Influx of the water was controlled by flow meters (GJ502, Gilmont Ins., Barrington, IL). At this flow rate, water flow from the two arms remained separately to the drain at the end of the Y-maze. Test newts were placed at the end of the maze behind a start gate for 3 min. After 3 min, the gate was slowly raised. Each newt was allowed 17 min to make an olfactory preference choice. After each trial, we washed the Y-maze using aged tap water. All test newts were selected arbitrarily from a pool of 50 individuals. No individuals were used more than once a day.

In this study, an initial olfactory response was considered when test newts initially moved toward odor sources by leaving the starting area at the end of the Y-maze. The latency for the initial olfactory response was defined as the time taken for test newts to leave the staring area after a 3-min adapting period. An olfactory choice was made when test newts have traveled more than 1/2 length of a given arm.

Olfactory responses of males and females to the sex pheromone extracts from the opposite sex

To extract crude pheromones from male and female cloacal and genial pheromonal glands, four males and five females were separately anesthetized in 4% ether. Cloacal and genial glands were excised, and incubated separately in 1 mL of 0.8 mM acetylcholine chloride (AchCl; pH 8.4) in distilled water for 30 min (Pool and Dent, 1977b; Rollmann et al., 1999; Park and Propper, 2002). This incubation caused the pheromone glands to release pheromones into the solution (Pool and Dent, 1977b). In control tests, 0.8 mM AchCl by itself did not affect olfactory preferences of test newts (Park and Propper, 2002). The glands were discarded and the resulting supernatant was centrifuged at 10,000 × g for 10 min. The supernatant was frozen at -80℃ and thawed before use.

We dissolved each cloacal and genial pheromone extract from four males and five females in 300 mL of aged tap water and used it in the olfactory preference tests. During the test, we delivered the extract solution into the one side arm of Y-maze, randomly chosen by tossing a coin, at a flow rate of 1.35 mL/min (for male cloacal and genial pheromone extracts) or 0.15 mL/min (for female cloacal and genial pheromone extracts) using an EP-1 Econo peristaltic pump (Bio-Rad, Hercules, CA). The other side arm of Y-maze received aged tap water alone. Thus, each test newt had an olfactory choice between either cloacal or genial pheromone extracts from the opposite sex and aged tap water. We selected different concentrations of male and female pheromone extracts because test females were only attracted to odors from more than 3 males, while one small female was enough to attract conspecific males (Dawley, 1984; Park and Propper, 2001). We measured the latency of initial olfactory responses and arm preference of each test newt. The data were analyzed using a t-test (applied when the data passed the normality test, Shapiro-Wilk, at $P > 0.05$) and a binomial

test, respectively (Sokal and Rohlf, 1981).

Pre-exposure to male sex pheromone extracts increases the likelihood of initial olfactory responses of females

To determine whether pre-exposure of test females to male cloacal and genial pheromone extracts increases the likelihood of initial olfactory responses, female newts were pre-exposed to control, male cloacal, or genial pheromone extracts and then were given a choice between odors from three male newts and aged-tap water. All test female newts used in this study were received a personal spot code to facilitate individual identification. Spot codes were assigned by visually dividing the animal down the spine and perpendicular to the spine at the fore and hind limbs. The number of red spots in each of these six segments was used together to create its identification code.

For the experiments, we arbitrary selected 12 female newts from a pool of 40 females and randomly allocated them into either a control or cloacal-exposure group by drawing a ticket from a hidden box on the first day of the experiment. Each test female newt from either group was separately exposed to a control solution or male cloacal extracts (see below) and had an olfactory choice between odors from three males and tap water in random order. On the second day, each test female newt was allocated into the group in which she did not serve on the first day of the experiment. For example, if one test female served as a control group on the first day, the female was allocated into a cloacal-exposure group on the second day. After that, each test female was exposed to one of the odors and had an olfactory choice in random order. Thus, each of 12 test females served as both control and cloacal-exposure groups in two consecutive days. Using the same protocol, we also determined whether pre-exposure to male genial pheromone extracts affects olfactory responses of test females to male odors.

For the exposure, we kept each test female newt in a glass Petri dish (diameter 12 cm) containing 30 mL of male pheromone extracts (prepared by dissolving 1 mL of either cloacal or genial pheromone extracts, collected using the same method described above, in 300 mL of aged tap water) for 1 h. To assure that females were exposed to male pheromone extracts, we confirmed that each female's nostrils were submerged in the extract solutions during that time. Females generally became quiescent when exposed to male pheromone extracts and hardly lifted up their heads from the solution. Control test females were exposed to 30 mL of control solution (prepared by dissolving 1 mL of 0.8 mM AchCl solution in 300 mL of aged tap-water) for 1 h in the Petri dish. After that, control females and females pre-exposed to male pheromone extracts were given an olfactory choice between odors from three males and aged tap water in random order. We measured the latency for initial olfactory responses of each test female and recorded arm preference of the female.

To analyze the rate of initial olfactory responses between control and pre-exposed test females, we excluded test females that did not show initial olfactory responses in both control and pre-exposure experiments. Because of small sample size of olfactory choice, we did not analyze the data. In analyses of the latency for initial olfactory responses, we included only test females that responded to male odors as both control and pre-exposure group. A t-test (applied when the data passed the normality test, Shapiro-Wilk, at $P > 0.05$) and a chi-square test were used to analyze differences in the latency for initial olfactory responses and the rate of initial olfactory responses between control and pre-exposed test females, respectively (Sokal and Rohlf, 1981).

Results

We have investigated olfactory responses of male and female red-spotted newts to each cloacal and genial pheromone extract of the opposite sex using Y-maze olfactory preference tests. We found that female and male cloacal extracts and female genial extracts induced initial olfactory responses from the opposite sex, but only female cloacal extracts made test males complete olfactory choice. More test females that were previously exposed to male sex pheromone extracts initiated olfactory responses to male odors than control females. These findings are described in detail below.

Olfactory responses of test males to the female sex pheromone extracts

Twenty one out of 23 test males, when exposed to female cloacal extracts against tap water, and 9 out of

Table 1. Olfactory responses of male and female newts to the opposite sex's pheromone extracts

Experiment (No. of trials)	Subjects tested	Odor sources	Number of times chosen	One-tailed binomial *P*
I (23)	Male	Female cloacal pheromone extracts	18	0.0005
		Plain tap water	3	
II (11)		Female genial pheromone extracts	4	0.500
		Plain tap water	4	
III (35)	Female	Male cloacal pheromone extracts	16	0.163
		Plain tap water	10	
IV (18)		Male genial pheromone extracts	3	0.500
		Plain tap water	2	

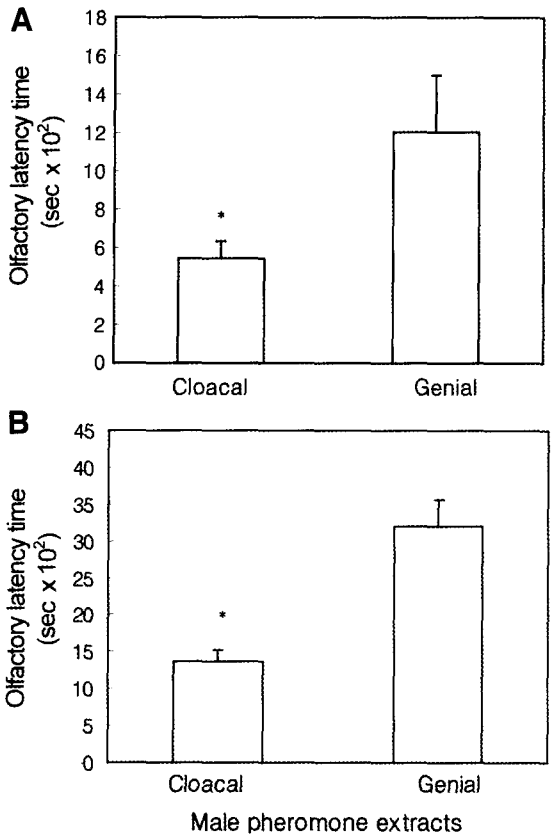

Fig. 1. Latency for initial olfactory responses of male and female newts to the opposite sex's pheromone extracts. The latency for the initial olfactory responses of both test male (A) and female (B) newts was shorter when exposed to the opposite sex's cloacal pheromone extracts than to the genial pheromone extracts. Vertical bars represent standard errors of mean. * $P < 0.01$.

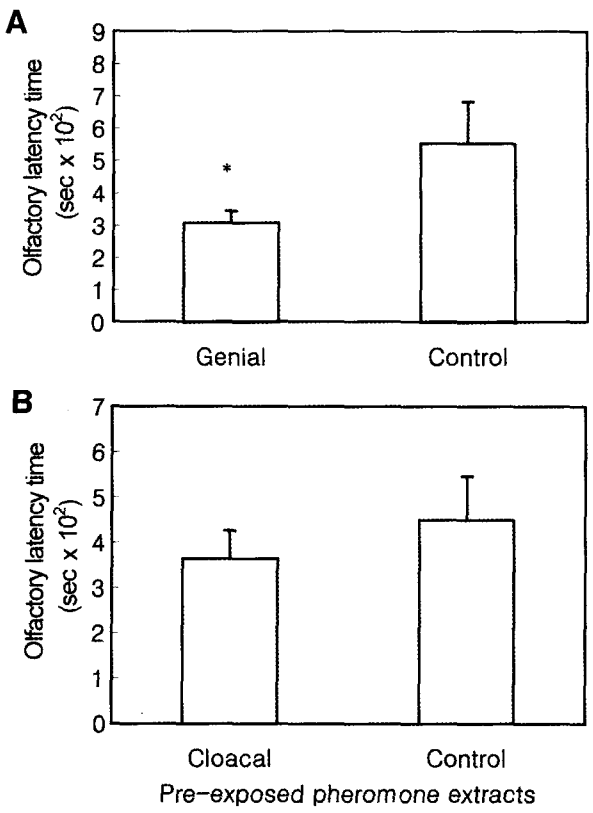

Fig. 2. Latency for initial olfactory responses of test female newts to odors from three males against tap water after pre-exposing to male genial (A) and cloacal (B) pheromone extracts. Male genial pheromone extracts significantly decreased the latency for the initial olfactory responses of test females (A), but male cloacal pheromone extracts did not (B). Vertical bars represent standard errors of mean. * $P < 0.05$.

11 test males, when exposed to female genial extracts against tap water, initiated olfactory responses by leaving the starting area in the Y-maze (Table 1). The rate of the initial olfactory responses to different female pheromone extracts was not significantly different (Chi-square test, $P = 0.36$). The latency for initial olfactory responses of test males was significantly shorter to female cloacal extracts against tap water than to genial extracts against tap water (Fig. 1A; $t = 2.77$, $df = 29$, $P = 0.010$). Test males successfully completed olfactory choice when female cloacal pheromone extracts were introduced into a side arm of Y-maze against tap water (Table 1I), but they could not complete the choice when exposed to female genial extracts against tap water (Table 1II). Interestingly, 4 out of 11 test males displayed 'hula' courtship displays in the main stem of Y-maze when female genial pheromone extracts were presented.

Olfactory responses of test females to the male sex pheromone extracts

Significantly more females showed initial olfactory response by leaving the starting area in the Y-maze when exposed to male cloacal pheromone extracts against tap water than when exposed to male genial pheromone extracts against tap water (26 of 35 test females to cloacal extracts and 5 of 18 test females to male genial pheromone extracts, Chi-square = 8.76, $P = 0.003$). The latency for initial olfactory responses of test females was significantly shorter when they were exposed to male cloacal pheromone extracts against tap water than to male genial pheromone extracts against tap water (Fig. 1B; $t = 5.24$, $df = 40$, $P < 0.001$). However, test females did not successfully complete olfactory choice to either male cloacal or genial pheromone extracts against tap water (Table 1III, IV).

Pre-exposure to male sex pheromone extracts increases the likelihood of initial olfactory responses of females

More test females that were previously exposed to male sex pheromone extracts initiated olfactory responses when exposed to male odors against tap water than control test females (16 of 17 pre-exposed test females and 9 of 17 control test females, Chi-square = 5.44, $P = 0.02$). The latency for initial olfactory respon-

ses of test females that were previously exposed to male genial pheromone extracts was significantly shorter than that of control test females (Fig. 2A; t = 2.248, df = 11, $P = 0.046$), but the latency of test females previously exposed to male cloacal pheromone extracts was not shorter than that of controls (Fig. 2B; t-test, $P = 0.457$).

Discussion

To investigate olfactory responses of male and female red-spotted newts to the opposite sex's pheromones, we conducted a series of Y-maze olfactory preference tests using crude pheromone extracts from male and female cloacal and genial glands. We found that male newts initiated olfactory responses to both female genial and cloacal pheromone extracts, but only female cloacal pheromone extracts made test males complete olfactory choice. Females also initiated olfactory responses to the exposure of male cloacal pheromone extracts, but neither male cloacal nor genial pheromone extracts made test females complete olfactory choice. Females previously exposed to male pheromone extracts were more likely to respond to male odors than control females. These results suggest that different pheromones may have different functions in olfactory responses.

Female cloacal pheromones may function to attract conspecific males. During our Y-maze olfactory preference tests, most subject males initiated olfactory responses when exposed to female cloacal pheromone extracts against tap water by leaving the starting area in the Y-maze. Test males successfully completed olfactory choice. In the early stage of mating, male newts generally approach females from a distance (Verrell, 1982). During this initial orientation, female cloacal pheromones may function to lead courting males toward her, although male newts are also likely to use visual cues (Verrell, 1982).

Female genial pheromones may function to induce male sexual responses. Previously, function of female genial pheromones in olfactory responses has not been reported. In our study, most test males also initiated olfactory responses when faced with female genial pheromone extracts against tap water, but they did not complete olfactory choice, suggesting that although female genial pheromones stimulate male's olfactory responses, it does not attract males. Interestingly, about half of test males (4 out of 11 individuals) showed the courtship display, known as 'hula', when exposed to female genial pheromones. This observation implies that female genial pheromones may increase male sexual responses. Considering that the number of openings of female genial glands on the cheeks is individually variable within a population (Pool and Dent, 1977a), females who have more genial glands may more readily induce male sexual responses. In the situation where males' initial sexual responses are critical for a successful mating, the function of female genial pheromone could be essential. However, in this species since the operational sex ratio (OSR: the ratio of sexually active males to responsive females at any one time; Emlen and Oring, 1977) is generally male-biased, ranging from one to 4.7 (Hurlbert, 1969; Gill, 1978) and males generally initiate courtship displays, practical function of female genial pheromones to induce male sexual responses remains to be tested.

Most test females initiated olfactory response when exposed to male cloacal pheromone extracts against tap water, but did not initiate when male genial pheromone extracts were presented against tap water. The latency for initial olfactory response was significantly shorter to male cloacal pheromone extracts than to genial pheromone extracts. Most test females could not successfully complete olfactory choice to either male cloacal or genial pheromone extracts introduced against tap water. These results demonstrate that male sex pheromones do not function to attract females, although the male pheromones could stimulate female olfactory responses (Verrell, 1988).

Initial olfactory responses of females to male cloacal pheromones may play important roles in mating. In the early stages of the mating, when a male approaches a female, the male will exhibits 'hula' display if the female stays close or approaches the male (Verrell, 1982). Females' responses are dependent on the level of sexual responsiveness (Verrell, 1982). Our result demonstrates that male cloacal pheromones may lead females to approach the male via stimulating the females' olfactory sensing. In addition, females' olfactory responses to male cloacal pheromones could be functional at the late stages of mating. In this species, successful mating is achieved when courting females pick up a spermatophore deposited by courting males. Courting males will deposit spermatophores only when courting females follow a male's movement closely and nudge the male's cloacal region (Arnold, 1977). In this step, to guide herself toward the male cloacal region, a courting female may use chemical cues from the male cloacal glands as well as visual cues (Arnold and Houck, 1982).

Male genial pheromones may increase female olfactory responses. More test females initiated their olfactory responses when they were previously exposed to male pheromone extracts than when exposed to control solution. Particularly, pre-exposure to genial pheromone extracts significantly reduced the latency of initial olfactory responses of test females. Considering that females who have high sexual responsiveness stay close to and often approach to courting males (Arnold, 1977; Verrell, 1982), our results suggest that male genial pheromones may increase female sexual responsiveness. The function of male genial pheromones in increasing female sexual responsiveness has been previously reported. Holding a female to the openings of male genial glands made her readily follow courting

males (Rogoff, 1927). Males with plugged genial glands could not cause females to be quiescent (reviewed in Verrell, 1988).

Red-spotted newts may use both sex attractants and courtship pheromones during their mating. Sex attractant is defined as the pheromone that attracts a potential mate to the individual emitting the chemosignals (Houck, 1998). Amphibian proteinaceous sex attractant, approximately 1 kDa protein, was previously purified and behaviorally verified in male red-bellied newts, *Cynops pyrrhogaster* (Kikuyama et al., 1995). Females exposed to 10 ng of the protein were strongly attracted to the source. In our study, cloacal pheromones of female red-spotted newts may fall into this category because males exposed to this pheromone completed olfactory choice by entering the side arm which contained the pheromones.

Male and female genial pheromones may fall into the category of courtship pheromones. Courtship pheromones are defined as chemosignals delivered to a mating partner during the course of courtship interactions (Arnold and Houck, 1982; Houck, 1986) and often function to increase sexual responsiveness of the partner. Recently, amphibian courtship pheromones were purified from the male terrestrial salamander, *Plethodon jordani*, (Rollman et al., 1999). In behavioral studies, approximately 20 kDa protein from male cheek glands increased female sexual responses (Rollman et al., 1999). In our study, female genial pheromones induced 'hula' display from test males and male genial pheromones increased female olfactory responses, suggesting that these pheromones may increase sexual responses of the opposite sex.

In conclusion, our results suggest that different types of amphibian sex pheromones may induce different olfactory responses and may differentially function in a specific stage of mating. To date, little is known about the features of chemical communications in Korean salamanders although previous studies have suggested that Korean salamanders may also use pheromones during their mating (Park et al., 1996; Park and SR Park, 2000). This study should stimulate further studies about pheromonal communications in Korean salamanders.

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