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Male *Hynobius leechii* (Amphibia: Hynobiidae) Discriminate Female Reproductive States Based on Chemical Cues

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Abstract: A series of no-choice olfactory response tests using water pre-conditioned with females, with intact and surgically removed ventral glands, at various reproductive states were conducted to determine whether male *Hynobius leechii* discriminates among females in different reproductive states based on chemical cues. Similarly, ventral gland extracts were tested, and ventral glands were examined histologically. Males' responses to putative odors of females in four (ovulating, ovulated, ovipositing, and oviposited) reproductive states were independently measured by: i) the latency time to initiate male behavioral response, ii) the arrival time at a fixed point of putative odor source, and iii) the staying time close to the odor point source. Male salamanders showed significant olfactory responses to recently ovulated and ovipositing female odors by quickly arriving at odor sources and staying longer at the origin of the source, but the olfactory responses to the earlier staged ovulating females and the later stage of already oviposited females were not different from controls. Olfactory responses of test males to water preconditioned by intact females or females with ventral glands excised were not different. In addition, ventral gland extracts did not induce significant olfactory responses of test males although the lumens of alveoli in ventral glands of oviposited females were smaller than those of ovulated females. These results indicate that male *H. leechii* recognizes ovulated and ovipositing females based on chemical cues released but not from the ventral glands.

Key words: Salamander, *Hynobius leechii*, external fertilization, olfactory, ventral gland

More than 90% of the salamander species internally fertilize eggs. Only species in the families Cryptobranchidae, Sirenidae, and Hynobidae display external fertilization (Salthe, 1967). Since most newts and salamanders use chemical signals during their mating regardless of the mode of fertilization (Verrell, 1985; Hasumi, 1996), comparative studies in the use of the chemical cues among species with internal and external fertilizing modes could give valuable insights on the evolution of chemical signals in the group. Although numerous studies on internally fertilizing species have been performed there is little information on the use of chemical signals in mating behavior in externally fertilizing forms.

In order to increase mating success, *Hynobius leechii* males with a territory need to attract females who are close to ovipositing into their territory, while males without a territory should adopt a strategy of closely following those females in order to take over deposited egg sacs and possibly fertilize eggs (Tanaka, 1989). For both groups of *Hynobius* males, knowledge of a female's reproductive state such as readiness to oviposit should increase male mating success by concentrating females with a higher mating success probability. Since visual cues for nocturnal activities in underwater breeding habitats are limited in *Hynobius*, males may discriminate female reproductive states based on chemical cues.

In this study, we conducted a series of no-choice olfactory response tests using female conditioned waters and ventral gland extracts combined with a histological study of ventral glands to determine 1) whether male *H. leechii* discriminate female reproductive states based on chemical cues and 2) if they do, whether female ventral glands release odors inducing male olfactory behaviors.

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MATERIALS AND METHODS

Animal collection and housing

Salamanders used in this study were collected from mountain streams located at Cheongwoon-myeon, Yangpyeong-gun, Kyeongki-province (37° 31' 56.6" N, 127° 42' 16.52" E) and at Gaduck-myeon, Cheongwon-gun, Chungbuk-province (36° 33' 09.8" N, 127° 35' 18.0" E), South Korea. Thirteen females and 104 males and 11 females and 32 males were collected between March 10 and 13, 2005 at each locality, respectively. Upon arrival at the laboratory, each individual was sexed based on the degree of cloacal gland swelling and all males were toe-clipped for individual identification (Donnelly et al., 1994).

Males and females were kept in separate aquaria (for males, 100 cm long, 74 cm wide, 35 cm high; for females, 33 cm long, 20 cm wide, 25 cm high) containing approximately 50 or 25 liters of aged tap water at a density of no more than 50 males or 10 females per tank. Water was circulated among the male aquaria by a pump (PB-38E-D, Hanil Co.). Half of the water in each aquarium was changed weekly. Water temperature of the aquaria was kept between 8-10°C by a cooler (DHI-150, Daeho) and the photoperiod followed a local photoperiod of approximately 12D : 12L. Wet paper towels on rocks that extended above the water line and pieces of broken pottery were provided for hiding places. Salamanders were fed with freshwater amphipod *Gammarus* and chopped earth worms *ad libitum*. All males used in this study were in full breeding condition, as evidenced by swollen cloacae and fully developed tailfins (Park and Park, 2000).

All experimental procedures followed the Guidelines for use of live amphibians and reptiles in filed and laboratory research advised by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists, 2004.

Information about putative odor-donor females

A series of no-choice olfactory response tests were conducted between March 16 and 24, 2005. For the tests, we collected preconditioned waters as stimuli from each of three ovulating, three ovulated, three ovipositing, and three oviposited females from both intact and ventral gland-excised groups (Table 1). The ovulating females were those who had at least some eggs in the middle of the oviducts. The ovulated females were those who had eggs only within the dilatable ovisacs (Hasumi, 1996). In Hasumi's study (1996), *H. nigrescens* females started ovulation 16hrs after a single injection of 600IU human chorionic gonadotropin (HCG) and completed ovulation 50 hrs after the injection, resulting in all eggs located out of the oviducts and in the ovisacs. We could easily determine the location of eggs both in the oviduct and ovisacs through the slightly translucent abdomen. Only those females confirmed later by dissection to be in the assumed condition were used in the analysis (Table 1). The ovipositing females were those who oviposited eggs within 10 hrs. The oviposited females were those who laid eggs one day before. For the oviposited females, we re-used the ovipositing females which were used one day earlier as ovipositing females during their oviposition in order to save the number of females used in the study (Table 1). Since those ovipositing and oviposited groups were not independent, we did not directly compared responses between ovipositing and oviposited female groups. Also, one ovipositing female in the ventral gland-excised group did not oviposite on the day of odor collection. So, we excluded this female as an odor-donor for ovipositing odors, but the female was used as an oviposited female (Table 1).

Table 1. A summary of the number of odor-donor females used to collect odor-conditioned water. Each one odor-donor female both in the intact ovulated female and excised ovipositing female groups was excluded for wrong decision of reproductive state and for not ovipositing on the odor collecting day, respectively. For the oviposited odor-donor females (*), we re-used ovipositing females from both intact and excised groups

Odor-donor groups	Female condition	
	Intact females	Ventral gland excised females
Ovulating	3	3
Ovulated	3(-1)	3
Ovipositing	3	3(-1)
Oviposited	3*	3*

Ventral gland excision of putative odor-donor females

Hynobius salamanders have ventral glands within their cloacal walls exposed externally so that removing the ventral glands is relatively easy (Sever, 1991). To excise the ventral glands from external cloacal walls, three ovulating and six ovulated females were first anaesthetized in 0.1% tricaine methanesulfonate (MS222) for 8-10 min. Three out of six ovulated females were used as ovipositing females after ventral gland excision (Table 1). We excised external regions of the cloacae using a dissecting scissor and a stereomicroscope. We sterilized surgery area using Phobidon (Dongin Medicine Co.) to prevent possible fungal and/or viral infections similar to previous studies (Sever, 1991; Park et al., 2001). We assured that all ventral glandular material was removed from the external cloacal parts during surgery by histological examination of the excised tissues. Each sample of excised cloacal tissue was separately fixed in 10% neutral formaldehyde for later histological study. After surgery, we covered females with wet paper towels and placed them in an individual box (15 cm long, 10 cm wide, 5 cm high) at 4°C filled with aged tap water to a 1 cm depth. After females recovered from

anaesthesia usually within 10 min, we kept them in the box at room temperature for surgical-recovery for a day. In our previous study (Rohr et al., 2005), such a procedure often prevented bleeding and induced faster recovery. In our experiments, most females did not bleed and we couldn't find any abnormalities in behaviors such as a change in moving activity. Since in our previous study (Rohr et al., 2005) we did not detect any alarm responses when test males were exposed to prepared cloacal extracts which also contains cloacal external skin extracts, we did not include sham surgery controls in this study.

Odor collection from putative odor-donor females

Putative odor-containing waters from ovipositing females were first collected. We induced oviposition in three ventral gland-excised and three ventral gland intact ovulated females by allowing them to mate with two males each. An aquarium (33 cm long, 20 cm wide, 25 cm high) for mating was set up by covering the bottom with a layer of sand of about 3cm and including a "V" shaped twig (33 cm long and 1 cm diameter) as an egg-attaching site. Approximately half of the twig was 1-2 cm above the water surface and the other half was submerged. Tanks were filled with aged tap water to about 4.5 cm in depth. Each mating proceeded under less than 0.1 lux of light measured by an illumination meter (YL 102, UINS). We confirmed occurrence of oviposition on a monitor (Samsung, Co.) connected to a low-light waterproof B/W camera (Model: 10IR LED). We took the female from the mating tank within 10 min after oviposition, and placed her in fresh tap water for 3 min to wash out possible male odors attached to the female's skin. The female was then moved to an odor-collecting box (15 cm long, 10 cm wide, 5 cm high) which contained 25 ml of aged tap water. The next morning (10 hrs after putting the female in the box), we collected putative odor-containing water (hereafter called putative-odors). Fifteen ml of the 25 ml putative-odor solution was reserved at 4°C and used within 4 days. The remaining 10 ml was immediately frozen and kept at 20°C and used within 8 days. All putative odors induced olfactory response from test males. To remove possible individual variations, we mixed equal amounts of putative odors from two or three females before using them in no-choice olfactory tests.

Odors from ovulating, ovulated, and oviposited females were collected on a single day. Each female was placed in an odor-collecting box which contained 25 ml of aged tap water at 2200 h. Water was collected at 0800 h. the next morning. No fecal contamination occurred. Collected putative odors were treated as described above. Control odors were obtained from water that was kept for 10 hrs in the same odor-collecting box without having placed any females. After collecting putative odors, all females were sacrificed with an over dose of MS222 and dissected to confirm their reproductive state. The external cloacal tissues of intact females were excised and fixed in 10% neutral formaldehyde for histological study of ventral glands.

Conducting no-choice olfactory response test with the collected odors from intact and ventral gland excised females

To investigate responses of male salamanders to female odors, we performed no-choice olfactory response tests using a rectangular box constructed of Plexiglas with a distance scale on the wall of the main body (58 cm long, 4 cm wide, 5 cm high). The box was continuously infused with aged tap water by a peristaltic pump (Fisher) at a flow rate of 100 ml/min from a 3 liters reservoir. Test males could sample the odors while staying behind a perforated starting gate. After each trial, the box was washed using aged tap water. A test male began the trial in a start location partitioned off (8 cm long, 4 cm wide, 5 cm high) at the bottom of the one side of the box. A cotton ball (0.25 g) soaked with 1.5 ml of putative odor solution was placed behind a perforated plastic mesh barrier at the other end of the box. Controls were prepared from control solutions in a similar manner.

After a 3-min adaptation period, the starting gate was slowly raised to allow the test males to enter the main body of the testing box for 5 min. Three time parameters were measured to evaluate olfactory responses to five different odors. (1) The latency time was defined as the time required for the tip of the snout of a test male to pass a 5 cm line from the starting gate. An animal that failed to cross the line within 5 min was removed from the box, and the data for that animal were not included in the analysis. (2) The arriving time was defined as the time required for the tip of the snout of a test male to pass a 40 cm line from the 5 cm latency line. An animal traveled between the 5 cm and the 40 cm lines within 5 min received 300 sec. - the latency time, because we believed short arriving time reflects high attractiveness of the odors in the no-choice olfactory tests (Livermore and Laing, 1998). (3) The staying time was defined as the time the male's head stayed within 40-45 cm of the treated cottons. The staying time of an animal that did not cross the 40 cm line after passing the latency line was 0 sec.

The odors were randomly presented in the no-choice olfactory trials by tossing paper sheets which have numbers on it. We tested each male only once in a day. Each male was tested with only one or a few of the different odors but no males were exposed to the same odors twice. All experiments were conducted under less than 0.1 lux light between 1900 and 0100 hrs. Males' olfactory responses within the no-choice test box were observed and recorded on the screen of a monitor. Detailed experimental sample sizes for each condition in the experiment are presented in Table 2.

Table 2. A summary of the number of test males tried, initiated, and completed olfactory responses when exposed to different odors

Odor donors	Donor conditions					Chi-square test (P)	
	Control	Ovulating female	Ovulated female	Ovipositing female	Oviposited female		
Intact	9 completed out of 16 initiated (total 19 trials)	13/14 (19)	13/13 (17)	16/17 (20)	12/15 (16)	0.20	1.00
Gland excised		8/14 (16)	12/13 (16)	13/14 (17)	11/13 (14)	0.09	
Fisher-exact test (P)		0.78	1.00	1.00	1.00		

The number of completed and initiated (the statistical results are not shown) males were not different across different reproductive states regardless of presence or absence of the ventral glands (Chi-square test, $P > 0.05$ for all cases). In addition, within each different reproductive state group, presence or absence of the ventral glands did not affect the number of males completed and initiated (the statistical results are not shown) olfactory responses (Fisher-exact test, $P > 0.05$ for all cases).

We first determined whether test males showed different olfactory responses to odors of females in different reproductive states. For this analysis, we used data only from the controls and intact female trials. We used the Kruskal-Wallis test to determine if odor-containing waters collected from females who were in different reproductive states induce different odor responses from test males because our data did not meet the normality assumption of parametric statistical tests (Kolmogorov-Smirnov test, $p < 0.05$). For the *post-hoc* test, we followed the Siegel and Castellan (1988) method. Second, to determine whether ventral gland excision affected olfactory responses of test males in each reproductive state group, we used the Mann-Whitney U-test with a Bonferoni correction because the data of intact females were already used once in the analysis and the data did not meet the normality assumption of parametric statistical tests (Kolmogorov-Smirnov test, $P < 0.05$). All data in this study were analyzed using SPSS statistical software (v.11.5; SPSS 2002).

Preparing ventral gland extracts and conducting no-choice olfactory response test with the extracts

To determine whether the ventral gland itself is the site of releasing male attractants, we performed the no-choice olfactory tests using ventral gland extracts. To extract substances from the ventral glands, we anesthetized two ovulating and two ovulated females with 4% ether. External cloacal parts were excised, and incubated in 0.65 ml of 0.8 mM acetylcholine chloride (AchCl; pH 8.4) in distilled water for 30 min. This incubation has been shown to cause the cloacal and mental glands to release male pheromones in newts and terrestrial salamanders (Pool and Dent, 1977; Rollmann et al., 1999). As a control, 0.8 mM AchCl solution dissolved in distilled water was used. The cloacal tissues were discarded and the crude extracts were centrifuged at 10,000 × g for 10 min. The supernatant was used in the no-choice olfactory response tests after diluting 200 μl of extracts in 20 ml of aged tape water. Similar concentration of cloacal extracts in a newt was successful in inducing olfactory responses in a previous study (Rohr et al., 2005).

No-choice olfactory tests were performed as described above. We measured the latency time, arriving time, and staying time of test males when exposed controls (n = 14) and ventral gland extracts (n = 13). We used a conservative Mann-Whiteny U-test to analyze the data.

Histological study of the ventral glands

We compared the size of ventral glands of 17 cloacal tissue samples from each of six ovulating, five ovulated, and six oviposited females. The left and right side of each cloacal tissue was paraffin-embedded, sectioned at 15 μm, stained following the Harris hematoxyline-eosin method (Presnell and Schreibman, 1997), and observed under a light microscope. We measured the longest diameter of all alveoli and their lumens within each 15th section up to 0.01 um. We calculated average diameter of the alveoli and lumens of ventral glands for each individual and then analyzed the data using Kruskal-Wallis test and followed the Siegel and Castellan (1988) method for the *post-hoc* test.

RESULTS

In response to female odors, the number of test males initiated (crossed only 5 cm line) and completed (crossed the 40 cm line) was not different among different reproductive state groups, regardless of presence and absence of the ventral glands (Chi-square test, $P > 0.05$ for the cases, Table 2). Within each reproductive state group, the number of initiated and completed males was not different between intact and ventral gland-excised groups (Fisher-exact test, $P > 0.05$ for the cases, Table 2). In the Table 2, statistical results for completed test males were presented only.

Olfactory responses of test males to odors from intact females who were in different reproductive states were significantly varied in arriving time (Kruskal-Wallis test, $X^2 = 10.54$, df = 4, $P = 0.032$, Fig. 1B) and in staying time (Kruskal-Wallis test, $X^2 = 17.41$, df = 4, $P = 0.002$, Fig.

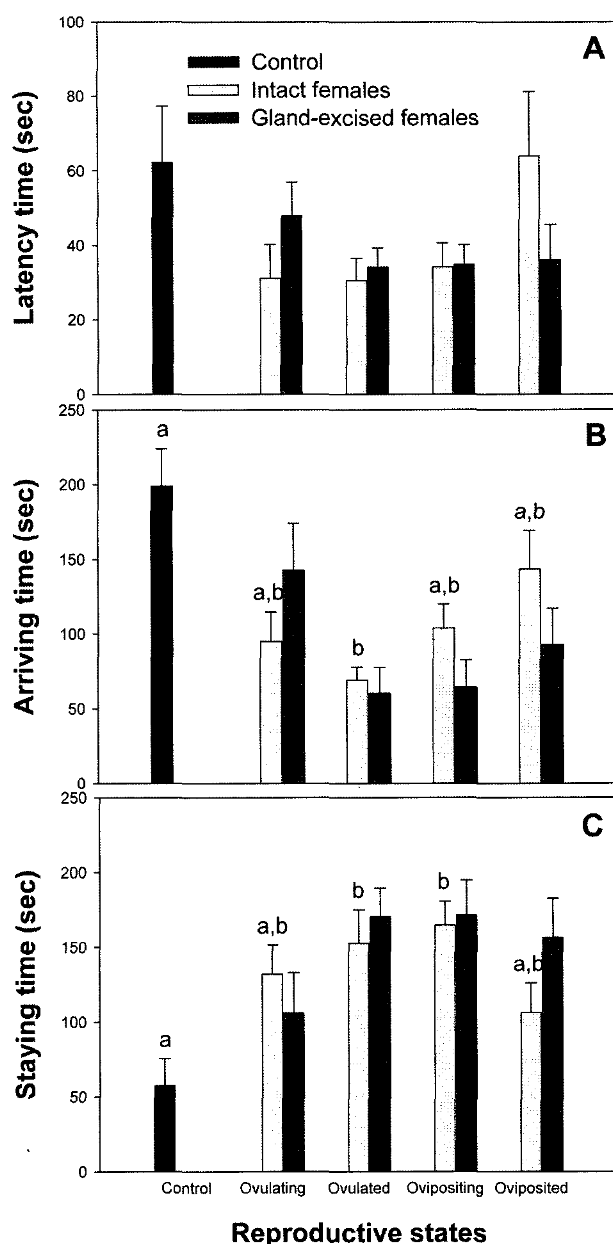

Fig. 1. No-choice olfactory response tests. In the analyses of intact and control groups, test males' responses to the odors of females who were in different reproductive states were significantly varied in arriving time (B, Kruskal-Wallis test, $P = 0.032$) and staying time (C, Kruskal-Wallis test, $P = 0.002$), but not in latency time (A, Kruskal-Wallis test, $P = 0.170$). Especially, arriving time to ovulated female odors and staying time to ovulated and ovipositing female odors were significantly different comparing to the responses to the controls (B, C; groups that do not share the same letters differed significantly from one another, *Post-hoc* test, $P < 0.05$). Within each reproductive group, none of males' responses to intact and ventral gland-excised female odors were significantly different in all measured parameters (Mann-Whitney U test, $P > 0.05$ for all cases, the statistical results are not shown in the figure). We did not compare males' responses to controls and ventral gland-excised female odors.

1C), but not in latency time (Kruskal-Wallis test, $P = 0.170$, Fig. 1A). In special, test males arrived at the 5 cm latency line earlier to ovulated female odors than to controls (*Post-hoc* test, $P < 0.05$, Fig. 1B). Also, males stayed longer close to the odor sources from ovulated and ovipositing females than to controls (*Post-hoc* test, $P < 0.05$ for both cases, Fig. 1C). Other comparisons were not significant (*Post-hoc* test, $P > 0.05$ for the cases). Olfactory responses of test males to odors from the females whose ventral glands were intact and whose ventral glands were excised were not different in the latency, arriving, and staying time within each reproductive group of ovulating, ovulated, ovipositing, and oviposited (Mann-Whitney U test, $P > 0.05$ for the cases, Fig.1A, B, C).

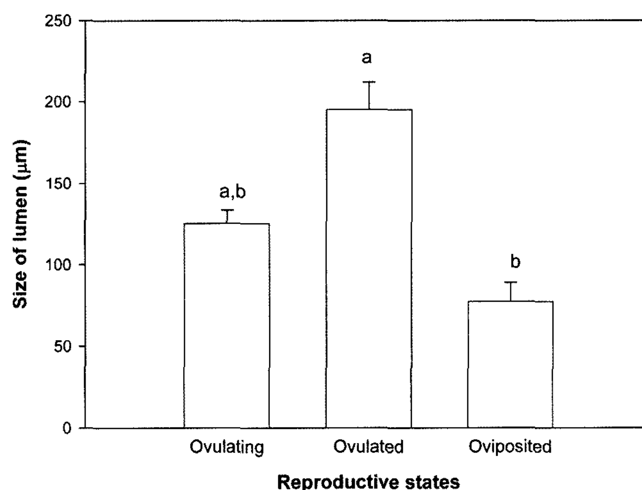

Fig. 2. The size of lumens of alveoli in the ventral glands among ovulating, ovulated, and oviposited females was significantly different (Kruskal-Wallis test, $P = 0.002$). Data were presented as mean ± SE. Groups that do not share the same letters differed significantly from one another (*Post-hoc* test, $P < 0.05$).

Olfactory responses of test males to controls and ventral gland extracts were not different in latency time (control; 57.08 ± 18.31SD, extracts; 31.43 ± 9.84SD, Mann-Whitney U-test, $P = 0.28$), arriving time (control; 161.62 ± 29.51SD, extracts; 136.43 ± 29.40SD, Mann-Whitney U-test, $P = 0.58$), and staying time (control; 72.15 ± 20.01SD, extracts; 106.43 ± 25.73SD, Mann-Whitney U-test, $P = 0.46$).

The size of alveoli of the ventral glands was not different among ovulating, ovulated, and oviposited females ($P = 0.072$), but the size of lumens of the alveoli was significantly different among those groups (Kruskal-Wallis test, $X^2 = 12.56$, df = 2, $P = 0.002$, Fig. 2). In special, the lumens of alveoli of oviposited females were significantly smaller than those of ovulated females (*Post-hoc* test, $P < 0.05$, Fig. 2). Other comparisons were not significant (*Post-hoc* test, $P > 0.05$ for the cases).

DISCUSSION

Our results indicate that male *H. leechii* respond to ovulated and ovipositing female odors, but not to ovulating and

oviposited female odors and that the ventral glands do not release male attracting odors unlike it has been believed. These results suggest that *Hynobius* males discriminate among females based on their chemical cues associated with reproductive states and that these chemical cues may play a role in mating.

Males approached to the odor sources from ovulated and ovipositing females faster and stayed longer near the source than to controls. In several urodele amphibians, odors from females attracted males (Verrell, 1985; Park and Propper, 2001), but whether or not female salamanders who are in different reproductive states release different odorants or release different amounts of odors is not yet well documented. The use of different odorants in different reproductive states has been studied in several fishes. Odors of gravid female mosquitofish, *Gambusia affinis*, facilitated male's mating behaviors, while odors of parturient females increased male aggressive behaviors without affecting mating behaviors (Park and Propper, 2002). In goldfish, before ovulation, females release 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) as a major primer pheromone, which stimulates testicular 17,20β-P synthesis and increases searching and swimming behaviors in males, while females after ovulation release prostaglandins that stimulate male courtship behaviors, resulting in successful mating (Sorensen and Stacey, 1999; Stacey, 2003). In the current study, we focused on immediate attractive effects of female odors. The possibility that ovulating female odors may induce long-term effects in male physiology remains to be tested. To elucidate whether *H. leechii* females in different reproductive states release different chemical cues, additional studies are necessary.

Male olfactory responses to ovulated and ovipositing female odors have important behavioral implications. In *Hynobius*, males approaching a female not ready to oviposit eggs are unable to successfully fertilize ova because of the nature of external fertilization. In *H. nigrescens*, when a female was close to oviposition, she often approached a male and/or to the site where a male defended a territory for egg-laying (Usuda, 1997). For enhancing successful fertilization, a male salamander without a territory should involve identifying females ready to oviposit and fertilizing egg sacs during oviposition, while a male with a territory should successfully attract females who are close to ovipositing into their territories. In both cases, prior identification of a female's reproductive state by a male could greatly increase the probability of successful fertilization because the male would be able to focus all mating effort on the ovipositing female.

The ventral glands of *H. leechii* females do not seem to release male attractants. Olfactory responses to female odors between with ventral glands excised and with ventral glands intact did not differ among males, nor did males show significant olfactory responses to ventral gland extracts. Some studies have reported that the cloacal glands of several newts release sex attractants. Substances extracted from the cloacae of female red-spotted newts, *N. viridescens*, attracted males (Rohr et al., 2005). Sodefrin purified from the cloacae of male red-bellied newts, *C. pyrrhogaster*, attracted females (Kikuyama et al. 1995). While, in axolotls, *A. mexicanum*, bile acids (i.e. allocholic acid) and taurochenodeoxycholic acid induced strong electro-olfactogram responses from males, suggesting that organs other than cloacal glands are able to release sex attractants (Ziobro et al., 2004). In several fishes that externally fertilize eggs, ovary, testis, oviduct, and bile were all suggested as sources of attracting pheromones (Partridge et al., 1976; Zhang et al., 2001; Li et al., 2002; Stacey, 2003). Finding an organ responsible for the release of sex attractants in *H. leechii* could give valuable insights on the evolution of pheromone producing organs and behaviors among salamanders with external and internal fertilization systems.

The ventral glands of *H. leechii* seem to release substances probably involved in attaching egg sacs. In this study, although the presence and absence of the ventral glands of females did not affect male olfactory responses to female odors, oviposited females had significantly smaller lumen of alveoli in their ventral glands than ovulated females had. This result indicates that ventral glands probably release some substances during oviposition but we do not know their nature. Based on the histological study of *H. nigrescens*, Hasumi (1996) suggested that male *Hynobius* salamanders may follow some substances released from the ventral glands of female salamanders. However, he did not perform behavioral experiments. Male red-bellied newts, *C. pyrrhogaster*, have two cloacal glands. One is the abdominal gland which releases female-attracting pheromones. The other one is the lateral gland which secretes substances that constitute the spermatophore sac (Kikuyama et al., 1980). Male and female hynobiids possess only ventral glands that secrete a glycoprotein (Sever, 1991). In our study considering that the ventral gland extracts did not induce male olfactory response, and females with excised external cloacal walls (with associated ventral glands) could not attach their egg sacs to the twig which we placed (Personal observation) suggests the substances from the ventral glands may play a role in egg attachment. In previous studies, if appropriate substrates such as rocks and twigs were presented, females always successfully attached their egg sacs to the substrate.

Studies on chemical communication in primitive urodeles with external fertilization that live in East Asia and Northern Europe are relatively uncommon although such studies could provide valuable evolutionary insights about chemical communication among salamander species with

external and internal fertilization. *Hynobius* that externally fertilize eggs and use chemical cues in mating are an excellent model system for further elucidating behavioral and physiological functions of chemical cues in primitive urodele mating behavior.

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