

Ovulated female salamander (*Hynobius leechii*) respond to water currents

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To determine whether or not the female Korean salamander, *Hynobius leechii*, responds to water currents and, if so, whether those responses depend on their reproductive conditions, we evaluated the responses of ovulated and oviposited females to 1-Hz water currents generated by a model salamander with and without the placement of a transparent water current blocker between the model and the test females. The ovulated females responded to water currents by turning their heads toward, approaching, and/or making physical contact with the model. When the water current blocker was in place, the number of salamanders that approached the model was reduced significantly. The approaching and touching responses of ovulated females were greater than those of oviposited females, whereas the other measurements evidenced no differences. None of the responses of the oviposited females to water currents was affected by the presence of the blocker. Our results indicate that female *H. leechii* responds to water currents via a mechanosensory system.

Key words: body undulation, courtship behavior, mechanosensory system, oviposition, ovulation, water current

INTRODUCTION

In many animal taxa, the mechanosensory system performs a critical function in a variety of activities, including conspecific and individual recognition, alarm response, foraging, and reproduction (Vogel and Bleckmann 1997, Baker and Montgomery 1999, Hill 2001, Quirici and Costa 2007, McHenry et al. 2009). The functions of the mechanosensory system in reproduction have been assessed in different animal species, including rotifers (Joanidopoulos and Marwan 1999), spiders (Maklakov et al. 2003, Quirici and Costa 2007) and salmon (Satou et al. 1994a); such studies demonstrated that a healthy mechanosensory system is crucial for successful reproduction. Urodela species possess a well-developed mechanosensory system (Lannoo 1987, Lee and Park 2008b), the functions

of which in foraging and predation have been relatively well-studied (Fritzsche and Neary 1998). However, the reproductive functions of this system have yet to be well-established.

Unlike the majority of other salamanders, salamanders in the Hynobiidae fertilize eggs externally (Salthe 1967). When a *Hynobius* male and female encounter one another, the male salamander performs body undulations when its snouts are in contact with the female's skin (Tanaka 1986, 1987, Park and Park 2000, Kim et al. 2009). Body undulation is defined as an intermittent undulation of the body trunk and tail, which generates water currents. Male *H. takedai* respond to surface water currents generated by a glass rod, and *H. nigrescens*

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responds to the body undulation of other males (Tanaka 1987, Usuda 1995). A recent study also demonstrated that *H. leechii* males respond to water currents generated by other males using their mechanosensory system (Park et al. 2008, Kim et al. 2009).

Previous studies have demonstrated that when *Hynobius* females are nearing oviposition, they approach a tree twig or rocks, rub their cloaca on that site, and attach their oviposited eggs to the sites (Sato 1992, Hasumi 1994, Park et al. 1996, Kim et al. 2009). Additionally, female *H. nigrescens* have been shown to approach a glass rod that generates water currents, and their response is intensified when they are close to oviposition (< 2 hours to oviposition) (Usuda 1995). Furthermore, female *H. leechii* have been shown to approach a tree twig next to which males are performing body undulation, thereby suggesting that females might respond to water currents caused by the body undulations of the males (Kim et al. 2009). However, in the former study, the authors did not control for visual signals during their experiments, whereas in the latter study, the authors did not conduct control experiments. Therefore, it has yet to be established clearly whether or not female *Hynobius* salamanders respond to water currents using their mechanosensory system.

In order to determine whether the female Korean salamander, *H. leechii*, responds to water currents and, if so, whether their responses are dependent on their reproductive condition, we evaluated the responses of ovulated and oviposited females to 1-Hz water currents generated by a model salamander.

MATERIALS AND METHODS

Animal collection and husbandry

The salamanders employed in this study were collected from small ponds in the Research Forest of Kangwon National University (N 37°46'52.9", E 127°48'55.4") located at Donsan-myeon, Chuncheon-si, Kangwon, South Korea several times from early March to mid-April 2008. Females were determined on the basis of the presence of ovulated eggs in the slightly transparent abdominal cavity, and were individually maintained in small plastic boxes (13 cm long × 7.5 cm wide × 4.5 cm high) filled with aged tap water to a depth of 3 cm and provided with wet paper towels in which the animals could conceal themselves. The boxes were maintained in an environmental chamber, the air temperature of which ranged between 8-10°C. The humidity was approximately 70%, and the

photoperiod was 12D:12L.

Experimental procedures

The responses of the females to water currents were measured under both ovulated and oviposited conditions. In each condition, females were tested randomly with and without a water current blocker placed between them and the model generating water currents to determine whether the females respond to water currents using their mechanosensory system. In a previous study (Park et al. 2008), the blockage of water currents allowed for the successful determination of whether or not the mechanosensory system plays a role in male salamanders' responses to water currents. We determined the order of the experiments (with and without the blocker) by flipping a coin, and the females had at least a 2-hour rest period between the two experiments. After the experiments on ovulated females (which were conducted between the 24 March and 28 March, 2008), all of the ovulated females were permitted to mate with males. After the completion of mating, between 29 March and 3 April, 2008, we tested the oviposited females in the same fashion as the ovulated females. All experiments were conducted between 1900 and 0200 under dim light, < 0.1 Lux (YL102; UINS, Seoul, Korea).

Because detailed information regarding the generation of water currents and the measurement of salamanders' responses to water currents has been published previously, along with diagrams (Park et al. 2008), we briefly describe our methods here. We placed sand at the bottom (~5 cm deep) of an experimental tank (48 cm long × 27 cm wide × 30 cm high) and filled the tank with aged tap water to a depth of 5 cm above the sand. We placed a plastic model salamander similar to a real male salamander on a tree twig (~1 cm in diameter) positioned such that it crossed the tank from one lower corner to the opposite upper corner. The lower body of the model salamander was submerged; the upper body remained out of the water. In this posture, the model salamander could generate water currents similar to the water currents generated by male body undulation during mating. The model was controlled by a transparent fishing line connected to a hand-made vibration generator. In the experiments, we employed 1-Hz water currents because this frequency had been successfully used in previous experiments with males (Park et al. 2008). Additionally, we placed a wire circle (15 cm in diameter) centered on the cloaca of the model salamander at 5 cm above the water surface in order to determine whether females ap-

proached the model and to measure the amount of time they remained within the circle.

Prior to beginning the experiments, each female was provided with a 5-minutes period to acclimate to the experimental tank without any water vibration. The female was then exposed for 5 minutes to 1-Hz water currents while its responses were recorded with a low-light video camera (10IR LED, SLCC). After each experiment, the tank walls and sands were rinsed thoroughly using tap water to avoid any possible olfactory interference. In the analysis, we counted the number of times the female's head was oriented toward the model, the number of times it approached and/or touched the model, and the amount of time it remained within the 15-cm circle. Head orientation was considered to be achieved when the female turned its head toward the model at an angle of greater than 45 degrees from the body trunk axis. Approach to the model was achieved when the female's snout entered the 15-cm circle. The time of stay was the amount of time from the moment the female's snout entered the circle to the moment it exited the circle. If the female's snout was within the circle when the initial current was generated, we measured the time of stay from the beginning of the experiment. Touching of the model was recorded whenever the female's snout made physical contact with any part of the model.

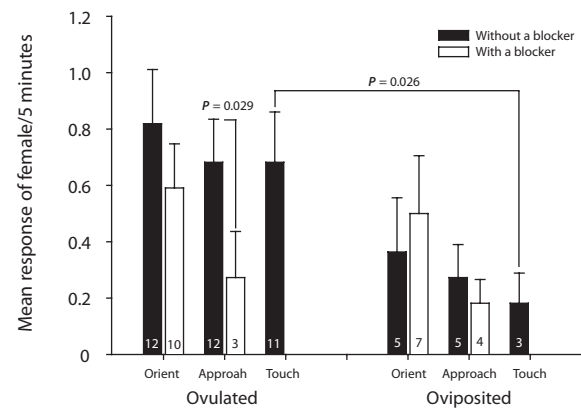
The water current blocker (13 cm long × 6 cm wide × 5 cm high, thickness 1.5 mm) was constructed of transparent acrylic plates and completely surrounded the model salamander at a distance of approximately 2-3 cm. The blocker allowed the model to undulate, but did not allow water currents to spread. In experiments conducted with a blocker, we measured only the number of head orientations and approaches toward the model. Due to the presence of the blocker, female salamanders could not remain within the circle or touch the model.

Data analysis

We compared the responses of the two groups of salamanders – those that had ovulated and those that had oviposited – to the water currents by a model salamander with and without the transparent current blocker. We employed a chi-square test to compare the number of responding individuals, and used a Wilcoxon's signed-rank test to compare the number of responses and the time of stay within the circle between the groups. All data were expressed as means ± SE.

RESULTS

The responses of 22 ovulated (2.09 ± 0.06 days before oviposition) and oviposited (3.09 ± 0.06 days after oviposition) females (snout-vent length, 6.26 ± 0.09 cm; body weight, 5.15 ± 0.21 g) to 1-Hz water currents were measured. Among the 22 ovulated females tested, 12 performed head orientation and approached the vibration model, and 11 touched the model (Fig. 1). However, only 3 females approached the model when the water currents were blocked (chi-square test, $X^2 = 8.20$, $df = 1$, $P < 0.01$), whereas 10 females still oriented their heads toward the model (chi-square test, $P = 0.55$). In experiments with oviposited females, 5 females executed head orientation and approached the model, and 3 females touched the model. With the water current blocker, 7 and 4 females evidenced head orientation and approached the model, respectively, and the effect of the blocker was not significant (chi-square test, $P > 0.05$ for both cases). When the ovulated and oviposited females were compared, it was determined that more ovulated females evidenced head orientation (chi-square test, $X^2 = 4.70$, $df = 1$, $P = 0.030$), approached the model (chi-square test, $X^2 = 4.70$, $df = 1$,



Females' responses and their reproductive conditions

Fig. 1. The responses (mean ± SE: number of head orientations and approaches toward the model and the number touching the model) of Korean female salamanders, *Hynobius leechii*, to 1-Hz water currents generated by a salamander model both with and without a transparent water current blocker. The numbers in the bars present the number of females responding among the 22 tested.

$P = 0.030$), and made contact with the model (chi-square test, $X^2 = 6.71$, $df = 1$, $P < 0.01$) than oviposited females.

Ovulated females executed 0.82 ± 0.19 head orientations, 0.68 ± 0.15 approaches, and 0.68 ± 0.18 touches over the 5-minutes test period (Fig. 1). With the water current blocker, the number of approaches was reduced signifi-

cantly (0.27 ± 0.16 , $Z = 2.18$, $P = 0.029$), but the number of head-orientations was not (0.59 ± 0.16 , Wilcoxon-signed rank test, $P > 0.05$). Ovulated females remained within the 15-cm circle for 20.23 ± 5.37 seconds. Oviposited females performed 0.36 ± 0.19 head orientations, 0.27 ± 0.12 approaches, and 0.18 ± 0.11 touches during the 5-minute test period. The placement of the water current blocker did not influence the number of head orientations (0.50 ± 0.21) and approaches (0.18 ± 0.08 , Wilcoxon-signed rank test, $P > 0.05$). Oviposited females stayed within the circle for 9.50 ± 4.54 seconds. As compared to the oviposited females, ovulated females performed more touches ($Z = 2.23$, $P = 0.026$), but we noted no differences in the numbers of head orientations ($Z = 1.64$, $P = 0.10$), approaches ($Z = 1.90$, $P = 0.058$), or staying time ($Z = 1.48$, $P = 0.14$) (Fig. 1).

DISCUSSION

In our experiments, the ovulated females performed head orientations and approached the vibration model; they also touched the model. Approximately 53% of ovulated females responded to water currents, similar to the previous observation that 56% of male *H. leechii* (Park et al. 2008) and roughly 44.5% of female *H. nigrescens* (Usuda 1995) responded to water currents. However, when a water current blocker was placed between the vibration model and the test females, the ovulated females approached less frequently. Thus, our results show that ovulated females respond to water currents using their mechanosensory system. In previous experiments conducted on males, both the physical blockage of water currents and pre-treatment of the salamanders with 1 mM Co^{2+} to damage the mechanosensory system significantly attenuated the responses of male salamanders to water currents (Park et al. 2008). These results indicate that ovulated female *H. leechii* could respond to water currents using their mechanosensory systems. On the other hand, in this experiment, the blockage of vibration currents did not influence the head orientations of the ovulated females, thereby implying that for head orientation, female *H. leechii* might employ both visual and vibration signals from males, as is also the case with Himé salmon (Satou et al. 1987).

In particular, the model-touching response of ovulated females to water currents was more profound than that of the oviposited females. Unlike the ovulated females, only approximately 14% of oviposited females responded to water currents. Also, the placement of a water current

blocker did not affect any of the responses of the oviposited females. These results indicate that the reproductive condition of females influences their responses to water currents. Similar results have been reported in previous studies. For example, the responses of ovulated *H. nigrescens* females to water currents increased gradually as the time for oviposition grew closer, although the responses of oviposited female salamanders were not evaluated (Usuda 1995). In salmon, female and male interactions using vibration signals occurred only prior to the oviposition of the female (Satou et al. 1994a, 1994b).

Why do females respond to water currents? Two possible explanations have been proposed for this phenomenon. The first is that ovulated females may simply wish to confirm the presence of males who would fertilize their eggs. In our experiments, ovulated females laid their eggs an average of 1.23 days after the experiments, meaning that females did not approach the model salamander because they were close to oviposition. In a previous study, females approached body-undulating males throughout the mating period, although the frequency of approaches increased gradually, beginning approximately one hour prior to oviposition (Kim et al. 2009). In the field, unfertilized eggs were frequently detected in the early breeding season (personal observation), probably because females were unable to find appropriate males to fertilize their eggs, or vice versa. In order to prevent the failure of egg fertilization, early confirmation of the presence of males at a site that could be readily approached by females may prove important.

The second explanation is that female *H. leechii* might use the currents to select a male, probably a large male, to fertilize their oviposited eggs. In a previous study, large males evidenced more body undulations than small males, and generated more water currents (Kim et al. 2009). During mating, *Hynobius* males generally excluded other males from a site or a tree twig at which they performed body undulations (Tanaka 1986, 1987, Usuda 1997, Lee and Park 2008a). Female salamanders approached a tree twig more frequently when large males executed body undulations than when small males did (Kim et al. 2009). The largest numbers of female eggs were deposited and fertilized on such sites (Usuda 1997), demonstrating that females selectively approached such sites for oviposition. In terrestrial salamanders, the female's choice of male mates is frequently based on chemical cues deposited by males on their territories (Marco et al. 1998).

The mechanosensory system performs a crucial function in the reproduction of many different species (Satou

et al. 1994b, Joanidopoulos and Marwan 1999, Quirici and Costa 2007), but relatively little is currently known regarding urodeles. The results of our previous (Park et al. 2008, Kim et al. 2009) and current studies indicate that the water currents generated by a male's body undulation might be employed for male-male competition and male-female interactions. Considering that pheromones from female *H. leechii* could induce male body undulation (Park and Sung 2006, Eom et al. 2009), this species, which fertilizes its eggs externally, could therefore furnish an appropriate model for the study of neurobehavioral and neuroendocrinological aspects of pheromone signaling in evolutionarily primitive urodeles.

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