



F-series prostaglandin function as sex pheromones in the Korean salamander, *Hynobius leechii*

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ABSTRACT

In order to test whether prostaglandins (PGs) function as sex pheromones in *Hynobius leechii*, a salamander that externally fertilizes its eggs, we conducted electro-olfactogram (EOG) studies with 19 PGs, liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses of female and male holding waters, and behavioral tests on selected PGs. Of the 19 PGs tested, only three induced strong EOG responses from both males and ovulated females: 15-epi-prostaglandin F₂α (15(R)-PGF₂α), 15-keto-prostaglandin F₂α (15K-PGF₂α), and 13,14-dihydro-15-keto-prostaglandin F₂α (13,14-dh-15K-PGF₂α). In the LC-MS/MS studies, samples of holding water from ovulated females contained higher concentrations of 15(R)-PGF₂α, PGF₂α, and 13,14-dh-15K-PGF₂α than those from males or oviposited females. In the behavioral tests, only 15(R)-PGF₂α and ovulated female holding water induced significant reproductive behavior from male salamanders. These results suggest that F-series prostaglandins function as sex pheromones in amphibians.

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1. Introduction

Pheromones play many roles in the lives of aquatic and terrestrial vertebrates, including fish (Stacey, 2003; Stacey and Sorensen, 2006; Burnard et al., 2008), amphibians (Kikuyama et al., 2002; Houck, 2009), reptiles (Mason, 1992; Halpern and Martinez-Marcos, 2003), and mammals (Brennan and Zufall, 2006). Because most urodeles, such as salamanders and newts, mate in an underwater environment with greatly limited visual and auditory stimuli (Dodson et al., 1994; Rohr et al., 2005), chemical cues are critical to successful mating. To date, four different proteinaceous urodele sex pheromones have been purified (Touhara, 2008). These pheromones attract females or increase female receptivity to mating and are released from specialized pheromonal glands, such as the abdominal glands (Kikuyama et al., 1995; Yamamoto et al., 2000; Nakada et al., 2007), the mental glands (Rollmann et al., 1999), and the rostral and parotid glands (Wabnitz et al., 1999).

Although only proteinaceous sex pheromones have been identified in amphibians to date, several different groups of pheromone compounds have been detected in teleost fish. These compounds include steroids (Dulka et al., 1987; Murphy et al., 2001), steroidal metabolites (Sorensen et al., 1995), aminosterols (Sorensen et al., 2005), bile acids (Doving et al., 1980; Li et al., 2002; Zhang and Hara,

2009), amino acids (Yambe et al., 2006), and prostaglandins (Stacey and Goetz, 1982; Sorensen et al., 1988; Sorensen and Goetz, 1993). For example, prostaglandins (PGs) that are involved in the ovulation and oviposition of vertebrates induced sexual behaviors in several vertebrates such as goldfish (*Carassius auratus*), cobitid loach (*Misgurnus anguillicaudatus*), and masu salmon (*Onchorhynchus masou*) (Sorensen et al., 1988; Ogata et al., 1994; Yambe et al., 1999).

The Korean salamander, *Hynobius leechii*, uses external fertilization like all hynobiids (Salth, 1967; Houck and Arnold, 2003). Fertilization is preceded by a number of courtship displays (e.g., snout contact, body undulation, fertilization, and post-fertilization; Park et al., 1996). Because the fertilization in this species takes place externally, knowing the time of a female's oviposition is critical for males to successfully fertilize eggs by allowing a male to concentrate his mating efforts on a higher probability-of-success female (Salth, 1967; Houck and Arnold, 2003). Thus, it is possible that male *H. leechii* might detect chemicals released from the females who are ovulated and close to oviposition. A recent study showed that male salamanders were quicker to approach the odor source where ovulated and ovipositing females were kept and stayed near that source longer, than when exposed to control tap waters. The result was not dependent on the existence of cloacal glands (Park and Sung, 2006), suggesting that possible internal secretions such as PGs and/or steroids might be responsible for the result.

In this study, we tested the hypothesis that PGs or their metabolites might function as sex pheromones in *H. leechii* by conducting electro-

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olfactogram (EOG) recordings, liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses of both the female and male holding water, and behavioral tests.

2. Materials and methods

2.1. Animal collecting and maintenance

For the EOG studies, we used a hand net to collect seven male and seven female *H. leechii* (Hynobiidae) from three small ponds (N 37° 46' 19", E 127° 48' 56") located in the Research Forests of Kangwon National University in Chuncheon, Kangwon, South Korea in early March 2007. An additional 48 males and 24 females were collected for behavioral tests and LC-MS/MS studies from the same ponds between late February and mid-March 2008. We kept the salamanders in refrigerated boxes (55 cm long, 35 cm wide, 35 cm high; 5–10 °C) to transport them to the laboratory. Upon arrival at the laboratory, the salamanders were separated based on their sex, which was determined by wide tails for males and the presence of eggs in the abdomen of females. They were then relocated to aquaria at a density of less than ten individuals per tank. The aquaria (33 cm long, 20 cm wide, and 25 cm high), containing approximately 25 L of aged tap water, were placed in an environmental chamber. We fed the salamanders bloodworms (*Limnodrilus gotoi*) and changed half of the water once every third day. The water temperature of the aquaria was kept between 6 and 8 °C, and the photoperiod was modeled after the local photoperiod of approximately 12:12 h (L: D). Wet paper towels and dead leaves collected from the field ponds provided hiding places. All males used in this study were in breeding condition, as was evident by their swollen cloacae and wide tailfins. All of the experimental procedures followed the guidelines for the use of live amphibians and reptiles in field and laboratory research (ASIH, 2004).

2.2. Electro-olfactogram recording

2.2.1. Stimulus compounds

In the first EOG study, we used 19 different PGs, ovulated female holding water, L-lysine, and charcoal-filtered tap water. The ovulated female holding water was used to confirm that the olfactory organ was responding to a stimulus. All PGs were purchased from the Cayman Chemical Company (Ann Arbor, MI, USA) and were as follows: prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F1 α (PGF1 α), 13,14-dihydro-prostaglandin F1 α (13,14-dh-PGF1 α), prostaglandin F2 α (PGF2 α), 16,16-dimethyl-prostaglandin F2 α (16,16-dimethyl-PGF2 α), 16-pheynyl-tetranor-prostaglandin F2 α (16-phenyl-tetranor-PGF2 α), 5-trans-prostaglandin F2 α (5-trans-PGF2 α), 11 β -prostaglandin F2 α (11 β -PGF2 α), 15-epi-prostaglandin F2 α (15(R)-PGF2 α), 15-keto-prostaglandin F2 α (15K-PGF2 α), prostaglandin F2 β (PGF2 β), prostaglandin F3 α (PGF3 α), U-46619, I-BOP, thromboxane B2, U-44069, 13,14-dihydro-15-keto-prostaglandin F2 α (13,14-dh-15K-PGF2 α), and 13,14-dihydro-prostaglandin F2 α (13,14-dh-PGF2 α).

We selected these 19 PGs because they had been tested at least once with EOG or behavioral experiments with teleost fish (Sorensen et al., 1988; Kitamura et al., 1994a,b; Moore and Waring, 1996; Sveinsson and Hara, 2000; Laberge and Hara, 2003). On the day they were received, all of the PGs were aliquoted at 100 μ g in 100 μ l of ethanol and stored at –80 °C until use. L-lysine was purchased from Sigma-Aldrich, St. Louis, MO, USA and it was dissolved daily in charcoal-filtered tap water at 10^{–4} M for the first EOG study and 10^{–5} M for the second EOG study. We used L-lysine as a positive control in EOG studies because, like female holding water, it induced strong EOG responses in urodele species (Toyoda and Kikuyama, 2000). Ovulated female holding water was collected for 10 h by keeping three ovulated females in separate chambers containing 10 mL of distilled water. The water was pooled, filtered using a syringe filter (0.45 μ m pore size, Millipore, USA), and then stored in 1 mL centrifuge tubes at –20 °C.

2.2.2. Recording EOG responses

For the EOG experiments, the salamanders were anesthetized with pH-corrected 0.1% 3-aminobenzoic acid ethyl ester (MS-222, Sigma, pH 7.5) in distilled water for 20 to 30 min and then immobilized with an intra-muscular injection of the neuromuscular blocker, gallamine triethiodide (Flaxedil, Sigma; 0.1 mg/100 g body mass, pH 7.6), dissolved in 0.6% amphibian saline (Park et al., 2004). During the experiments, supplemental doses of MS-222 were delivered as necessary into the salamander's skin along the main trunk of its body.

The main olfactory epithelium (MOE) was exposed by removing the tissue dorsal to the nasal capsule immediately before EOG recordings. To record electrical field potentials, a glass capillary electrode (100–200 μ m tip diameter) was filled with 3 M KCl in 0.6% amphibian saline with 1% agar bridged to a chloride-coated silver wire. An Ag–AgCl reference electrode was placed under the skin on the head. The electrodes were coupled to a pre-amplifier (IDAC-2, Syntech, Netherlands) that was controlled by the Syntech EagPro program, which also displayed and recorded the EOG signals. The MOE received a continuous 4 mL/min flow of charcoal-filtered tap water through a flow meter (FR-55S, Warner Ins. USA) by a gravity feeding system with a reservoir. In a test using dye, 50 μ l of stimulus samples were carried to the epithelium about 10 s after injection into the carrier stream, and they remained on the epithelium for 2 to 3 s.

We conducted the first EOG study to determine which of the 19 PGs were effective odorant for this species. In this experiment, we used 10^{–6} M of each PG, the collected ovulated female holding water, and 10^{–4} M L-lysine. Stimuli (50 μ l) were injected into the carrier stream. Before every trial, we prepared each stimulus in charcoal-filtered tap water and kept it on ice throughout the experiment. On the MOE, we selected a site where 10^{–4} M L-lysine induced a good EOG response but charcoal-filtered tap water induced, at most, a very small response. This area was often located at the edge of the MOE, which is close to the vomeronasal organ (VNO). Although previous studies have shown that the VNO often exhibits a larger EOG response to pheromone than the MOE (Toyoda and Kikuyama, 2000), we did not specifically record EOG responses from the VNO. The VNO in this species is small, and we often could not find an appropriate area to place a recording electrode. In addition, the female holding water generally induced large EOG responses in the MOE. When we placed the recording electrode on the MOE, we did not change its position until finishing all replicates of the EOG recordings.

This first EOG study was conducted in three females (Snout-vent length (SVL), 6.95 \pm 0.21 cm; body mass, 6.03 \pm 0.22 g) who were within 2 days of ovipositing. We injected 50 μ l of each PG, at random order, into the carrier stream using a micro-syringe (100 μ l syringe, SGE), with a 3-minute interval between injections. In the middle and at the end of the EOG recording window, we randomly recorded EOG responses to 10^{–4} M L-lysine and ovulated female holding water to confirm that the area was still responding appropriately to the stimulus. If 10^{–4} M L-lysine and ovulated female did not induce any EOG responses from the recording site, we did not include the recorded data in our analysis. EOGs of some PGs were recorded on two different MOE sites on the same female. In this case, the electrode position was moved between the different recording sessions, although females were used on only one occasion. The response magnitude of the EOG was defined as a relative peak phasic displacement measured from the baseline in millivolts. In this experiment, the magnitudes of EOG responses to the PGs were not adjusted by subtracting EOG responses to the charcoal-filtered tap water.

The second EOG study focused on better evaluating the response of males and females to PGs. Based on the results from the first EOG study, we selected five PGs (15(R)-PGF2 α , 15K-PGF2 α , U-46619, 13,14-dh-15K-PGF2 α , and 13,14-dh-PGF2 α) because these were the only tested PGs that induced large EOG responses in the first EOG study. For male responses, we also included PGF2 α because it is a

common pheromone in fish even though it did not induce large EOG responses in the first EOG study (Sorensen et al., 1988). In the second EOG study, we used 10^{-12} to 10^{-6} M of each PG as stimuli. We also used matched vector control solutions (ethanol in charcoal-filtered tap water). As with the first EOG study, we first determined a site where 10^{-5} M L-lysine induced the largest EOG responses, while charcoal-filtered tap water induced a minimal response. In this experiment, we used 10^{-5} M L-lysine instead of 10^{-4} M L-lysine because the lower concentration of L-lysine is more proper as a positive control in the experiments where we use very low concentrations of PGs as stimuli. For the recordings, we randomly selected a PG stimulus and applied 50 μ L amounts at concentrations ranging from 10^{-12} M to 10^{-6} M. After recording the EOG responses of seven different concentrations of a PG stimulus, we also recorded EOG responses to the exposure of vector controls. The EOG responses to 10^{-5} M L-lysine were recorded at both the beginning and the end of the application of each PG stimulus set. We then averaged the values to obtain a mean EOG response to 10^{-5} M L-lysine exposure as a positive control. There was a 3-minute interval between any two EOG recordings throughout all the experiments. We recorded EOG responses to the exposure of each of the seven different concentrations of the five PGs in seven ovulated female salamanders or six PGs in seven male salamanders from seven male and seven ovulated female salamanders. During the analysis, when EOG response magnitudes elicited by PGs were smaller than those of the control, we considered the response to be zero. In this experiment, the magnitudes of EOG responses were not corrected by subtracting EOG responses to vector controls or charcoal-filtered water. To compare EOG magnitudes among different PGs at each given concentration, we used a Kruskal–Wallis test because the data did not pass the normality test (Kolmogorov–Smirnov, $P < 0.05$). If the result was significant, we conducted the post-hoc test according to the Siegel and Castellan (1988) method. All of the statistical analyses were performed using SPSS (ver. 14.0, SPSS, USA). The significance level was $\alpha = 0.05$ for a two-tailed test, and the data were presented as mean \pm SEM.

2.3. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

2.3.1. Sample preparation

For the LC-MS/MS study, we collected the holding water of males (SVL 7.03 ± 0.26 cm; body mass 7.75 ± 0.85 g, $n = 12$) and both ovulated (SVL 7.15 ± 0.30 cm; body mass 8.50 ± 1.20 g, $n = 12$) and oviposited (SVL 7.23 ± 0.34 cm; body mass 6.49 ± 0.77 g, $n = 12$) females by keeping them in 40 mL of charcoal-filtered tap water for 12 h. The collected salamander holding water samples were filtered separately with a syringe filter (0.45 μ L pore size, Millipore) and then lyophilized (PVTFD-10R, Ilshin, Seoul). After that, the samples were stored at -80°C .

F-series prostaglandins (PGFs) were extracted from the holding water samples with a solid-phase extraction (SPE) technique based on the technique used by Liang et al. (2003). For each sample, a C18 SPE (Resprep, tube size 3 mL, USA) cartridge was rinsed first in 5 mL of ethanol and then in 5 mL of distilled water. The lyophilized sample was dissolved in 500 μ L of distilled water and then spiked with 35 ng of an internal standard PGF (PGF2 α -D4) to calculate the PGF extraction efficiency. After loading the sample onto a C18 SPE cartridge, it was eluted with 5 mL of distilled water, 5 mL of ethanol: distilled water (5:95 v/v), 1 mL of hexane, and 2 mL of ethyl acetate. The extracted sample was filtered (pore size 0.22 μ m, Millipore), evaporated using a nitrogen stream, and dissolved with 200 μ L of acetonitrile: distilled water (20:80 v/v). All of the organic solvents in this experiment were HPLC grade and obtained from either Carlo Erba (Italy) or Sigma-Aldrich (USA). The water was purified using an 18.2 M Ω cm filter (Millipore, USA).

2.3.2. LC-MS/MS analysis

Ten microliters of the reconstituted PGF samples were injected onto a YMC-Pack Pro C18 RS column (150 \times 2.0 mm, particle size 5 μ m, Japan). The sample was delivered at a flow rate of 200 μ L/min. The mobile phase consisted of 0.1% formic acid in distilled water (A) and 2 mM ammonium acetate with methanol:acetonitrile (2:1 v/v) (B). The LC separation was carried out with a linear solvent gradient program of: 60% A to 40% B at 0 min, 57% A to 43% B at 4 min, 0% A to 100% B at 10 min, and 60% A to 40% B at 15 min. The total LC running time was 15 min.

The LC system was interfaced directly with a triple stage quadrupole TSQ Quantum Ultra mass spectrometer (Thermo Scientific, USA) equipped with a heated electro-spray ionization (H-ESI) probe. The mass spectrometer was run in the negative ion mode ($[\text{M}-\text{H}]^-$) and used as the means of ionization with a capillary temperature of 350°C , a spray voltage of 5.0 kV, a vaporized temperature of 250°C , a sheath gas pressure of 60 psi, and an auxiliary gas pressure of 40 psi. The source collision-induced dissociation (CID) was set at 37 eV for PGF2 α , 15(R)-PGF2 α , 13,14-dh-15K-PGF2 α , and 15K-PGF2 α and at 30 eV for PGF2 α -D4. Instrument control, data acquisition, and data analysis were carried out with the Xcalibur™ Data System analysis software. Nitrogen was used as the collision gas. The mass spectrometer was optimized in the selected reaction monitoring (SRM) mode by diffusing 1 μ L/mL of PGF2 α , 15(R)-PGF2 α , 13,14-dh-15K-PGF2 α , and 15K-PGF2 α standard solutions and 1 μ L/mL of PGF2 α -D4 as the internal control. An ion pair of m/z 353.2/193.1 was used to monitor PGF2 α (Fig. 4), 15(R)-PGF2 α , 13,14-dh-15K-PGF2 α , and an ion pair of m/z 351.16/289.15 was used to monitor 15K-PGF2 α . In addition, an ion pair of m/z 357.2/197.1 was used to monitor PGF2 α -D4. The retention time of each PGF standard was different at this SRM mode (Fig. 5). A four to five point, linear calibration curve was established using standard prostaglandins. During the process, 35 ng of the internal standard, PGF2 α -D4, were also added to the 200 μ L calibration samples.

In the data analysis, we first determined if the female or male holding water samples contained a detectable level of each PGF using a standard curve. If the PGFs were detectable, we calculated the specific amounts of each PGF as ng ind $^{-1}$ h $^{-1}$. In the analysis of 15K-PGF2 α , one value from the ovulated female water samples was excluded as an outlier based on a stem-and-leaf plot. To determine whether a different number of ovulated and oviposited female and male holding water samples had detectable PGFs, we applied the Chi-squared test. Because most data did not pass the normality test (Kolmogorov–Smirnov, $P < 0.05$), comparing the amount of PGFs among the sample groups required the Kruskal–Wallis test, followed by post-hoc tests (Siegel and Castellan, 1988).

2.4. Behavioral tests of four PGFs as potential sex pheromones

2.4.1. Sample preparation

To determine if the four different PGFs (PGF2 α , 15(R)-PGF2 α , 15K-PGF2 α , and 13,14-dh-15K-PGF2 α) that were selected from the EOG studies and detected in salamander holding waters in the LC-MS/MS study could affect the reproductive behavior of male salamanders, we conducted behavioral experiments. In this phase of our study, we used ovulated female holding water as a positive control and charcoal-filtered tap water as a negative control. We excluded U-46619 because of its low potency in EOG studies.

The female holding water was prepared by keeping an ovulated female in 10 mL of distilled water for 10 h (from 22:00 to 08:00). We collected the female holding water from 23 ovulated females and pooled the samples together to remove individual variations from the stimulus. Following this pooling, the water was aliquoted in 10 mL centrifuge tubes, kept at -20°C , and used within 10 days. On the last day of the experiment, the female holding water was still active enough to induce responses from male salamanders. The aliquots

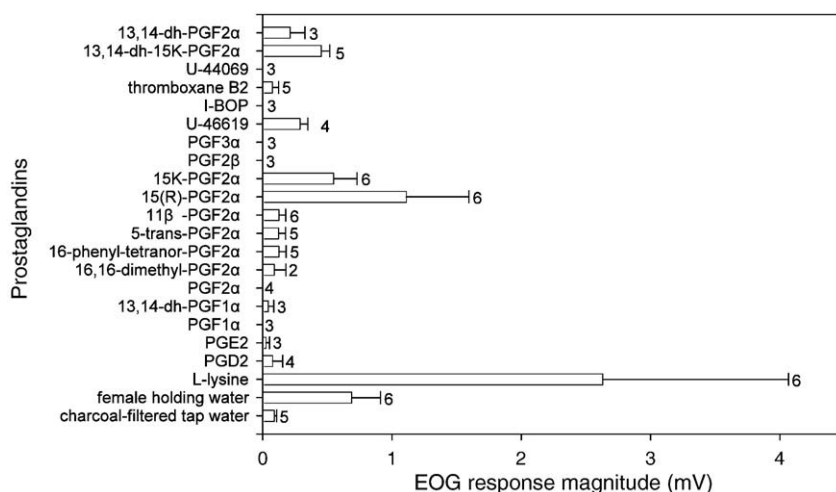


Fig. 1. The EOG response magnitude (mean \pm SEM) of three oviposited female *Hynobius leechii* exposed to 10^{-6} M concentrations of 19 different prostaglandins, ovulated female holding water, and 10^{-4} M L-lysine. Numbers on the each bar indicate sample size.

(100 μ g/100 μ L EtOH) of the four PGFs were dissolved separately in 10 mL of charcoal-filtered tap water and placed on ice just before starting each experiment. The final concentration of each PGF used in the experiments was 10^{-8} M in 260 mL of charcoal-filtered tap water.

2.4.2. Behavioral tests

For the experiment, we followed a modified Latin square experimental design. On the first day, each pair of male salamanders was randomly designated for one of the six stimulus groups. After the trial, each pair of males was kept for 24 h in a shoe box (135 mm wide, 75 mm long, 50 mm high) that contained aged tap water at a depth of approximately 4 cm and a paper towel for hiding. On the second day, the same pair of males was introduced into one of the other stimulus groups. We repeated this process until each pair of male salamanders had been exposed to all six stimulus groups. Each behavioral test began at 1900 under the 6.1 lux dim light (YL102, UINS, Seoul) between March 18th and 29th, 2008. In total, 18 pairs of male salamanders completed the responses for the six different stimuli during the behavioral tests.

To facilitate interactions following a previous study (Park et al., 2008), we put a pair of test males (SVL 5.93 ± 0.7 cm; body mass 8.05 ± 0.27 g, $n=36$) into a Petri dish (155 mm diameter, 20 mm high) containing 250 mL of charcoal-filtered tap water and let them acclimate for 10 min. Following the acclimation period, we recorded all of the behavioral interactions between the two males for 10 min using a camcorder (DCR-SR65, Sony, Japan). Next, we dissolved one test stimulus in 10 mL of charcoal-filtered tap water and gently combined it with the 250 mL of charcoal-filtered tap water in the Petri dish using a glass rod. After 1 min, we recorded an additional 10 min of behavioral interactions between the two males. The Petri dishes were washed using hot charcoal-filtered tap water after each trial.

In this experiment, we used two males instead of a male and female pair for several reasons. First, the females collected in the field sites were already undergoing ovulation. In this species, ovulation status can be easily confirmed through the semi-transparent abdomen skin that is observed during breeding season. Because the degree of the females' ovulation in *Hynobius* was fairly advanced, we could not use females at the same reproductive status for pre- and post-experimental design studies. Second, in preliminary experiments, when female holding water was added to a container where two males were present, they showed signs of courtship behaviors towards each other. Finally, although we are currently developing a

system where one can use a plastic model salamander for the tests, the system was not completed at the time of the experiments.

To determine whether the PGFs affect the reproductive behaviors of this salamander, we determined the number of snout contacts and the number of individuals who conducted body undulation. Snout contact and body undulation are typical reproductive behaviors of male *H. leechii* (Park et al., 1996). Snout contact includes touches between the snout of a male and the head, trunk, cloaca, and tail of another individual, suggesting that the behavior might facilitate gaining olfactory information about the sex and/or female reproductive conditions. Body undulation is a display during which a male stiffens his body intermittently, so his tail and the hind part of his body move from side to side (Park et al., 2008) and attracts females (Kim et al., 2009). We also measured the mean velocity of the two males before and after being introduced to the stimuli in the Petri dish. During this procedure, we used Ethovision software (ver. 2.3, Noldus, Netherlands) to determine if PGFs affect the general activity of male salamanders. The differences among instances of snout contacts and the mean velocity before and after the stimulus treatments were determined using the Wilcoxon-Signed Rank test because the data did not pass the normality test (Kolmogorov-Smirnov, $P<0.05$). To compare the number of males who conducted body undulations before and after the stimulus treatments, we applied Fisher's Exact test.

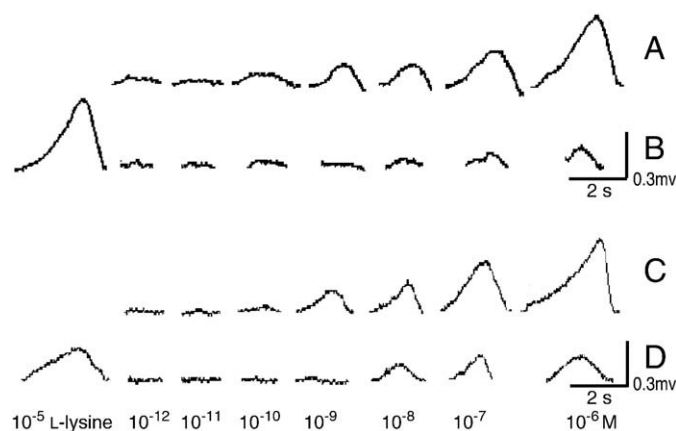


Fig. 2. Representative EOG responses of a *Hynobius leechii* male (A, B) and a female (C, D) to 15(R)-PGF2α (A, C) and vector control (B, D). 10^{-5} M L-lysine was used as a positive control.

3. Results

3.1. Electro-olfactogram recording

During the first EOG study, we recorded mean 4.11 times EOG recordings for each PG (range: 2–6 recordings) from three oviposited females. The EOG magnitudes from 10^{-4} M L-lysine and female holding water were 2.63 ± 1.44 mV and 0.69 ± 0.22 mV, respectively. Comparing these with the EOG responses from charcoal-filtered tap water, 15(R)-PGF 2α , 15K-PGF 2α , U-46619, 13,14-dh-15K-PGF 2α , and 13,14-dh-PGF 2α induced relatively larger EOG responses from the oviposited females than other PGs (Fig. 1).

In the second EOG study, 10^{-5} M L-lysine induced a 0.36 ± 0.04 mV EOG response in males ($n = 41$) and 0.22 ± 0.03 mV EOG response in females ($n = 38$, Fig. 2). The EOG response magnitudes among different PG stimuli were significantly different between 10^{-10} M and 10^{-6} M doses in males and between 10^{-9} M and 10^{-6} M doses in females (Kruskal–Wallis test, $P < 0.05$ for all cases). In particular, EOG response magnitudes in response to 10^{-8} M to 10^{-6} M 15(R)-PGF 2α , 10^{-8} M to 10^{-6} M 15K-PGF 2α , and 10^{-6} M 13,14-dh-15K-PGF 2α

were found for males, while females exhibited response magnitudes in response to 10^{-9} to 10^{-6} M 15(R)-PGF 2α and 10^{-9} M 13,14-dh-15K-PGF 2α . These responses were significantly greater than those recorded from matched vector control stimuli (post-hoc test, $P < 0.05$ for all cases, Figs. 2 and 3). Other comparisons were not significant (post-hoc test, $P > 0.05$ for all cases).

3.2. LC-MS/MS analysis

We obtained four standard curves for the PGFs ($y = 0.0322x - 0.0938$, $r = 0.988$ for PGF 2α ; $y = 0.0355x - 0.0622$, $r = 0.985$ for 15(R)-PGF 2α ; $y = 0.0146x - 0.6741$, $r = 0.990$ for 15K-PGF 2α ; $y = 0.0024x - 0.0210$, $r = 0.974$ for 13,14-dh-15K-PGF 2α). The extraction efficiencies from holding water samples were 90.67% ($n = 5$) for PGF 2α , 94.59% ($n = 5$) for 15(R)-PGF 2α , 90.74% ($n = 8$) for 15K-PGF 2α , and 99.86% ($n = 6$) for 13,14-dh-15K-PGF 2α . In at least one sample from ovulated and oviposited female and male holding waters, we detected four different PGFs: PGF 2α , 15(R)-PGF 2α , 15K-PGF 2α , and 13,14-dh-15K-PGF 2α (Figs. 4–6). The amounts of each PGF in the water samples were significantly different among the three water groups (Kruskal–Wallis test, $P < 0.001$ for all cases, Table 1). In particular, ovulated female holding water samples contained much higher concentrations of PGF 2α , 15(R)-PGF 2α , and 13,14-dh-15K-PGF 2α than samples from males or oviposited females (post-hoc test, $P < 0.05$ for all cases). Any other comparisons were not significantly different (post-hoc test, $P > 0.05$ for all cases).

3.3. Behavioral tests

The instances of snout contact between males significantly increased when they were exposed to female holding water ($Z = 2.49$, $n = 18$, $P = 0.013$) and 15(R)-PGF 2α ($Z = 2.57$, $n = 18$, $P = 0.010$), but exposure to the other stimuli had no effect on these behaviors ($P > 0.05$, for all cases) (Fig. 7A). More male salamanders conducted body undulations after being exposed to the female holding water than before (Fisher Exact test, $P = 0.02$), but the PGFs and the charcoal-filtered tap water control did not induce significant changes (Fisher Exact test, $P > 0.05$ for all cases, Fig. 7B). The mean velocity of the two males did not significantly change after being exposed to control water, ovulated female holding water, 15(R)-PGF 2α , 15K-PGF 2α , or 13,14-dh-15K-PGF 2α (Wilcoxon–Signed Rank test, $P > 0.05$, for all cases).

4. Discussion

In this study, we show that several PGs induce EOG responses from both male and female salamanders. Four PGFs were detected in salamander holding water, and 15(R)-PGF 2α increased reproductive behaviors of male salamanders, suggesting that PGs might function as sex pheromones in *H. leechii*, a primitive salamander that externally fertilizes eggs. This is the first study to detect several PGFs secreted in urodeles, and it is also the first study to show that PGFs function as sex pheromones in amphibians.

Screening revealed that, of the 19 PGs previously tested in teleost fish as potential sex pheromones, five induced large EOG responses from oviposited female salamanders: 15(R)-PGF 2α , 15K-PGF 2α , 13,14-dh-15K-PGF 2α , U-46619, and 13,14-dh-PGF 2α . In the second EOG study, 15(R)-PGF 2α , 15K-PGF 2α , and 13,14-dh-15K-PGF 2α induced larger EOG responses, in both male and female salamanders, than U-46619 and 13,14-dh-PGF 2α did. The potency of various PGFs as an olfactory stimulant has been tested in many different teleost fishes. 15K-PGF 2α induced large EOG responses in goldfish and lake whitefish (*Coregonus clupeaformis*) (Kobayashi et al., 2002; Laberge and Hara, 2003). 13,14-dh-15K-PGF 2α functions as a potent olfactory stimulant in several Cypriniformes (Kitamura et al., 1994b). PGF 2α is known as a potent sex pheromone that induces significant EOG responses in many different teleost fish, such as Atlantic salmon

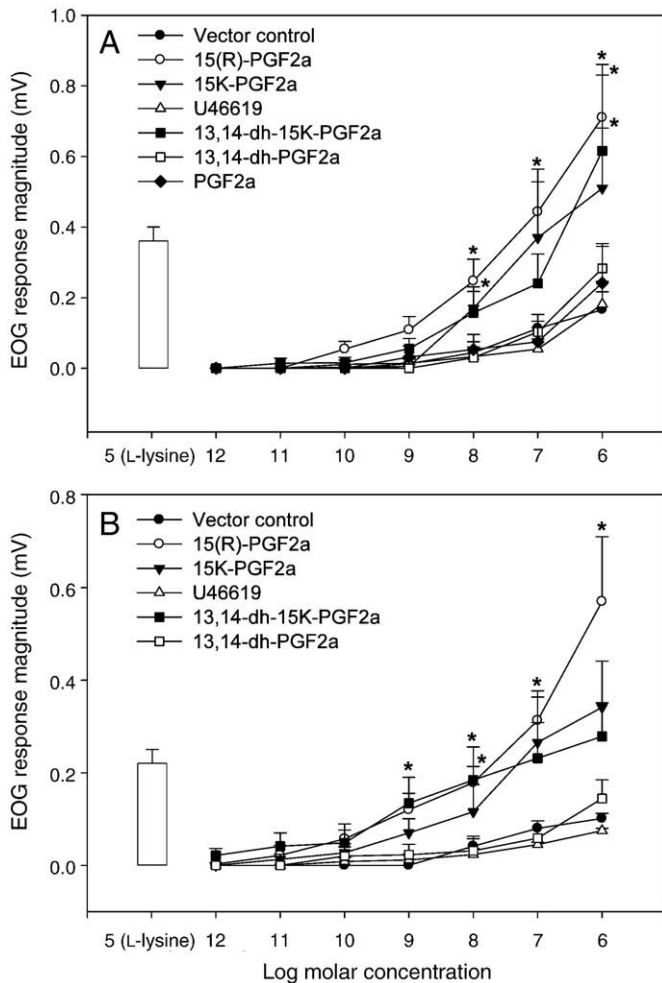


Fig. 3. The concentration–response relationships of EOG responses (mean \pm SEM) of male (A) and ovulated female (B) *Hynobius leechii* to vector controls and different prostaglandins: PGF 2α , 15(R)-PGF 2α , 15K-PGF 2α , U-46619, 13,14-dh-15K-PGF 2α , and 13,14-dh-PGF 2α , and 10^{-5} M L-lysine. The sample size was seven for all prostaglandin and vector control treatments, except for 15K-PGF 2α in male and U-46619 in female recordings; wherein these treatments, the sample size was six. The sample size of L-lysine was 41 in males and 38 in females. PGF 2α was tested only in male salamanders. *, significant difference to the vector control ($P < 0.05$).

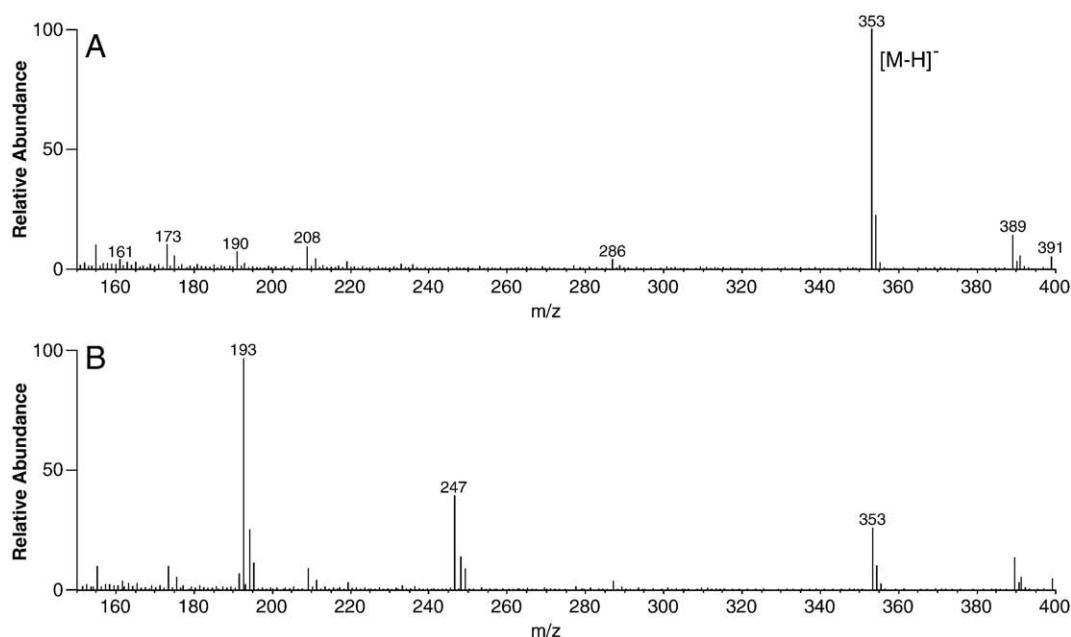


Fig. 4. H-ESI mass spectra of PGF2 α at a concentration of 1 $\mu\text{g/mL}$: full scan mass spectrum (A) and daughter ion spectrum (B) of m/z 353. The collision energy was 37 eV.

(*Salmo salar parr*), goldfish, and lake whitefish (Moore and Waring, 1996; Sorensen et al., 1988; Laberge and Hara, 2003). In addition to these three PGFs, several other PGFs, such as 5-trans-PGF2 α , 11-beta-PGF2 α , and PGF2 β , also activated olfactory systems in teleost fish, although they were much less stimulatory than the other PGFs (Sveinsson and Hara, 2000). To date, the potency of 15(R)-PGF2 α as an olfactory stimulant has been tested only in one species (Arctic char, *Salvelinus alpinus*). In that study, 10^{-8} M 15(R)-PGF2 α did not induce any EOG responses (Sveinsson and Hara, 2000). Although PGF2 α , a representative olfactory stimulant in teleost fishes, did not induce EOG responses in this salamander, our EOG study results suggest that PGFs may be potent olfactory stimulants in urodeles such as fish.

In the second EOG study, the most potent EOG stimulant was 15(R)-PGF2 α , followed by 15K-PGF2 α and 13,14-dh-15K-PGF2 α . The EOG threshold concentration of 15(R)-PGF2 α was 10^{-8} M in males and 10^{-9} M in females. In freshwater fish, the EOG threshold concentrations of PGFs that function as a sex pheromone generally range from 10^{-12} M to 10^{-8} M. For example, mature male Atlantic salmon and brown trout (*Salmo trutta*) were sensitive to PGF1 α and PGF2 α , with a detection threshold concentration of 10^{-11} M (Moore and Waring, 1996; Moore et al., 2002). Also, the exposure of male brown trout to concentrations of 10^{-8} M PGF2 α resulted in significant increases in the level of milt volume (Moore et al., 2002). These results suggest that exposure to 15(R)-PGF2 α at the threshold concentrations could produce potential pheromone-induced behaviors in this salamander. Nevertheless, considering that a relatively low concentration of the PGFs (0.05 ng–9.64 ng ind $^{-1}$ h $^{-1}$ which are approximately 10^{-12} M– 10^{-10} M) was detected in ovulated female holding water in the LC-MS/MS study, the EOG threshold concentrations are quite high. In particular, the EOG threshold concentrations of 15K-PGF2 α and 13,14-dh-15K-PGF2 α are high and varied greatly, although they induced large EOG responses. Large standard deviations of EOG magnitudes among the recordings are caused by several factors and might be largely responsible for the results. First, olfactory sensitivities might be varied across test individuals because of variations in waning reproductive conditions in laboratory housing conditions. Second, our delivery system, where we manually injected stimulus into the carrier stream, may also be responsible for large variations in EOG responses. Finally, we recorded EOG responses on the MOE, not on the VNO, where sex pheromones often evoke larger

responses in urodeles (Toyoda and Kikuyama, 2000); using the MOE probably resulted in small EOG responses with larger variations.

In urodeles, we first detected several PGFs from salamander holding water using the LC-MS/MS method. These results indicated that, as with teleost fish (Sorensen et al., 1988; Appelt and Sorensen, 2007), urodele salamanders also release significant amounts of PGFs and/or their metabolites into the water. Release of PGFs and their metabolites through urine has been observed in several fish species, including Atlantic salmon (Moore and Waring, 1996), masu salmon (Yambe et al., 1999), and goldfish (Sorensen et al., 1988; Appelt and Sorensen, 2007). Although no study has directly measured PGFs in individual amphibian holding water, Gobbetti et al. (1991) measured the plasma concentration of PGF2 α in female crested newts, *Triturus cristatus*, applying a radioimmunoassay, suggesting that it might also be released into the water via urine such as fish. In this study, ovulated female holding water of *H. leechii* had significantly higher amounts of PGFs (approximately two times higher for 15(R)-PGF2 α and approximately ten times higher for other PGFs) than oviposited female and male holding water did. Such different concentrations of PGFs based on different reproductive conditions or sex have previously been reported in several fish (Moore and Waring, 1996; Yambe et al., 1999) and newts (Gobbetti et al., 1991). For example, female crested newts (*T. cristatus*) contained approximately 5 ng PGF2 α mL $^{-1}$ plasma sample during a breeding season, while the concentration was less than 2 ng PGF2 α mL $^{-1}$ plasma sample during a non-breeding season (Gobbetti et al., 1991). In Atlantic salmon, ovulated females had 18.2 ng PGF2 α mL $^{-1}$ urine, while males had 0.7 ng PGF2 α mL $^{-1}$ urine. Our detection of PGFs in ovulated female holding water implies that, in this salamander, the PGFs may also be produced during ovulation and released into the water or, inversely, that an increased concentration of PGFs triggers ovulation and maintains a certain concentration level when released into the water (Guillette et al., 1991). In addition, because females in an externally fertilizing species are only valuable as mates when they have eggs, the release of these pheromonal substances might be limited to ovulated and ovipositing females.

In behavioral tests, only 15(R)-PGF2 α significantly increased the number of snout contacts; however, it did not affect the mean velocity of the males or the number of males who conducted body undulations. This finding indicates that 15(R)-PGF2 α specifically

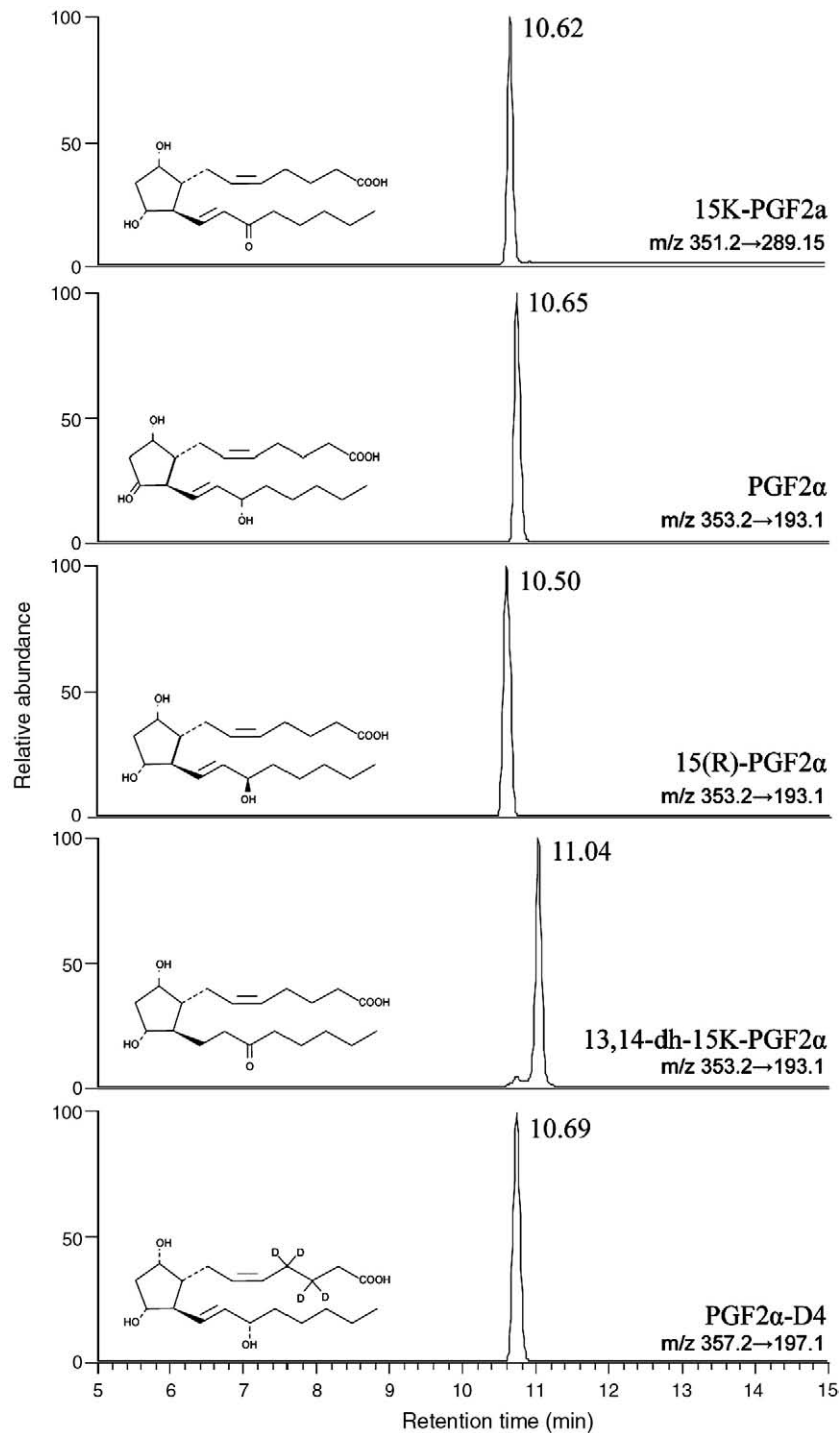


Fig. 5. LC-MS/MS results for the prostaglandin standards: 15K-PGF2 α , PGF2 α , 15(R)-PGF2 α , 13,14-dh-15K-PGF2 α , and PGF2 α -D4 (an internal control).

affects the snout-contact display of male *H. leechii*. During mating, male *H. leechii* frequently contact females, and the frequency of the snout contacts progressively increased up to the time of female oviposition, implying that snout contact may be important to get olfactory information about female's ovipositing time (Park et al., 1996; Kim et al., 2009). The female holding water significantly increased both the number of snout contacts and the number of individuals who conducted body undulations. Both 15(R)-PGF2 α and 13,14-dh-15K-PGF2 α also induced some changes in body undulations, although these changes were not statistically significant. Evoking behavioral responses by PGFs has been reported in several freshwater

fishes. For example, PGF2 α induced sexual behavior in male goldfish (Sorensen et al., 1988). 13,14-dh-15K-PGF2 α elicited male sexual behavior in the cobitid loach (Ogata et al., 1994). In addition to our current results, we might need additional studies to determine the detailed functions and the exact potency of 15(R)-PGF2 α and other PGFs as pheromones for several reasons. First, in behavioral studies, we have used a relatively high concentration of PGFs, and we only tested a single concentration. Second, we did not include females in behavioral tests. Finally, because several PGFs induce pheromonal priming effects in male freshwater fish (Moore and Waring, 1996; Moore et al., 2002), we need to measure potential pheromonal

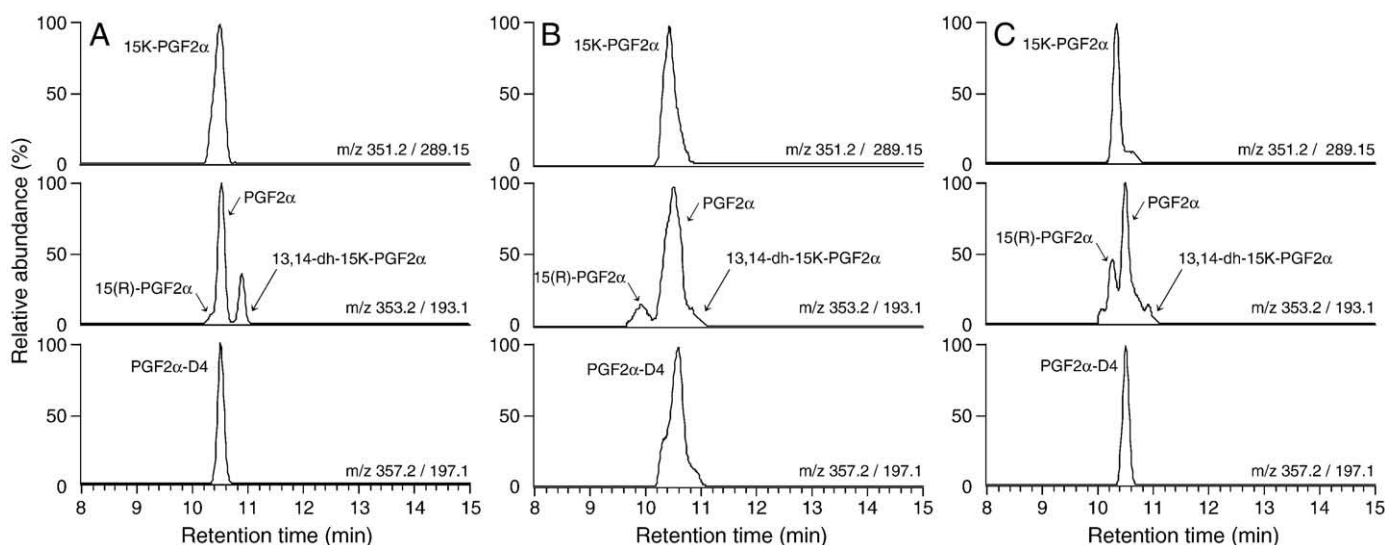


Fig. 6. The sample LC-MS/MS results of ovulated female (A), oviposited female (B), and male holding water (C).

responses in various ways, such as measuring hormonal changes after exposure to PGFs. In particular, there are still possibilities that 15K-PGF 2α and 13,14-dh-15K-PGF 2α might induce other aspects of reproductive behavior and/or physiology in male salamanders. Although 15K-PGF 2α and 13,14-dh-15K-PGF 2α did not induce significant behavioral changes, they evoked large EOG responses in both males and females and were also detected in high concentration in ovulated female holding water.

Our study provides the first evidence that any urodele species uses PGFs as sex pheromones. Because these results are similar to those found in fish, they might provide insight into how pheromonal communication systems in urodeles changed as salamanders evolved from an external to an internal mode of fertilization. Although PGs act as pheromones in this clade, other compounds, such as steroids, amino acids, peptides, and proteins, may also act as sex pheromones in

this primitive urodele species. Lastly, different chemical classes of pheromones may play significant roles in inducing appropriate behavioral and physiological responses in urodeles.

Table 1

The number of ovulated female, oviposited female, and male holding water samples with detectable levels of PGF 2α , 15(R)-PGF 2α , 15K-PGF 2α , and 13,14-dh-15K-PGF 2α , and the amounts (ng ind $^{-1}$ h $^{-1}$) of each prostaglandin in each sample group as determined by LC-MS/MS analyses.

Prostaglandins investigated	Salamanders	Ovulated female (n = 12 inds)	Oviposited female (n = 12 inds)	Male (n = 11 inds)	Statistics
PGF 2α	Number of individuals detected	12	7	5	$\chi^2_2 = 5.36$, $P = 0.069^a$
	Amount	4.02 ± 1.13	0.36 ± 0.10	0.18 ± 0.04	$\chi^2_{2,24} = 17.49$, $P < 0.001^b$
15(R)-PGF 2α	Number of individuals detected	12	12	11	$\chi^2_2 = 0$, $P = 1.000$
	Amount	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	$\chi^2_{2,35} = 16.56$, $P < 0.001$
15K-PGF 2α	Number of individuals detected	11	11	8	$\chi^2_2 = 0.82$, $P = 0.664$
	Amount	3.82 ± 1.44	0.25 ± 0.08	0.91 ± 0.34	$\chi^2_{2,20} = 9.64$, $P = 0.008$
13,14-dh-15K-PGF 2α	Number of individuals detected	12	11	11	$\chi^2_2 = 0.08$, $P = 0.959$
	Amount	9.64 ± 2.24	0.24 ± 0.03	0.41 ± 0.13	$\chi^2_{2,34} = 22.46$, $P < 0.001$

^a Chi-square test.

^b Kruskal-Wallis test.

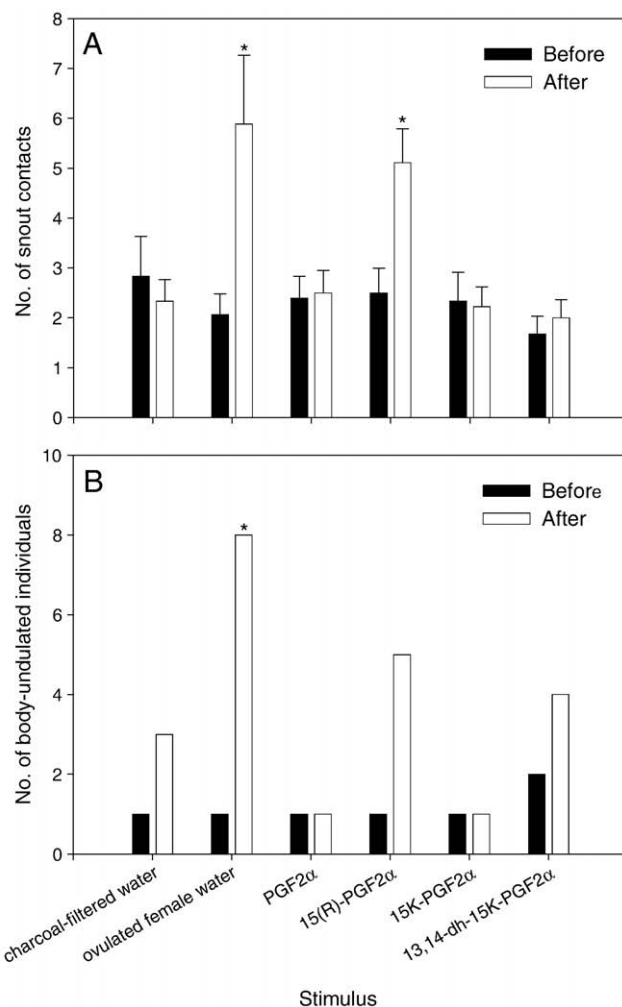


Fig. 7. Male salamanders exposed to 15(R)-PGF 2α exhibited more snout contacts (mean \pm SEM, A), and ovulated female holding water induced significant changes in both snout contacts (A) and the number of individuals (B) who conducted body undulations. *, significant difference ($P < 0.05$).

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