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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.

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 rostal 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 F1α (PGF1α), 13,14-dihydro-prostaglandin F1α (13,14-dh-PGF1α), prostaglandin F2α (PGF2α), 16,16-dimethyl-prostaglandin F2α (16,16-dimethyl-PGF2α), 16-pheynyl-tetranor-prostaglandin F2α (16-phenyl-tetranor-PGF2α), 5-trans-prostaglandin F2α (5-trans-PGF2α), 11β-prostaglandin F2α (11β-PGF2α), 15-epi-prostaglandin F2α (15(R)-PGF2α), 15-keto-prostaglandin F2α (15K-PGF2α), prostaglandin F2β (PGF2β), prostaglandin F3α (PGF3α), U-46619, I-BOP, thromboxane B2, U-44069, 13,14-dihydro-15-keto-prostaglandin F2α (13,14-dh-15K-PGF2α), and 13,14-dihydro-prostaglandin F2α (13,14-dh-PGF2α).

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 μg in 100 μl 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 μm 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 um 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 μL 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 μL) 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 ± 0.21 cm; body mass, 6.03 ± 0.22 g) who were within 2 days of ovipositing. We injected 50 μL of each PG, at random order, into the carrier stream using a micro-syringe (100 μL 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)-PGF2α, 15K-PGF2α, U-46619, 13,14-dh-15K-PGF2α, and 13,14-dh-PGF2α) because these were the only tested PGs that induced large EOG responses in the first EOG study. For male responses, we also included PGF2α because it is a