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Females of the four-eyed frog, *Pleurodema thaul* (Anura, Leptodactylidae), respond behaviourally to conspecific male scent

Valentina Rojas[1], Antonieta Labra[2,3], José Luis Valdés[4,5], Nelson A. Velásquez[1]

1 *Laboratorio de Comunicación Animal, Facultad de Ciencias Básicas, Universidad Católica del Maule. San Miguel 3605, 3480112, Talca, Chile*
2 *Centre for Ecological and Evolutionary Synthesis, University of Oslo, P.O. Box 1066, Blinder GNO-0316, Oslo, Norway*
3 *NGO Vida Nativa, Santiago, Chile*
4 *Departamento de Neurociencia, Facultad de Medicina, Universidad de Chile, Independencia 1027, 8380453, Santiago, Chile*
5 *Biomedical Neuroscience Institute (BNI), Facultad de Medicina, Universidad de Chile, Independencia 1027, 8380453, Santiago, Chile*

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Corresponding author: Valentina Rojas (vaaaleee.13@gmail.com)

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Abstract

Among amphibians, conspecific chemical communication has been widely studied in Caudata. Adult anurans, by contrast, have received less attention. Recently, it was shown that chemical scents are also relevant for adult anuran intraspecific communication. In this context, we evaluate whether females of the four-eyed frog (*Pleurodema thaul*) respond to conspecific male scents. We carried out a double choice experiment in a Y-maze. Females were repeatedly presented with the scents of several males versus distilled water. To extract the scent from males, we acoustically stimulated males and then used the water from their aquaria for the experiments. Our data suggest that females are capable of responding behaviourally to male scents, since they spent longer periods in the zones with male scent, rather than in zones with water. We propose that under natural breeding conditions, females of *P. thaul* may use either their chemical sense or chemical cues to facilitate their encounters with males.

Key Words

anuran communication, chemical communication, female choice, male scent

Introduction

Animal communication is a process by which information is transferred from a sender to a receiver, provoking in the receiver a behavioural or physiological reaction (Dusenbery 1992). This communication process is essential for social interactions (e.g. agonistic interactions, courtship rituals), and can depend on different channels (e.g. acoustic, visual, vibrational, chemical), which might be utilised independently or in association (Partan 2004; Bradbury and Vehrencamp 2011).

Chemical communication is one of the oldest systems of communication in the animal kingdom, and it has been studied across ontogenetic stages and in different social situations across several taxa (Gorman and Mills 1984; Roberts et al. 2003; Wyatt 2014). Particularly in amphibians, most knowledge regarding conspecific chemical communication has been developed for Caudata, where scents have a significant role in different aspects of their social life (Park and Sung 2006; Houck et al. 2007, 2008). For instance, salamanders of the family Plethodontidae have been widely studied for the use of chemical signals in their social interactions (Guillaume 2000; Rollinson and Hackett 2015). It was observed that males of *Plethodon shermani* produce a sexual pheromone denominated Plethodon Receptivity Factor (PRF) that

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increases the female`s receptivity (Rollmann et al. 1999). In addition, courtship behaviour involving pheromone delivery (Houck 2009), the genetic components for sex pheromone productions and their evolution across amphibian groups, have been widely described (Watts et al. 2004). The genus *Cynops*, has received strong attention for their intersexual interactions mediated by chemosignals (Toyoda et al. 2004). Kikuyama et al. (1995), discovered that males of the fire belly salamander (*C. phyrrogaster*) release a female-attracting pheromone (denominated Sodefrina) and males of *C. ensicauda* release a peptide from their abdominal gland called Silefrin that attracted females (Yamamoto et al. 2000). The fire belly salamander and their Sodefrin pheromone have become a model for the study of sex pheromones in aquatic salamanders, resulting in the discovery of a multi-component pheromone system (Van Bocxlaer et al. 2016).

Anurans have been described as acoustically dependent; several studies have, however, by now demonstrated that scents can mediate conspecific recognition (Kiseleva 1996; Carlson et al. 2015), provide information about kinship identity (Waldman 1985) and play a role in predator-prey interactions in the larval stages of anurans (Pearl et al. 2003; Chivers and Ferrari 2013; Troyer and Turner 2015). Even learning of chemical cues at embryonic stages has been described (Hepper and Waldman 1992). Additionally, chemical signals are also important in different social contexts in adult anurans (Kam 2002; Still et al. 2019). For instance, *Leiopelma hamiltoni* releases a chemical compound in the faeces that provides information to conspecific individuals about the size of the sender (Lee and Waldman 2002). In courtship situations, it has been observed that males of *Litoria splendida* release a sexual pheromone called Splendipherin that elicits changes in postural and permanence times in females (Wabnitz 1999). A recent study has contributed knowledge about anuran chemical communication through the study of compound secretions (Brunetti et al. 2019) and also several investigations provided evidence for their role in social interactions (Pearl et al. 2000; Asay et al. 2005; King et al. 2005). For example, Byrne and Keogh (2007) showed that the Australian terrestrial *Pseudophryne bibronii* toadlets use scents to identify and locate potential mates. In addition, the frog *Lithobates pipiens* shows differences in their behavioural responses to adult male and female chemical cues (Graham et al. 2020). Compared with other amphibian groups, however, more investigation describing chemical communication in adult anurans is still needed.

Adult males of the four-eyed frog, *Pleurodema thaul*, emit advertisement calls during the reproductive season, inflating their vocal sacs while floating on the surface of slow-flowing streams or pools (Penna and Veloso 1990; Velásquez et al. 2014). Research has shown that females do not exhibit a preference for males based on their calls (Velásquez et al. 2015). Alternatively, their preference may be guided by male scent. Currently, the only information available on chemosensory recognition in this species focuses on tadpoles using chemicals under predation risk (Pueta et al. 2016; Pueta and Perotti 2016), but the question of whether adults utilise scent recognition has not been studied yet. In this context, and considering the available information on sexual scents involved in mate attraction in other anurans (Wabnitz et al. 1999), we aimed to determine whether female *Pleurodema thaul* responded behaviourally to male scents.

Methods

Subjects

We collected a total of 13 individuals (3 males and 10 females) of *Pleurodema thaul* at Puente Negro (34°40'S, 70°53'W) in central Chile, and transported them to the laboratory in Talca, Chile. All specimens were separated by sex and housed individually in glass aquaria (15 × 20 × 30 cm) with water and gravel. The individuals were maintained in an animal room with an inverted photoperiod (12Light: 12Dark), with a temperature of 21 ± 1 °C (mean ± SD), and were fed tenebrionid larvae once a week, according to the described care instruction for laboratory animals (National Research Council 2010). After finishing all experiments and re-adapting the frogs to their normal photoperiod, we returned all of them in a healthy condition to their geo-referenced collecting point.

Conspecific male scents solution

To obtain male scent during their reproductive state (see Chu and Wilczynski 2001), we acoustically stimulated males as follows: after one week of acclimation, each male *P. thaul* was isolated for 10 days in an aquarium (20 × 8 × 15 cm), containing 50 ml of distilled water and previously washed gravel. To simulate the acoustic conditions experienced by males at night during the breeding season, we placed all 3 males in their individual aquaria, into a semi-anechoic chamber (with the walls and ceiling covered with a 25 cm-high foam wedge) to avoid sound reverberation and external noises. The male advertisement call was continuously broadcasted for one hour in complete darkness. We repeated this for 10 consecutive days, according to Chu and Wilczynski (2001). For the playbacks, we used a personal computer (Macbook Unibody, Apple Inc. Cupertino, CA, USA), controlling the amplitude with a programmable attenuator (PA5, Tucker-Davis Technologies). The calls were amplified (NAD C326BEE, NAD Electronics, Pickering, Canada), and broadcasted via two loudspeakers (Behringer 1C, Behringer, Willich, Germany) with an intensity of 70 dB SPL RMS (Penna and Solís 1998; Penna et al. 2008) measured inside each aquarium with a sound level meter (Bruel and Kjaer 2238; Bruel & Kjaer Instruments, Inc., MA, USA). The aquaria were placed on an anti-vibration table and the

loudspeakers were located 20 cm away from the aquaria. After stimulating the males for 10 days, we followed the methodology previously tested by Waldman and Bishop (2004) and Schulte and Rössler (2013) to extract and store water samples from each male. Immediately at the end of acoustic stimulation, we took water samples from each male's aquarium (henceforth, *conspecific scent solution*), and stored them separately at 4 °C for three days, until the olfactory discrimination experiments.

Olfactory discrimination experiments

Experiments were carried out in a semi-anechoic chamber. Following Pearl et al. (2000), we tested the discrimination ability of females (N=10; mean ± SD; weight=4.32 ±1.79 g; snout-vent length= 37.16 ± 3.81 mm) using a Y-maze made out of plexiglass walls (Fig. 1). At the base of the Y-maze, in the starting zone (10 × 10 × 10 cm), we placed the focal female, one minute before the experiment. We installed an acoustic-transparent plastic mesh at the end of each arm behind which, we placed a loudspeaker. Along the floor of one arm of the maze (15 × 10 × 10 cm), we provided a scent trail using 5 ml of the conspecific scent solution, and in the other, we used 5 ml of water-control (see in Fig. 1, as yellow and blue arm, respectively). The position of each solution was interchanged throughout the experiments to avoid potential position bias.

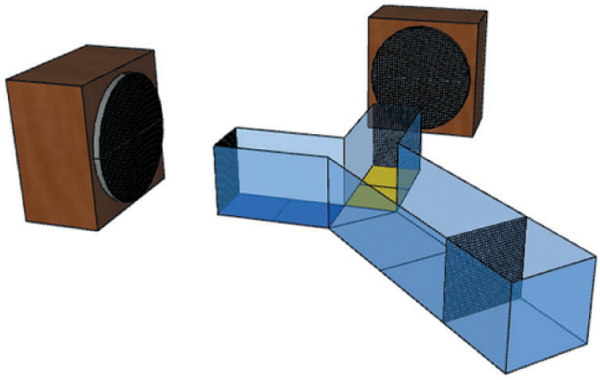

Figure 1. Graphical representation of the set-up of the Y-maze experiment. The maze was divided into different zones, where yellow represents the presence of conspecific scent solution and blue represents the control solution. The box at the base of the Y-maze represents the starting zone into which females were placed before the beginning of each experiment.

While females were in the starting zone of the Y-maze, we simultaneously broadcasted a conspecific advertisement call from each loudspeaker at the end of each arm of the maze for one minute, to stimulate movement in the females. After the acoustic stimulation, we lifted a gate, and monitored the female's reaction for five minutes (Fig. 1). We video-recorded the focal individual with a digital camera (SONY HDR-PJ760V, SONY Inc, Tokyo, Japan) placed in a zenithal position at 1 m above the centre of the maze. The experiments were carried out in darkness, only using an infrared LED bulb that was placed next to the digital camera (which had its infrared sensor activated). Experiments were monitored in real time from the outside of the semi-anechoic chamber using a computer screen connected to the digital camera. At the end of each experiment, the focal female was placed back into her aquarium and the Y-maze was washed with 70% ethanol and dried out to eliminate all chemical traces. Videos were downloaded to an Apple computer (iMac, Apple Inc. Cupertino, CA, USA) and formatted to AVI files with the VIRTUALDUB software (Copyright 1998–2009 by Avery Lee, version 1.9.7) for further analysis. The behavioural responses of the females were analysed with a custom-made MATLAB video tracking routine (Matlab 2007b, MathWorks, Natick, MA, USA). From these routines, we established different zones inside the Y-maze (Fig. 1), sectioning the base of the Y-maze into three squares and each arm into two squares, establishing a triangle at the junction between the base and the arms. Finally, we tracked the position of the females continuously during each trial.

The female's reaction to the conspecific scent solution was tested twice using the scent of each of the three males, resulting in a total of 6 experiments per female. We measured the female's choice towards the conspecific scent solution vs. water-control, by monitoring the first choice (i.e. the arm which a female entered first). We also measured the time spent in each zone across the maze (i.e. permanence time) of the Y-maze. Female choices towards the arm dowsed with conspecific scent solution out of all trial were analysed using the Binomial tests, while the differences in time spent within the arm including the conspecific scent solution versus the arm with the water-control were analysed using the Sign test. To compare the behavioural responses between the first and second trials we used a time index: (M-C/M+C), where M is the time spent in the zones with the conspecific scent solution and C the time spent in the water control zones. The indexes of both trials were compared using U-Mann-Whitney tests. Finally, to determinate whether the male identity influenced the female responses, we performed a Kruskal-Wallis test on the time indexes obtained from the female responses to each male. All statistics analysis were performed in STATISTICA 8.0 (StatSoft. Inc., Tulsa, OK, USA).

All the protocols of this study were previously approved by the Ethics Committee of the Universidad Católica del Maule (CICUAL 2018-04 FCBUCM) and complied with regulations for the animal care and conservation in Chile (SAG, Livestock and Agriculture Service, permit N° 5641).

Results

A total of 60 trials were done, and, in 80% of these cases, females showed a positive response toward the scent of

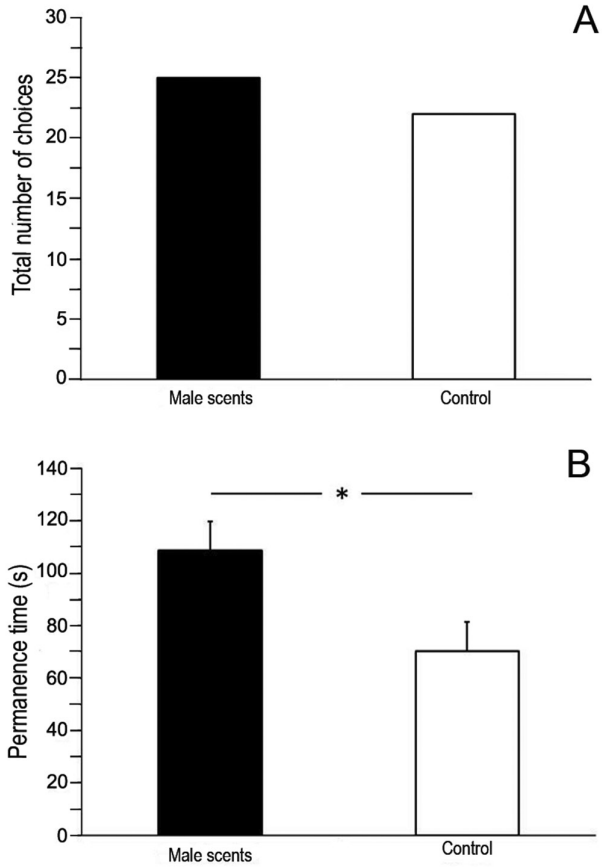

Figure 2. Total number of choices (**A**), and permanence time (**B**) shown by females of *Pleurodema thaul* in the Y-maze experiment. The asterisk indicates significant differences ($p < 0.05$).

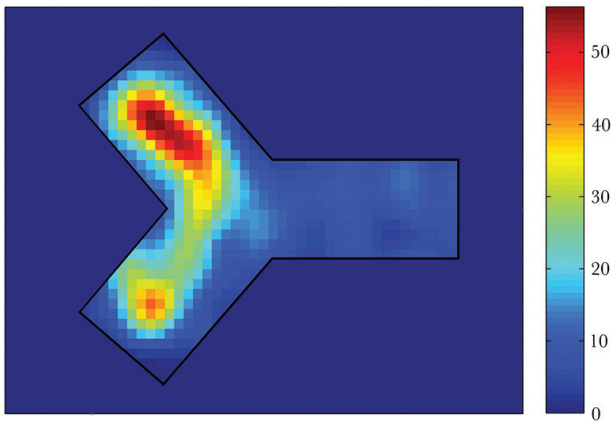

Figure 3. Heat-map showing the permanence time of females of *Pleurodema thaul* in the Y-maze. **A)** represent arm containing male scents and **B)** represent water-control arm. The lateral bar indicates the relation between colour and the permanence time (s). Heat-map is the outcome of overlaying track routine videos.

males by entering the corresponding arm. Despite this observation, females chose the arm with the conspecific male scent and the arm with the control solution indiscriminately (One-tailed Binomial test: $p = 0.385$, Fig. 2A), but they spent more time in the male scent solution than in the control solution arm (Sign test: $Z = 2.33$; $p < 0.05$, Figs 2B, 3). The analysis of permanence time indexes showed that females were consistent between replicates (U-Mann Whitney test: $Z = -0.483$; $p = 0.629$), and the identity of the male did not affect the response of the females (Kruskal-Wallis test: $H = 2.736$, $p = 0.741$).

Discussion

Females of *Pleurodema thaul* exhibit a behavioural response towards the scents of conspecific males by remaining in the arm containing the conspecific scent for a longer period compared to the arm containing water, suggesting that females of *P. thaul* are capable of detecting chemical cues originating from conspecific males. This, however, was not reflected in the first-choice behaviour of the females. Females seemed to choose both arms at similar rates.

The lack of initial choice for the arm dowsed with a male's scent may be explained by females depending on water-soluble compounds during mate choice. During the breeding season, male *P. thaul* congregate in pools to call and attract females (Penna and Veloso 1990; Velásquez et al. 2015). Females might need to be in close contact with water so as to show a behavioural response, similar to what was observed in *Litoria splendida* (Wabnitz et al. 2000). The water-soluble sexual pheromone Splendipherin produced by *L. splendida* males was effective in eliciting female attraction when the aquarium had a water column depth of 2 cm (Wabnitz et al. 2000). The lack of female choice for the conspecific scents in *P. thaul*, together with the longer period they spent in the arm with the conspecifics scent, suggests that females depend on water-soluble molecules to elicit specific behavioural responses, as it has been previously proposed (Wyatt 2010). Therefore, it is likely that similar experiments that include more water (e.g., Pearl et al. 2000; Asay et al. 2005) may trigger more robust behavioural responses in females of *P. thaul*.

Despite the lack of first choice preference, females remained for longer periods in zones containing the conspecific scent solution, hinting that females reacted to the scents produced by males. This result might indicate that chemical compounds promote intersexual interactions in *P. thaul.* Females may alter their locomotion or activity levels to move in the direction of the scent source in order to find a mate (Norris and Lopez 2011). Similar results were obtained in the dwarf African clawed frog (*Hymenochirus sp*) in which females were attracted to water containing male scent in a two-choice aquatic Y-maze and then remained for longer periods closer to the chemical scent source, suggesting an intersexual recognition mediated by scent (Pearl et al. 2000). Permanence time has been used to determinate the existence of conspecific chemical communication across different anuran species (Lee and Waldman 2002; Graham et al. 2020). Thus, considering the time spent in male scented zones and that conspecific male solution used in this experiment could resemble pool water during a breeding season, our results are in

concordance with previous studies that demonstrate how chemical communication plays an important role in conspecific interactions. A previous study showed that females of *P. thaul* do not exhibit a preference for males based on their calls (Velásquez et al. 2015). Potentially, females of *P. thaul* need both acoustic and chemical information to approach males, where acoustic signals can be used to find breeding ponds, and chemical signals to pick within a chorus (e.g., Rendall et al. 2009; Buxton et al. 2015). The consistency showed by females toward scents obtained from conspecific males (N=3; mean ± SD; weight=4.32 ± 0.67 g; snout-vent length= 31.98 ± 0.53 mm) could subtly suggest that: i) there were no differences between the males' characteristics or ii) in *P. thaul* frog, chemical and acoustic signals promote reproduction without involving sexual selection. Future studies must set up experiments that explore all factors influencing chemical behaviour, such as the breeding season and non-breeding season, largest sample size and intra and/or intersexual interactions. In this way, it would be possible to identify the exact role of the chemical compounds in male-female interactions and female preferences.

Conclusions

Our study demonstrates the capability of female *Pleurodema thaul* to discriminate conspecific male scents from a water control solution, since they remained for longer periods in zones with the conspecific solution compared to the control. Considering this result, we suggest that chemical compounds are involved in the intersexual interactions in this species helping females to find a mate. This study expands our knowledge on chemical communication, which is relevant to understand the factors and mechanism that take part in and modulate reproductive events.

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