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Female Attraction to Conspecific Chemical Cues in the Palmate Newt *Triturus helveticus*

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Abstract

Although chemosignals are largely used in sexual communication in urodeles, olfactometer studies in newts provided contrasting results about the sex specificity of female behavioural responses. Because long-range sexual advertisement is believed to be costly, some species might restrain this activity to close interactions with conspecifics. We tested chemical-mediated sexual attraction in female palmate newt (*Triturus helveticus*) by measuring the attraction to male and female odours in a linear water olfactometer. Unexpectedly, females were attracted towards conspecifics regardless of sex. They did not show attraction towards *Limnaea stagnalis*, a common sympatric aquatic gastropod. These results do not support the use of long-range male sexual signalling in the palmate newt. Instead, conspecific attraction is likely to promote aggregation of males and females in breeding ponds. Observations in the field and in the laboratory tend to support the aggregative behaviour of this species. We discuss the possible function of conspecific attraction in this context. Heading towards any conspecific would increase the probability of finding potential mates. Chemical cues do not need to be sex-specific at that stage so that long-range sexual advertisement might be unnecessary. This work emphasizes the need for studies investigating the evolutionary relationships between sexual signalling systems and population-distribution patterns.

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Introduction

A primary goal of sexual signalling is detection, from a distance, by potential mates. Because they allow a large broadcasting across air or water, acoustic and chemical signals are particularly suited to achieve this goal (Bradbury &

Vehrencamp 1998). However, sexual advertisement is believed to be an energy-consuming activity (Kotiaho et al. 1998; Gil & Gahr 2002), so that not all species invest in the production of costly long-range signals. Among amphibians, anurans make extensive use of acoustic signals (Ryan & Rand 1990; Andersson 1994; Krebs & Davies 1997; Doty & Welch 2001), whereas urodeles mostly rely on chemical and visual signalling (but see Diego-Rasilla & Luengo 2004). In the latter order, chemical communication has been described in several groups and in species with different life styles, from fully aquatic to fully terrestrial (Jaeger et al. 1986; Guillaume 2000). Its function ranged from territorial or sexual advertisement to social or age recognition (Dawley 1984; Simon & Madison 1984; Horne & Jaeger 1988; Yamamoto et al. 2000; Park & Propper 2001; Kikuyama et al. 2002; Gautier et al. 2004). In particular, sexual signalling has been reported in several species (Malacarne & Vellano 1987; Houck & Reagan 1990; Kikuyama et al. 2002).

In urodeles, male pheromones have been shown to increase either receptivity, orientation, or attraction in females (Houck & Reagan 1990; Rollmann et al. 1999). However, studies using olfactometers also reported unexpected results, namely females responding to chemical cues of other females. For instance, *Triturus carnifex* females placed in a Y-maze were tested in conspecific vs. water trials. Logically, they preferred the waterborne odour of a courting pair. However, they also displayed more 'sniffing' to female odours, whereas they did not respond to single male odours (Malacarne & Vellano 1987). More surprisingly, females from the complex of terrestrial species *Plethodon glutinosus–P. jordani* tested in an air Y-olfactometer, showed preference for female over male stimuli (Dawley 1984). These results do not fit the classical view of sexual signalling. An explanation might be that long-range sexual signalling is not used in some species. For instance, in *Triturus* newts, adults tend to aggregate on discrete breeding sites where populations can reach high densities (Griffiths 1996; J. Secondi pers. obs.). In such an ecological context, assessing and comparing the quality of different potential mates from the distance might be particularly unreliable and long-range sexual signalling might prove to be inefficient and costly. Obviously, this does not preclude the use of short-range chemical signals during sexual interactions.

We investigated long-range sexual signalling in the palmate newt, *T. helveticus*. In this genus, breeding occurs during the aquatic stage of the life cycle, which starts in early or mid-winter for western populations and extend to early summer. Breeding males develop fleshy ornaments, vivid colourations, and contrasting skin patterns. They also display a vigorous courtship during which they emit pheromones when facing a female at close range (Halliday 1977).

We experimentally quantified female sexual responses to male and female odours by measuring their displacements towards odour sources in a linear water olfactometer. Attraction to male stimuli only would suggest a sexual response whereas responses to both male and female stimuli would indicate conspecific attraction. To check the species-specificity of behavioural responses, we also tested the effect on female behaviour of an unrelated species frequently observed in the same ponds as the palmate newt, the great pond snail *Lymnaea stagnalis*.

Methods

Subjects and Stimuli

Capture, housing, handling and testing were in agreement with the *Guidelines for the Use of Animals in Research*. Mature female and male palmate newts were collected by dip netting in different ponds from western France near Angers (Maine-et-Loire) between Feb. and Apr. 2004 (Capture and Release Permit delivered by Préfecture du Maine-et-Loire). In the palmate newt, the breeding period spans from Jan. to Jun.–Jul. depending on climatic conditions and pond temperature (Griffiths 1996; ACEMAV et al. 2003). In western France where we sampled individuals, the highest abundance in breeding sites was observed between mid-Feb. and May (J. Secondi pers. obs.) and a peak of reproductive activity was expected during this period. All behavioural experiments were carried out between 11 Mar. 2004 and 3 May 2004.

Unisexual groups of five newts were transported in containers with a low water level (2–3 cm depth) and for a maximum transport duration of 1 h. They were transferred immediately to individual aquaria (20 cm length × 10 cm width × 15 cm height) filled up with 3 cm of dechlorinated tap water, and exposed to a natural photoperiod. This housing method was selected to avoid possible intra-sexual agonistic or inter-sexual interactions between individuals before testing. A support was provided so that individuals could leave or enter water at will. Room temperature was maintained between 14 and 17°C throughout the duration of the experiment. Individuals were fed twice a week with live chironomid larvae, but not on the day of experiments to avoid any decrease of activity.

They were maintained for durations ranging between 2 wk and 3 mo in the laboratory. Handling was limited to biometrical measurement, regular care-taking (water renewal and feeding), and testing. Newts were released into the pond from where they were caught. Before release, a finger tip was collected for later genetic analyses. This is a standard technique used in amphibians where other non-invasive sampling alternatives are not readily available. This caused no bleeding, and no behavioural modification could be noted even after a few days in the laboratory. Futhermore, some toe-clipped individuals were recaptured in their ponds several week after their release.

We tested 18 female palmate newts. In the first experiment, we used 14 females and 14 males as stimuli. All but the last female tested were also used as stimuli. For the second experiment, we caught six large great pond snails *Lymnaea stagnalis* from a pond where newts were also captured for this experiment. The snail is herbivorous and no competition with or predation upon palmate newts are expected in natural conditions so that we did not expect a snail odour to attract or repel subjects. In contrast, we did not use other newt species as control because interspecific interactions between species, and particularly intra-guild predation of adults upon larvae, might generate unpredictable behavioural responses, such as avoidance or aggression, from gravid female newts to other species. Females and males were not tested before 4–6 d after capture. The sexual receptivity of individuals (test and stimulus) was checked before testing. Males and females were placed in a water tank. An individual was selected for testing when it displayed one of the courtship behaviours as described in (Halliday 1977). All tests were conducted between 8.00 and 18.00 hours.

Experimental Apparatus

Displacements of females (direction, time spent in different zones) in a linear olfactometer (Fig. 1) were used to measure the response of female palmate newts to different olfactory stimuli. Two identical stimulus boxes (23 cm long × 17 cm width × 10 cm height), made out of plastic glass, were filled up with 2.5 l of dechlorinated tap water at room temperature. The first box contained a stimulus (newt or snail), the other one only water and was used as a control (see below). Stimuli were always placed in the same box. The main unit, the test chamber, was made of glass (120 cm long × 15 cm width × 10 cm height). To limit the influence of spatial cues on the behaviour of the subjects, all walls of the test chamber but the front one were covered with an opaque black casing. The wall facing the observer was left uncovered to allow the visual recording of behaviour. We delineated three zones in the test chamber (Fig. 1). A central zone (40 cm long) was used to retain individuals before testing (see section 'Test Protocol and Behaviour Recording' below). We defined a lateral zone (20 cm long) at each end of the olfactometer. Twenty centimetres separated each lateral zone and the central zone. The test chamber was filled up with 4.5 l of dechlorinated tap water at room temperature (water level 3 cm). Stimulus boxes were placed 60 cm above

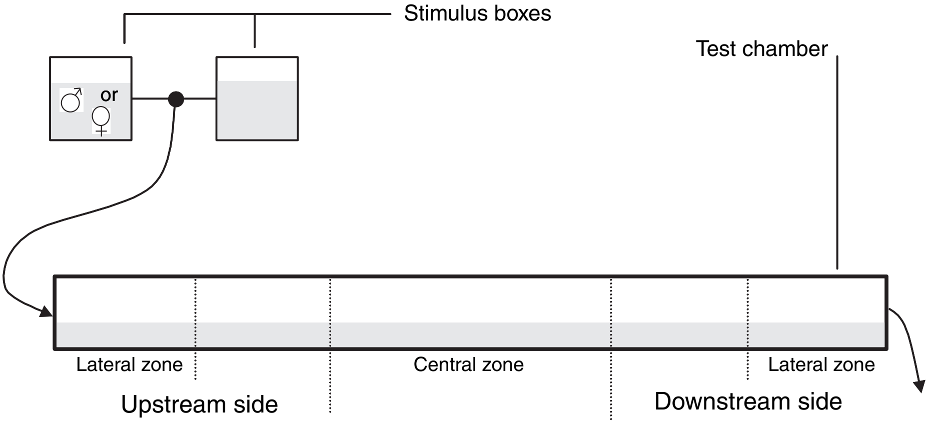

Fig. 1: Linear water olfactometer used to test attraction to chemical cues in the palmate newt *Triturus helveticus*

the test chamber to produce a waterflow. These could be plugged into the left or right end of the olfactometer so that waterflow direction could be changed between tests, which prevented any systematic side-preference effects. Water flowed out of the opposite side of the test chamber to maintain a constant water level. In addition, we could select from which stimulus box the flow would come and change at will. The flow (n = 5 measurements, mean value = 50.25 ± 3.58 ml/min) was adjusted visually using a medical drip.

Experiments were conducted in the dark. Only a tube light (1.20 m long) was placed behind the backwall of the chamber test, on the bottom of which a thin slit allowed light to enter the apparatus. This setup provided a uniform dim light in the chamber and maintained the observer in relative darkness.

Test Protocol and Behaviour Recording

The same protocol was used for all stimulus types (male, female, snail) throughout the experiment. A test proceeded as follows: (i) Familiarization period – The female is allowed to move freely in the chamber (40 min) but is not exposed to a waterflow. (ii) Control period – The female is placed back in the central zone and kept there between two wire-mesh plates under a constant flow of water coming out of the box with water only (5 min). Plates are removed and the female is allowed to move freely in the chamber. Its behaviour is continuously recorded (20 min). (iii) Test period – The female is placed back in the central zone and maintained between the two plates. The flow from the box with water only is shut down and the water from the box with either a newt or a snail is allowed to flow down into the apparatus. After 5 min, the wire-mesh plates are removed and the female behaviour in the chamber is continuously recorded (20 min).

A newt of the opposite sex to the stimulus individual was placed in a small plastic container filled up with water. The container was then deposited in the stimulus box. Even if no chemical communication could occur between them, the aim was to initiate sexual interactions and the production of sex pheromones from the stimulus individual. Female movements in the olfactometer were recorded continuously during the control and test periods. The time spent in each zone was recorded using a stopwatch. In the first series of tests (male/female), the presentation order of stimulus type was randomized between subjects. The direction of water flow was the same within a test and within a subject, but was alternated between subjects. At least a gap of 3 d was maintained between the first and the second test. In the second experiment, one great pond snail was picked up at random and placed in the stimulus box. Water flow direction was alternated between individuals. After completion of a test, the stimulus box and the test chamber were emptied, thoroughly rinsed with tap water and wiped.

Statistical Analyses

To measure attraction to stimuli, we considered the time spent in the lateral upstream and downstream zones. We computed the differences

($C_{upstream} - C_{downstream}$) and ($T_{upstream} - T_{downstream}$) where C is the time spent in the upstream or downstream lateral zone during the control period, and T the time spent in the upstream or downstream lateral zone during the test period. Positive values indicate that females increased the time spent in the upstream zone during the considered period. To test for an effect of stimulus on females, we compared the computed values for the control and test periods using Wilcoxon signed rank tests. We also compared female reactions to different stimulus type using Spearman rank correlation (Siegel & Castellan 1988). For all analyses, we used two-tailed tests with a significance level set at 0.05.

Results

Attraction to Male Stimuli

Females spent significantly more time in the downstream lateral zone than in the upstream lateral zone during the control period (n = 18, Z = 2.54, p = 0.012; Fig. 2). This was no longer the case during the test period (n = 18, Z = 0.588, p = 0.552). In contrast, the difference in time spent between the upstream and downstream zones was significantly higher during the test period than during the

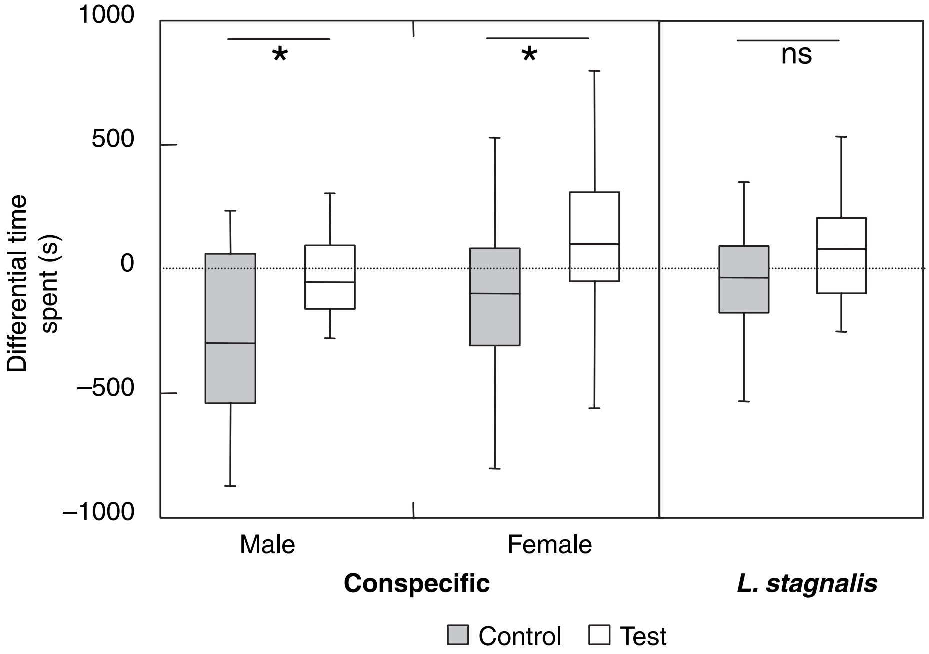

Fig. 2: Relative time spent (see 'Methods') by female *Triturus helveticus* in the olfactometer lateral zones when tested with a conspecific male or female stimulus (left panel), and a *Lymnaea stagnalis* stimulus (right panel). Positive values indicate that females increased the time spent in the upstream zone during the test period; $*p < 0.05$, ns: difference not significant

control period ($n = 18$, $Z = 2.025$, $p = 0.043$). Thus, females increased the time spent in the upstream lateral zone relative to the downstream lateral zone when water was flowing from a male stimulus (Fig. 2).

Attraction to Female Stimuli

As shown in Fig. 2, females did not spend more time in a lateral zone, upstream or downstream, both during the control ($n = 18$, $Z = 1.198$, $p = 0.231$) and test periods ($n = 18$, $Z = -1.720$, $p = 0.085$). However, the relative time spent in the upstream lateral zone was significantly higher during the test period than during the control period ($n = 18$, $Z = 2.352$, $p = 0.019$). Similar to that for male stimuli, females spent relatively more time upstream than downstream when water was flowing from a box with a female (Fig. 2). We detected no difference between the intensity of female responses to female and male stimuli ($n = 18$, $Z = -0.152$, $p = 0.879$). Responses to male and female stimuli were not correlated either ($n = 18$, $\rho = 0.220$, $p = 0.391$) (Fig. 3). We observed no relationship between female responses to male and female stimuli and the date of experiment (linear regression: females $F_{1,16} = 0.724$, $p = 0.407$; males $F_{1,16} = 1.700$, $p = 0.211$). Scatterplots of female response against test date did not show any kind of non-linear relationships, such as a quadratic relationship, either.

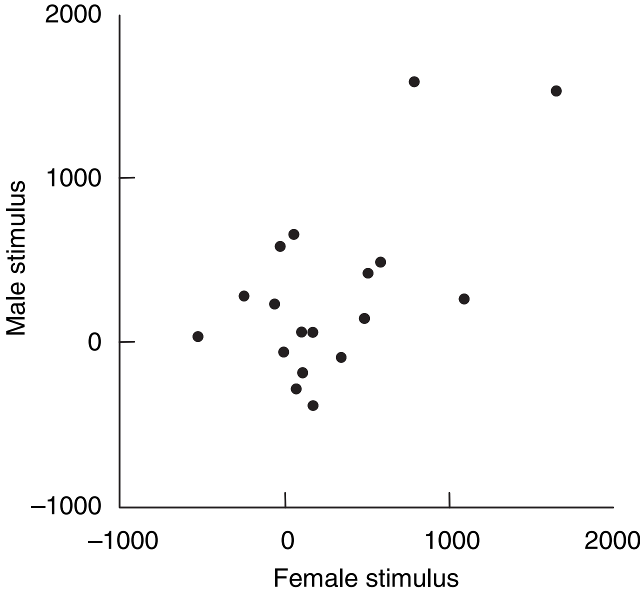

Fig. 3: Correlation between female *Triturus helveticus* responses to conspecific male and conspecific female stimuli. Positive values indicates that females increased the time spent in the upstream zone during the test period

Response to a Non-Specific Stimulus

We detected no difference in the time spent in upstream and downstream lateral zones (Fig. 2) both during control ($n = 17$, $Z = 0.734$, $p = 0.463$) and test periods ($n = 17$, $Z = -0.686$, $p = 0.492$). Likewise, the difference in time spent between the upstream and downstream zones did not significantly vary between the control and the test periods ($n = 17$, $Z = .449$, $p = 0.652$) when testing for the gastropod *L. stagnalis* (Fig. 2). We did not find any significant correlation between the response to snail and male ($n = 17$, $\rho = 0.120$, $p = 0.642$) or female ($n = 17$, $\rho = -0.154$, $p = 0.553$) stimuli either.

Discussion

We investigated the use of chemical cues in intersexual interactions by female palmate newts. Unexpectedly, females were attracted to either sex with equal intensity, which indicates that they did not respond to a long-range male sexual signal. Thus, according to our results, females did not discriminate between sexes or did not show diffential responses if they discriminated. Nevertheless, they showed no attraction or avoidance to the non-related sympatric gastropod species *L. stagnalis*, which discards the possibility that female newts moved towards any odour source or simply upstream when placed in a flow. Altogether, the combined results of these two experiments support the existence of an attraction towards conspecifics in the palmate newt.

At present, we do not know which molecules are used in the long-range detection of conspecifics in the palmate newt. Because stimulus individuals had visual contact with opposite sex newts, they could have released sexual pheromones in the medium. We cannot discard this explanation although it seems unlikely. First, it does not explain female–female attraction and, secondly, we hardly saw any interaction between the stimulus subjects and the other individuals placed in the container. Faecal pellets can repel or attract conspecifics in urodeles (Guillaume 2000). We did not quantify faeces deposition in the stimulus box but its frequency was very low and would unconvincingly explain the observed pattern. From our data, we cannot definitively demonstrate that the response is absolutely species-specific. Female might have reacted to odours from other newt species. This hypothesis could be tested using a closely related and sympatric species like the smooth newt *T. vulgaris*, but then again a lack of response for this species would not ascertain that palmate newts would not react to another one. More tests are required to identify which chemical cues are involved in sexual signalling and what are their level of species-specificity. Nevertheless, the present study shows no evidence that sex-specific sexual signals are produced in absence of close contact between individuals.

Still, female newts were able to detect conspecifics from the distance. Two explanations, not necessarily exclusive, could explain what they use this information for: (i) newts could benefit from using chemical cues when dispersing to a new site – detecting the presence of conspecifics would limit the risks of selecting a site with inappropriate ecological characteristics, or without potential sexual partners (Cogalniceanu 1992; Woody & Mathis 1997); (ii) chemical cues could serve as an aggregation pheromone. Laboratory observations suggest little aggressiveness between individuals which is in agreement with high adult densities sometimes observed in the field (Raxworthy 1989; Griffiths 1996). In addition, repeated dip nettings within a site usually yield a high variance in the number of individuals captured, which is very suggestive of an aggregative distribution (J. Secondi pers. obs).

There is no strong support for food-driven aggregation in this species. *Triturus* newts are rather generalist feeders (Nöllert & Nöllert 2003) and spatial variability in food availability might be rather lower in ponds than in other aquatic habitats. In contrast, sex driven aggregation is possible. Hedlund & Robertson (1989) described lekking behaviour in the great crested newt *T. cristatus*, and Höglund & Alatalo (1995) suggested that most species of the genus *Triturus* mate in leks. In a lek, females could reduce the costs of mate detection (searching time, predation risk), by heading towards any conspecific, regardless of its sex.

Our results might explain some unexpected female behaviours observed in other olfactometer studies in newts. Both long- and short-range chemical cues are probably used for locating and selecting mates in urodeles, but sex recognition might not be required at all stages. In gregarious species, the continuous production of long-range pheromones seems unnecessarily costly if other cues can be used to locate conspecifics. In contrast, sex pheromones might be emitted when partners have visual contact, and be used for mate choice. This outlines how little is known in this group about the relationships between the evolution of chemical signalling and ecological parameters such as the distribution of individuals in breeding habitats.

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