

# Conspecific male chemical cues influence courtship behaviour in the male newt *Lissotriton boscai*

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(Accepted: 12 January 2009)

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## Summary

Chemical information can have a direct or indirect influence beyond dyadic interactions. How chemical cues can alter communicating interactions where the transmitters of chemical cues do not participate is poorly understood. Chemical information plays an important role in the sexual behaviour of urodeles. Previous studies of the newt *Lissotriton boscai* and other salamandrids showed that males modify their courtship in presence of competitors to avoid courtship interferences. The aim of this study was to test whether *L. boscai* males assess the level of competition through chemical cues and adjust their courtship accordingly. In a first experiment, male courtship displays were recorded in aquaria containing their own chemical stimuli, or from another male. The duration and/or the number of several courtship displays were scored, and found that males decreased their courtship effort when the water contained chemical stimuli from another male. This experiment showed that semiochemicals can be sufficient to modify courtship displays without visual contact with transmitters of the chemical information, suggesting that males may adjust their courtship display to reduce reproductive costs. An additional experiment resembling chemically mediated residence asymmetries was then performed to examine whether territoriality is compatible with results of experiment 1, and found no evidence.

*Keywords:* Bosca's newt, chemical cues, courtship display, audience effects, *Lissotriton boscai*, amphibia.

## Introduction

Information transfer among individuals beyond the classical dyadic interaction can result in complex interrelationships among individuals in the context

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of information network systems (McGregor, 2005). For instance, individuals can extract information from social interactions in which they do not participate and subsequently modify their behaviour, and in turn their presence may alter those social interactions they are eavesdropping (Doutrelant et al., 2001). More generally, many studies on information networks have focused on visual and acoustic signals (mammals; Bernal et al., 2007; birds; Naguib & Kipper, 2006; amphibians; Diego-Rasilla & Luengo, 2004; fish; Oliveira et al., 2001), but the role of chemical information integrated within this framework has been paid less attention (Johnston, 2005; Wisenden & Stacey, 2005). Whereas it has been shown that chemical cues reflecting social or predator–prey interactions can influence the behaviour of perceivers that did not participate in those interactions (Chivers & Smith, 1998; Johnston, 2005; Wisenden & Stacey, 2005; Aragón et al., 2006), how chemical cues can modify communicating interactions where the transmitters of chemical cues do not participate is less understood (Johnston, 2005). Extracting information from conspecifics in the absence of the transmitters of that information may be advantageous as it may allow individuals to avoid costly encounters (e.g., Aragón et al., 2001). Many organisms are able to adjust their behaviour, physiology and/or morphology in response to chemical cues resulting in increased fitness. In amphibians, chemical cues may serve for a variety of functions such as conspecific attraction (Aragón et al., 2000; Gautier et al., 2006), sexual behaviour (Rohr & Madison, 2001; Rohr et al., 2005), territorial defence (Mathis & Simons, 1994), competitive ability assessment (Mathis, 1990), or predator avoidance (Gonzalo et al., 2007).

The sexual behaviour of male newts and salamanders is influenced by the presence of rival males to reduce reproductive costs (Uzzell, 1969; Verrell, 1983, 1984; McWilliams, 1992; Uzendoski et al., 1993; Verrell & Krenz, 1998). On the other hand, it has been demonstrated in urodeles that courtship responses to chemical cues can be modulated as a trade-off between mating and predator avoidance (Rohr & Madison, 2001). Thus, male newts might also use chemical cues to adjust their courtship behaviour according to trade-offs in other contexts. The aim of this study was to examine whether individuals can assess through chemical cues the level of competition, and consequently adjust their courtship behaviour to reduce reproductive costs. Thus, I examined the potential role of chemical cues from potential rivals on the courtship behaviour of the male *Lissotriton boscai*. I selected this species as a model of aquatic vertebrate because (1) the courting behaviour of this species

has been described in detail (Arntzen & Sparreboom, 1989; Faria, 1993), and observations of free living populations showed that courting behaviour in nature is similar to that observed in laboratory studies (Mouta-Faria, 1995); (2) male-to-male competition by courtship interference is very intense in *L. boscai* and other urodeles (Raxworthy, 1989; Verrell, 1989); (3) longer sexual sequences favour courtship interference in this species, which mainly occurs during the display phase (Mouta-Faria, 1995); (4) males of *L. boscai* are able to distinguish between chemical cues from males and females (Aragón et al., 2000); and (5) the courtship behaviour of this species is visually conspicuous. I performed a first experiment to test whether male *L. boscai* adjust their courtship behaviour depending on the social environment assessed through male conspecific chemical cues (own chemical stimuli *vs.* other male chemical stimuli). To better understand the potential mechanism involved in the results of experiment 1, I performed an additional second experiment. In experiment 2, pairs of males were faced to a residence asymmetry protocol by manipulating the same chemical cues (own chemical stimuli where focal males acted as residents *vs.* chemical stimuli from other males where focal males acted as intruders). This protocol enabled to test whether the pattern observed in experiment 1 can be compatible with a more general strategy of territorial behaviour or site defence regardless of the disputed resource, or the presence of females in experiment 1 are responsible of males' behavioural response.

## Methods

### *Study organism*

*Lissotriton boscai* is a small newt endemic to the Iberian Peninsula. It is one of the most aquatic newts, and inhabits shallow streams of slow-running clear water with aquatic vegetation. The sexual dimorphism in this species is less pronounced than in other newts (Montori & Herrero, 2004). Active adults can be observed in the water all year except for mid-summer when they become terrestrial for aestivation, and the breeding period encompasses all the aquatic phase (Caetano, 1982). The sexual behaviour of this species and other similar small-bodied newts consists of a courtship sequence where the main display is the so-called tail fanning behaviour (Halliday, 1990). This display is a quick undulation of the tail while it is positioned parallel towards

the body and oriented to the female. This display can be often followed by the creep and quiver, and/or flamenco displays before spermatophore deposition. The quiver consists of a shivering movement with the hind part of male's body (Halliday, 1974; Faria, 1993). The flamenco display is the raise of the male's tail up to 45–90°, and is considered to have a luring function (see Faria, 1993; Mouta-Faria, 1995 for a more detailed description of the sexual behaviour of this species).

In February 2008, I collected 41 adult newts (21 males and 20 females) from a stream located in Navia (Asturias province, northern Spain). After gently drying individuals, they were weighed to the nearest 0.01 g (males: mean  $\pm$  SE = 1.28  $\pm$  0.04 g; females: mean  $\pm$  SE = 1.79  $\pm$  0.05 g) and their lengths measured to the nearest 0.5 mm (males: mean  $\pm$  SE = 69  $\pm$  0.86 mm; females: mean  $\pm$  SE = 75.85  $\pm$  0.78 mm). Newts were individually housed for 2 weeks in aquaria (20  $\times$  30 cm) containing water (10 cm deep) and vegetation from the stream of origin. Feeding was standardized by placing a standard sized piece of *Lumbricus* in the front of the newts' snout once a day, which was eaten within few seconds.

### *Experiment 1: Courtship behaviour*

This experiment was performed to examine the courtship behavioural tactics of male newts in response to different social environments assessed from conspecific chemical cues. I staged male–female encounters (i.e., one-to-one) under two different conditions. The response of ten males to the presence of a female was recorded in the male's own home aquarium ('own chemical stimuli'), whereas the response of ten other males were recorded in the aquaria of other males ('conspecific male stimuli'). Another possibility of a non-competition condition could be a treatment using water with no chemical stimuli, instead of the 'own stimuli' treatment. However, it has been shown in mammals and reptiles that the use of treatments free of olfactory cues as controls may not be as innocuous as desired (Hurst et al., 1996; Aragón et al., 2006). Specifically, it has been shown that *L. boscai* avoids water ponds with no conspecific chemical stimuli (Aragón et al., 2000). Therefore, it should be more appropriate to include the 'own stimuli treatment' to avoid confounding effects. Before each trial began, males and females were gently confined to two opaque cylinders located on opposite sides of

the aquaria for five minutes. Cylinders served as shelters where all newts rested inside ensuring the same initial conditions through treatments. Therefore, differences in behavioural responses cannot be due to handling effects because all individuals were handled in the same way through treatments. All trials were performed in the morning and lasted for 10 min. To avoid a response due to size asymmetries, experimental individuals and donors of the chemical cues were size matched so that they were within 1 mm of the same size. No pair of newts was repeated. Among the male courtship displays of *L. boscai*, I selected the fanning, quiver, and flamenco behaviours because these are the most conspicuous. The fanning behaviour was also selected because it has the longest duration, and the flamenco behaviour because it represents the highest variability in the frequency of occurrence. The response variables scored were the latency to the first fanning event, the time spent fanning, and the number of fanning, quiver, and flamenco events.

The responsiveness of females to the males' courtships might affect the males' behaviour. For instance, males might modify their courtship behaviour depending on whether females remain motionless or flee. Therefore, two additional variables were scored to control for a potential effect of female behaviour during trials. I scored the number of times that females withdrew or slowly moved away from their male partners (avoidance events), and, as a measurement of activity, the total time spent moving by each female.

Data were analyzed using ANCOVAs with treatments as the between-subject factor, and the females' avoidance events and the time spent moving as covariates. The dependent variables were the latency to the first fanning event, the time that males spent fanning, or the number of fanning or quiver events. The interactions between the treatment and the females' behaviour were also included in the analyses to test whether the response of males to the social environment was dependent on females' responsiveness. I started with a general model including all of the main effects and their first-order interactions with the treatment. I then selected the most parsimonious model by backward selection (McCullagh & Nelder, 1989), dropping all non-significant terms starting with the interactions. I also used ANOVAs to verify that length, mass and corpulence of males and females, and that female behaviour did not differ between treatments. Corpulence was estimated by taking the residual score of the linear regression of body mass on the length. These residuals are size-corrected measures of mass. I used Pearson correlations to test whether the behaviours scored were associated with morphological traits.

*Experiment 2: Residence asymmetries*

According to the scent-matching hypothesis, individuals can assess residence status by comparing odours of a conspecific with those found in the vicinity (Simons et al., 1994). Therefore, in order to better understand the mechanism underlying the observed pattern of courtship behaviour, I staged encounters between two males. I used a repeated measures design in which each focal male was tested in two different treatments in a random and counterbalanced order of presentation. The response of focal males was recorded in their own home aquarium ('resident treatment'), and in the home aquarium of the partner male ('intruder treatment'). It was previously demonstrated that analogous protocols reliably induce asymmetries in residency, and that this can modulate the aggressive response of males in species of the order Urodela by means of chemical cues (e.g., Simons et al., 1994). Thus, focal newts acted both as residents and intruders in the experimental conditions. Pairs of males were size matched so that they were within 1 mm. For each trial, two male newts were located on opposite side of the aquarium. Each male responded to the treatments in different days and no pair of males was repeated. As in the other experiment, males were confined to two opaque cylinders for five minutes before each trial began. I performed 20 trials (10 focal paired males  $\times$  2 treatments). All trials were performed in the morning and lasted 10 min. As a measurement of the aggressive response depending on the treatments, I scored the number of agonistic interactions initiated by focal males, which consisted of single quick bites. Agonistic interactions never came to the point of injuries or persistent attacks and did not lead to sustained attempts to escape.

Additionally, I also scored other more subtle behaviours to explore whether there was an effect of the experimental treatment. Thus, as a measurement of avoidance behaviour, I scored the number of times that focal males withdrew or slowly moved away from their partners, and, as a measurement of activity, the time spent moving by focal males. Data were analyzed using repeated measures ANCOVAs with treatments as the within-subject factor, male length and corpulence as covariates, and the number of bites, avoidance events or time spent moving by focal males as the response variables. The interactions between the treatment and morphological traits were also included to test for an effect of male competitive ability. I then selected the most parsimonious model by backward selection.

Both experiments were performed with water at 13.5°C, which was within the temperature range measured in the stream where newts were collected (12.5–14°C), and within the range observed during the entire aquatic phase of this species (Mouta-Faria, 1995). Males were held with the same water for five days before using their home aquaria in trials, which is sufficient time to allow the detection and discrimination of conspecific chemical stimuli contained in water (Aragón et al., 2000). All trials were recorded with a camcorder aligned perpendicularly to the experimental aquarium. No pair of males was repeated through the experiments (neither by means of their presence nor their chemical cues). The order of presentation of experiments were randomized and counterbalanced. Newts participated in the experiments in different days. Behavioural responses in both experiments were not dependent on whether individuals have previously participated in the other experiment or not. Thus, when previous experience were included as treatments in the initial models (i.e., previous participation in the other experiment or not) they were dropped from all models through the backward selection procedure (all  $p > 0.27$ ). In concordance, other behavioural response of *L. boscai* was not dependent on the order of treatment presentation in another study (Aragón, 2009). For both experiments, data in the form of counts were square root transformed, and continuous variables were log-transformed when appropriate to meet the assumptions underlying linear models. Also for both experiments, normality and homocedasticity were tested with Kolmogorov–Smirnov and Levene's tests, respectively (Quinn & Keough, 2002). Unadjusted probabilities for each set of tests are reported, but significance was verified using the sequential Bonferroni adjustment of Rice (1989). Data were analyzed with the software STATISTICA 6.1 (StatSoft 2003). After the experiments, newts were returned healthy to their capture site.

## Results

### *Experiment 1: Courtship behaviour*

Males courted females in all trials. There were no significant differences between treatments in the latency to the first fanning event as a main effect ( $F_{1,14} = 0.75$ ,  $p = 0.40$ ; own stimuli: mean  $\pm$  SE = 91.90  $\pm$  19.15; conspecific male stimuli: mean  $\pm$  SE = 119.66  $\pm$  26.37). There were no significant effects of females' behaviour on the latency to the first fanning

event (time spent moving:  $F_{1,12} = 0.13$ ,  $p = 0.71$ ; avoidance response:  $F_{1,13} = 0.84$ ,  $p = 0.37$ ), and the interactions with the treatment were not significant (all  $p > 0.09$ ). In contrast, the time that males spent fanning in the presence of a female was significantly longer when males were in their own aquaria than when trials were performed in the aquaria of a conspecific male ( $F_{1,18} = 7.45$ ,  $p = 0.013$ ; Figure 1a). This response did not depend on the behaviour of females (time spent moving:  $F_{1,16} = 1.75$ ,  $p = 0.20$ ; avoidance response:  $F_{1,17} = 1.96$ ,  $p = 0.17$ ), nor did it depend on the interactions between female behaviour and the treatment (all  $p > 0.41$ ). The female behaviour did not differ between treatments (avoidance response:  $F_{1,18} = 1.28$ ,  $p = 0.27$ ; time spent moving:  $F_{1,18} < 0.0001$ ,  $p = 0.99$ ).

The number of fanning events performed by males was significantly higher in their own aquarium than in the aquarium of a conspecific male ( $F_{1,18} = 11.10$ ,  $p < 0.01$ ; Figure 1b). This response was not dependent on the female behaviour either as a main effect (time spent moving:  $F_{1,16} = 0.64$ ,  $p = 0.43$ ; avoidance response:  $F_{1,17} = 0.77$ ,  $p = 0.39$ ), or in interaction (all  $p > 0.31$ ). The number of quiver events performed by males was significantly higher in their own aquaria than in the aquaria of a conspecific male ( $F_{1,18} = 14.74$ ,  $p < 0.01$ ; Figure 1c). Again, this response was not dependent on the female behaviour either as a main effect (avoidance response:  $F_{1,16} < 0.001$ ,  $p = 0.98$ ; time spent moving:  $F_{1,17} = 0.41$ ,  $p = 0.53$ ), or in interaction (all  $p > 0.09$ ).

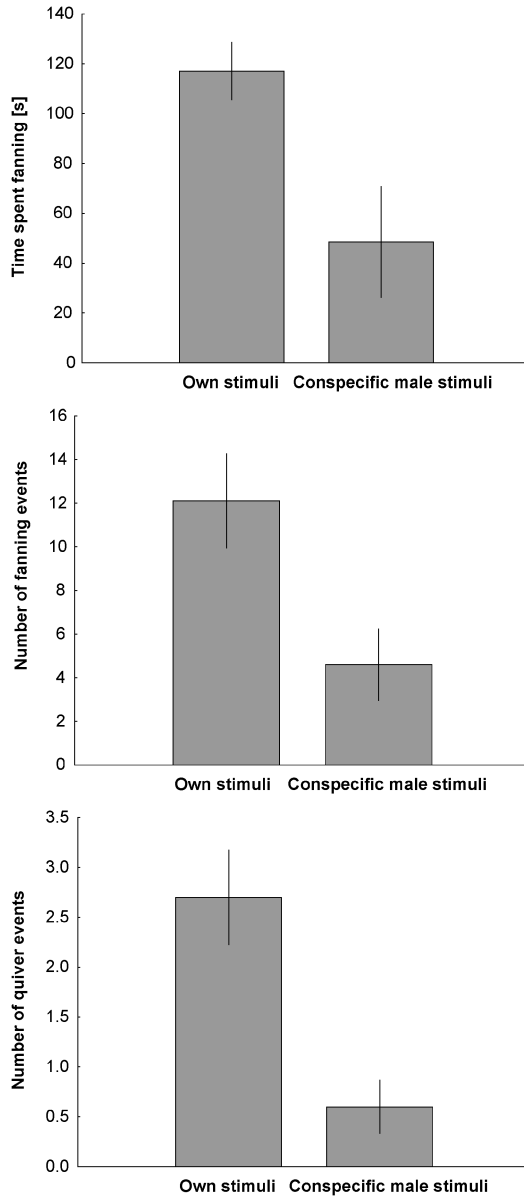
The proportion of trials in which the flamenco display was observed was 40% (four cases) in the own males' water and only 10% (one case) when males were in the water with chemicals from other males. The low variance in the 'conspecific male stimuli' treatment prevented any statistical analyses.

Body length, mass and corpulence did not significantly differ between treatments (male length:  $F_{1,18} = 0.30$ ,  $p = 0.59$ ; male mass:  $F_{1,18} = 0.56$ ,  $p = 0.46$ ; male corpulence:  $F_{1,18} = 0.29$ ,  $p = 0.59$ ; female length:  $F_{1,18} = 0.59$ ,  $p = 0.45$ ; female mass:  $F_{1,18} = 0.01$ ,  $p = 0.91$ ; female corpulence:  $F_{1,18} = 0.25$ ,  $p = 0.62$ ). Furthermore, these traits were not significantly associated with the males' responses ( $0.17 < \text{all } p < 0.98$ ).

### *Experiment 2: Residence asymmetries*

There were no significant differences between treatments in the number of bites performed by focal males as a main effect (within-subject factor:  $F_{1,9} = 1.49$ ,  $p = 0.25$ ; resident treatment: mean  $\pm$  SE =  $0.3 \pm 0.21$ ; intruder





**Figure 1.** (Top) Time (mean  $\pm$  SE,  $N = 20$ ) that *Lissotriton boscai* males spent fanning, (middle) number (mean  $\pm$  SE,  $N = 20$ ) of fanning display events and (bottom) number (mean  $\pm$  SE,  $N = 20$ ) of quiver events performed by males toward females in aquaria containing males' own chemical cues or those from other conspecific males during a period of 10 min.

treatment: mean  $\pm$  SE =  $0.8 \pm 0.51$ ). There were no significant effects of morphological traits (corpulence:  $F_{1,7} = 2.48$ ,  $p = 0.15$ ; length:  $F_{1,7} = 3.23$ ,  $p = 0.10$ ), and the interactions with the treatment were not significant (all  $p > 0.16$ ).

In the same way, there were no significant differences between treatments in the avoidance events as a main effect (within-subject factor:  $F_{1,9} = 0.35$ ,  $p = 0.56$ ; resident treatment: mean  $\pm$  SE =  $2.1 \pm 0.40$ ; intruder treatment: mean  $\pm$  SE =  $1.3 \pm 0.61$ ). There were no significant effects of morphological traits (corpulence:  $F_{1,8} = 1.26$ ,  $p = 0.29$ ; length:  $F_{1,7} = 0.14$ ,  $p = 0.71$ ), and the interactions with the treatment were not significant (all  $p > 0.30$ ). Similarly, the time that focal males spent moving was not significant (within-subject factor:  $F_{1,9} = 0.11$ ,  $p = 0.74$ ; resident treatment: mean  $\pm$  SE =  $68.8 \pm 14.04$ , range 29–165; intruder treatment: mean  $\pm$  SE =  $89.6 \pm 29.76$ , range 17–350), nor were the morphological traits as main effects (corpulence:  $F_{1,7} = 1.55$ ,  $p = 0.25$ ; length:  $F_{1,8} = 0.006$ ,  $p = 0.93$ ). The interactions were not significant (all  $p > 0.19$ ).

## Discussion

In experiment 1, the time that males *L. boscai* spent fanning when courting a female was longer in their own aquaria (containing chemical cues from themselves) than in the aquaria of other males. Moreover, the number of fanning and quiver events was higher in the males' own aquaria. The results cannot be attributed to females' responsiveness, since there were no significant effects of avoidance behaviour or time spent moving by females during trials. Also, there were no effects of absolute or relative competitive ability/size since individual length and corpulence were not significantly associated with male courtship, and pairs were size matched. Finally, the pattern in the proportions of flamenco displays was in agreement with the other variables, although the low variance in the 'conspecific male stimuli' treatment prevented any statistical analyses.

The results of experiment 2 did not provide evidence for site defence or territoriality. This is not an unexpected result since males of this species do not seem to avoid areas containing chemical cues from other males. This finding is supported by a previous experiment in which males selected ponds containing either their own chemical cues or chemicals from other con-

specifics in similar proportions (Aragón et al., 2000). Consistently, a previous study with the sibling species *L. vulgaris* did not find any evidence of site tenancy in the field, but a type of lek mating system similar to other small-bodied newt species (Verrell & McCabe, 1988). Thus, according to the results of experiment 2 and those of previous studies, prior residence does not appear to be involved in the response found in experiment 1. Regardless of whether residence asymmetries are involved or not, the contribution of this study is that males can modify their courtship displays depending on the social environment assessed through conspecific chemical cues and in absence of transmitters. Thus, this study demonstrates that chemical cues can modify courtship interactions in which other transmitters of chemical cues do not participate.

There are two potential non-exclusive explanations for the reduction of the courtship display efforts in presence of chemical cues from other conspecific males. A possibility is that chemical cues from other males might denote higher probability of previous inseminations by competitors, which might bear lower chance of paternity by subsequent courting males. Under this scenario, subsequent male courtship efforts should be reduced or ceased if the costs of courtship (Halliday, 1977) outweigh the fitness benefits. An experiment testing the order of matings in the newt *Taricha granulosa* revealed a previously uncharacterized vertebrate mechanism of sperm competition, where the second male fertilized less than half of female's eggs (Jones et al., 2002). However, similar sperm competition experiments with other species more closely related to the study species do not support this explanation. Thus, it has been demonstrated in *Triturus alpestris* and in the sibling species *L. vulgaris* that the paternity share between two competing males was independent of their order of insemination (Rafiński & Osikowski, 2002; Jehle et al., 2007).

An important component in the context of information network is the so-called audience effect, in which the signallers' behaviour is influenced by other extra potential receivers that do not take part in the signalling interaction (Doutrelant et al., 2001; Matos & Schlupp, 2005). The results of this study can perhaps be best explained by chemically mediated audience effects. Thus, as proposed by Johnston (2005), the audience effects might also extend to cues that can denote the proximity of a potential audience without necessarily implying their physical presence. Theory predicts that signallers should reduce the risk of being detected by competitors by shortening

their display time (Dabelsteen et al., 1998). In this study, the treatment effect on the latency to the first fanning event was not significant, suggesting that rather than beginning later, males reduce the time spent courting. In this and other newt species, sexual interference in nature is common and is mainly caused by males, which often end with the withdrawal of the female with no sperm transfer (Verrell & McCabe, 1988; Hedlund & Robertson, 1989; Halliday, 1990; Mouta-Faria, 1995). Interestingly, field observations showed that male-to-male courtship interference occurs mainly during the males' display phase in *L. boscai*, and that interference was significantly less frequent in shorter sexual sequences (Mouta-Faria, 1995). This suggests that the fanning display might be used as cues by competitor males to detect and interrupt courtships performed by other males. Accordingly, other studies have shown that male newts and salamanders modify their behaviour to avoid courtship interference (Vernell, 1983; Uzendoski et al., 1993). In agreement with the present results, males of *Ambystoma talpoideum* decreased the proportion of time courting females when rival males were present (Verrell & Krenz, 1998). Mouta-Faria (1995) also suggested that *L. boscai* males may reduce the probability of interference by shortening the duration of sexual encounters. However, if males reduce the frequency or length of their courtships, this may also imply a reduction of mating opportunities. For instance, higher frequencies of courtship displays by males of the salamander *Desmognatus ocoee* are associated with higher mating success (Vinnedge & Verrell, 1998). Thus, male newts and salamanders may be subject to a trade-off between advertising to females and limiting the information transfer to other competitor males. Similarly, behavioural adaptations that conceal the information on mate choice when there are costs associated with the physical presence of visual audience have been suggested in fish (Plath et al., 2008). The results of this study warrant future studies to examine whether other vertebrate species use conspecific chemical cues to assess trade-offs between using signals to mate and avoiding the attraction of male potential eavesdroppers.

### Acknowledgements

Financial support was provided to P.A. by an I3P-PC2005L postdoctoral contract. These experiments were performed with a license from the Consejería de Medio Ambiente, Ordenación del Territorio e Infraestructuras del Gobierno del Principado de Asturias (Spain). I thank J.M. Lobo for providing field work facilities.

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