

Chemosensory responses to alcohols found in femoral gland secretions of male Iberian wall lizards *Podarcis hispanica*

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In lizards, femoral gland secretions of males can have an important role in intraspecific communication. However, the chemosensory responses of lizards to specific compounds found in femoral secretions have been little studied. Steroids are the most abundant compounds in secretions, which have been shown to have a signalling function. However, other type of compounds might also be important. In this paper, we explored the role of alcohols in communication of Iberian wall lizards, *Podarcis hispanica*. We studied chemosensory behaviour, by measuring tongue flick rates, to test whether lizards could recognize several alcohols that are naturally found in femoral secretions. Results showed that lizards can discriminate and show chemosensory responses of different magnitude to different alcohols. The existence of high responses to alcohols suggest that, in addition to steroids, alcohols may also have a signalling function in femoral secretions of male *P. hispanica* lizards.

Key words: alcohols, chemical senses, chemoreception, femoral glands, *Podarcis hispanica*

INTRODUCTION

The chemosensory system and chemical communication are well developed in lizards and snakes (Mason, 1992; Mason & Parker, 2010). Behavioural experiments showed a prominent role of chemical signals in social relationships, where, for example, scent of males could convey information about social status and competitive ability (López & Martín, 2002; Carazo et al., 2007; Martín et al., 2007), or even transmit information that females may use to select mates (Martín & López, 2000, 2006c; Olsson et al., 2003; López & Martín, 2005a).

However, in spite of the potential importance of chemical signals in lizard social behaviour, the chemical composition of femoral gland secretions is known in only a few species of lizards (reviewed in Weldon et al., 2008; Mason & Parker, 2010; Martín & López, 2011). Femoral gland secretions are composed of both proteins and lipids. The latter is thought to be the main compounds involved in communication (Mason, 1992; Martín & López, 2011). Moreover, the chemosensory behavioural responses of lizards to specific compounds found in these secretions have been studied in few species (Martín & López, 2006b, 2006c, 2008; Khannoon et al., 2011b), or has been limited to chemicals used for prey identification in foraging rather than in social contexts (Cooper & Pérez-Mellado, 2001; Cooper et al., 2002). However, variations in the responses to different chemicals may be an important first indication of the potential role of some compounds as chemical signals.

The lacertid Iberian wall lizard, *Podarcis hispanica*, is a common lizard living in rocky habitats and on artificial walls of the Iberian Peninsula. This lizard uses multiple types of signals in social behaviour (i.e. colouration and chemical secretions from femoral glands), but chemical

signals seem to be important in sex recognition and rival recognition (López & Martín, 2001, 2002; López et al., 2002; Carazo et al., 2008). The femoral gland secretion of male *P. hispanica* is a mix of steroids, carboxylic acids, waxy esters, alcohols and other minor compounds (Martín & López, 2006a). Among steroids, cholesterol is the most abundant component, as it occurs in many lizard species (Weldon et al., 2008). Other major steroid in secretions is cholesta-5,7-dien-3-ol, which seems to function as a sexual signal in mate choice processes (López & Martín, 2005a; Martín & López, 2006c; López et al., 2009). A previous study showed that female *P. hispanica* can discriminate these two steroids and assess changes in their concentration (Martín & López, 2006b). However, although most previous works have examined the role of steroids as chemical signals, other main compounds in secretions, such as alcohols, may also have a role in chemical communication. In the rock lizard *Iberolacerta monticola*, two alcohols found in the femoral secretions of males (hexadecanol and octadecanol) seem to be a reliable status badge (i.e. signalling aggressive behaviour) (Martín et al., 2007). Similarly, long chain alcohols in femoral secretions elicit avoidance and aggression in male lizards *Acanthodactylus boskianus* (Khannoon et al., 2011b). These alcohols are also found in secretions of *P. hispanica*, so it is likely that alcohols could have a similar role in intraspecific communication of this lizard species.

In this paper, we explore the possible role of alcohols that are naturally found in femoral secretions of males in intraspecific communication of Iberian wall lizards, *P. hispanica*. We designed an experiment of chemosensory recognition, by measuring tongue flick rates, to test whether male and female lizards can recognize and show differential chemosensory responses to several alcohols

also found in males' femoral secretions. We used lizards from two distinct populations because they differ in the chemical composition of femoral gland secretions, and at least males can discriminate between populations based on chemical cues alone (Martín and López, 2006a). Thus, we aimed to test whether there were also inter-population differences in responses to alcohols.

MATERIALS AND METHODS

We captured *P. hispanica* lizards from two distinct populations from the north of the Madrid province (Central Spain); we caught 20 males and 19 females from granite rock-cliffs at the edge of a pine forest in the 'Fuenfría' Valley (40°47' N, 04°03' W; 1750 m altitude), and 18 males and 31 females on granite rocky outcrops in a large oak forest ('Golondrina') near Cercedilla village (40°44' N, 04°02' W; 1250 m altitude). All lizards were individually maintained in captivity at "El Ventorrillo" Field Station (Cercedilla, Madrid) following methods described in López et al. (2009). All lizards were healthy during the trials, without showing behavioural or physiological changes, and were returned to their exact capture sites at the end of experiments.

We used tongue-flick (TF) behaviour in response to chemical stimuli as a bioassay for measuring discrimination of chemical cues (Cooper & Burghardt, 1990). We measured TF rates of lizards (males and females) to stimuli arising from cotton applicators bearing scents of (1) cholesterol, (2) hexadecanol, (3) octadecanol (4) docosanol, (5) tetracosanol and (6) dichloromethane (DCM). The rationale for testing these compounds was that: (1) cholesterol is the most abundant compound in femoral secretions. We previously knew that *P. hispanica* lizards can discriminate and show high chemosensory responses to it (Martín & López, 2006b); thus, this compound was considered as a control indicating a high chemosensory response. (2) Hexadecanol and (3) octadecanol are present in the femoral secretions and could be putative chemical signals signalling male dominance status, as it occurs in other lacertid lizard species (Martín et al., 2007); the rest of alcohols, (4) docosanol and (5) tetracosanol, are also found in femoral secretions of males, and they might also have a signalling function as it occurs in other lacertid lizards (Khannoon et al., 2011b). Finally, (6) DCM was used as a control to gauge baseline TF rates under the experimental conditions because all compounds were dissolved in DCM for trials (see below).

We prepared chemical stimuli the day of the tests by dissolving 30 mg of each compound (authentic standards, GC grade, Sigma-Aldrich, St. Louis, MO) in 1 ml of DCM (Sigma-Aldrich Chromasol V plus for HPLC, purity >99.9%) in glass vials with Teflon-lined stoppers, and kept the vials in a refrigerator. Immediately before the trials, we prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) for 3 s in vials containing DCM alone, or the other chemicals dissolved in DCM. The DCM readily evaporated after a few seconds leaving the compounds deposited on the swabs, but we waited for 15 min before presenting the swabs to the lizards to ensure total evaporation of DCM.

We considered that this time was enough long as to allow DCM to evaporate completely. Moreover, the very low TF responses to swabs impregnated with DCM alone (see Results) suggested that either DCM was completely evaporated or that lizards did not react to DCM alone.

Every lizard was confronted to each stimulus in a randomized order, but only one trial (i.e. only one chemical stimulus) was conducted per day for each animal. Trials were conducted in outdoor conditions during April, which coincided with the mating season of lizards in their original natural populations (Martín & López, 2006a), and between 1100–1300 hours (GMT) when lizards were fully active and had attained optimal body temperatures.

To begin a trial, the experimenter (MG) slowly approached the terrarium and slowly moved the cotton swab to a position 1 cm anterior to the lizards' snout. Lizards usually did not flee from the swab, but explored it repeatedly by tongue-flicking or ignored it after the first TFs, but if the lizard moved, the swab was quickly and gently repositioned in front of the lizard. The numbers of TFs directed to the swab were recorded for 60 s, beginning with the first TF.

To examine differences in number of directed TFs (log-transformed) by individuals (males and females) among compounds presented, we used two or three-way repeated measures ANOVAs with chemical stimuli as a within factor, and with the sex (male vs. female) and population of origin (Golondrina vs. Fuenfría) of the responding lizard as between factors.

RESULTS

Lizards' TF rates towards chemical stimuli differed significantly (three-way repeated measures ANOVA, SCENT: $F=285.13$, $df=5,420$, $p<0.0001$). The sex and the population of origin of lizards had no significant effects on the overall number of TFs (SEX: $F=2.57$, $df=1,84$, $p=0.11$; POPULATION: $F=0.95$, $p=0.33$), but responses to the different chemicals varied significantly between males and females (SCENT*SEX: $F=7.00$, $df=5,420$, $p<0.0001$), and between populations (SCENT*POPULATION: $F=5.41$, $df=5,420$, $p<0.0001$). Thus, we analyzed separately the responses of males and females (see below). The interaction between sex and population was not significant (SEX*POPULATION: $F=0.38$, $df=5,420$, $p=0.86$) (Fig. 1). In all cases, the TF responses of lizards in response to any compound dissolved in DCM were significantly higher than in response to the solvent DCM alone (Tukey's test: $p<0.05$ for all).

In females, we found significant differences in TF rates among chemical stimuli (two-way repeated measures ANOVA, SCENT: $F=175.64$, $df=5,240$, $p<0.0001$) (Fig. 1A), but not between populations (POPULATION: $F=2.78$, $df=1,48$, $p=0.10$), and females from different populations responded differentially to the different chemical stimuli (SCENT*POPULATION: $F=3.63$, $df=5,240$, $p=0.003$). Females from Fuenfría made significantly more TFs in response to cholesterol, tetracosanol and hexadecanol than in response to octadecanol and docosanol (Tukey's tests: $p<0.001$ for all). There were no significant differences

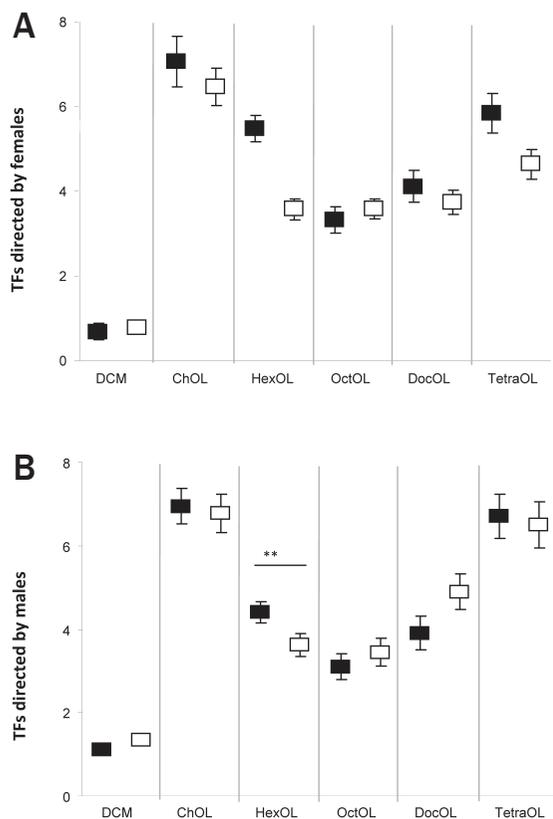


Fig. 1. Mean (\pm SE) tongue flick rates directed by (A) female or (B) male *P. hispanica* from two populations (Fuenfría in black and Golondrina in white) in response to several compounds: cholesterol (ChOL), hexadecanol (HexOL), octadecanol (OctOL), tetracosanol (TetraOL), docosanol (DocOL) and dichloromethane (DCM).

between responses to cholesterol, tetracosanol and hexadecanol ($p > 0.50$ for all) nor between octadecanol and docosanol ($p = 0.99$). In contrast, females from Golondrina showed a significantly higher number of TFs in response to cholesterol than to all the other compounds ($p < 0.001$ for all), and there were not any significant difference among the alcohols ($p > 0.08$ for all).

In males, there were significant differences among chemical stimuli in TFs directed (two-way repeated measures ANOVA, SCENT: $F = 123.90$, $df = 5, 180$, $p < 0.0001$) (Fig. 1B), the population of origin had no significant effects on overall TFs elicited by males (POPULATION: $F = 0.31$, $df = 1, 36$, $p = 0.58$), but the interaction between scent stimuli and population was significant (SCENT* POPULATION: $F_{5,180} = 2.27$, $df = 5, 180$, $p = 0.049$). Only for hexadecanol, we observed significant differences between populations; males from Fuenfría showed a significantly higher number of TFs in response to hexadecanol than males from Golondrina (Tukey's test: $p < 0.0001$).

Males made a significantly higher number of TFs in response to cholesterol and tetracosanol than to hexadecanol, docosanol and octadecanol (Tukey's test: $p < 0.03$ for all). In males from Golondrina, the TF rates in response to tetracosanol and hexadecanol were not significantly different ($p = 0.11$). There were no significant differences (in both populations) between cholesterol and

tetracosanol ($p = 0.99$ for both), nor between hexadecanol and docosanol (Fuenfría: $p = 0.95$; Golondrina: $p = 0.31$). There were significant differences in the responses to docosanol and octadecanol in males from Golondrina ($p = 0.048$) but not in males from Fuenfría ($p = 0.67$), and between hexadecanol and octadecanol in males from Fuenfría ($p = 0.02$), but not in males from Golondrina ($p = 0.99$).

DISCUSSION

This study first showed that *P. hispanica* lizards can discriminate between the solvent (i.e. DCM alone) and the tested compounds (cholesterol and several alcohols dissolved in DCM). This result indicated that chemosensory responses were elicited by these compounds alone, and not by the solvent. Furthermore, we used cholesterol as a reference. We knew that these lizards recognize and show a high chemosensory response to this steroid (Martín & López, 2006b), so the comparison with the responses to cholesterol could inform on the relative magnitude of the responses to other compounds. We found that lizards had similar high responses to some alcohols (hexadecanol and tetracosanol) than to cholesterol. These results strongly suggest that *P. hispanica* lizards could detect and discriminate alcohols presented in femoral secretions of males.

We observed the highest number of tongue flicks (TF) in response to tetracosanol or 'lignoceric alcohol'. This compound is a fatty alcohol with a hydrocarbon chain of 24 carbons that is also present in large amounts in secretions of lacertid lizards of the genus *Acanthodactylus* (López & Martín, 2005b; Khannoon et al., 2011a). This and other long chain alcohols elicit aggressive responses in male *A. boskianus* (Khannoon et al., 2011b), which suggest that these alcohols may function as scent marking pheromones. High levels of long chain alcohols in secretions might be related to the condition of a male, which could be an indication of his dominance status.

Similarly, *P. hispanica* lizards had relatively higher chemosensory responses to hexadecanol, but it seems that it was not clearly discriminated from octadecanol and docosanol. Nevertheless, it is likely that we could not find significant results in our experiment because differences in the responses to different alcohols were very small. Perhaps, not just one alcohol could have a signalling function, but the blend of several alcohols and/or the different relative proportions of each one could be the true signal (see Johnston, 2005). The concentration of hexadecanol, and also octadecanol, could be related to, and may signal the dominance status of a male as it occurs in the lizard *I. monticola* (Martín et al., 2007).

Interestingly, we found some inter-population differences in chemosensory responses to alcohols. Males from Fuenfría showed higher responses to hexadecanol than males from Golondrina. Also females from the two populations responded differently to the alcohols tested, with females from Golondrina showing lower responses to alcohols than to cholesterol. These two populations differ in morphology and chemical signals, and males discriminate between males of the two populations

based on femoral secretions alone (Martín & López, 2006a). Moreover, males from Fuenfría have greater proportions of alcohols in femoral secretions than males from Golondrina, although proportions of hexadecanol are similar (Martín & López, 2006a). Alcohols in femoral secretions might allow this discrimination and have different signalling roles in these populations. This is important because chemoreception is implied in sexual selection processes in this lizard (López et al., 2002; López & Martín, 2005a; Carazo et al., 2007), and differences in chemical signals and in chemosensory responses might lead to reproductive isolation between populations and further speciation processes. This might reflect or explain the high diversity of genetic lineages found in this lizard (Kaliontzopoulou et al., 2011). Thus, more experiments are necessary to analyze why lizards of these populations show differential responses to alcohols.

In summary, *P. hispanica* lizards can discriminate several alcohols found in male femoral secretions. We suggest that the concentration of some of these alcohols in secretions might be related to the condition or social status of individuals. Alcohols could signal this information alone or be associated with other chemical compounds in the femoral secretions, such as steroids, which also have been previously shown to have a signalling function (López & Martín, 2005a). Thus, alcohols presented in secretion of male *P. hispanica* may have a signalling function, providing more opportunities to signal a males' quality or condition via different circumstances, but also may reinforce the reliability of the signal when several types of compounds (i.e. steroids and alcohols) may be perceived simultaneously. However, more experiments are necessary to know the potential signalling role of alcohols in femoral secretions of lizards and the consequences of interpopulational variation in the responses.

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