Intersexual differences in chemosensory responses to selected lipids reveal different messages conveyed by femoral secretions of male Iberian rock lizards

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Abstract. The effects of intrasexual selection (i.e., male-male competition) and intersexual selection (i.e., mate choice) may result on the evolution of different secondary sexual traits. We tested whether chemosensory responses of male and female *Iberolacerta cyreni* lizards to femoral secretion of conspecific males (a chemical sexual trait used in social behavior) were eliciting by different chemical traits. Tongue-flick essays showed that males and females had similar chemosensory responses to the femoral secretions of males, but males and females differed in the magnitude of their chemosensory responses to the different chemicals found in secretions. Moreover, responses to chemicals related to body size depended on the own body size of the responding male, but did not in females. These results might support that femoral secretions of males convey different messages for male or female *I. cyreni* lizards.

Keywords: chemosensory recognition, femoral glands, *Iberolacerta cyreni*, Lacertids, sexual selection.

It is generally assumed that success in male-male competition reflects high quality and that female preference for dominant males should be widespread (Quarnström and Forsgren, 1998). Thus, traits reflecting male dominance, such as large body size or status badges, are expected to be the same traits also selected by females. However, many studies suggest that traits signaling male dominance are not always attractive to females because they might not be reliable indicators of better paternal care or genetic quality for females (Quarnström and Forsgren, 1998; Moore and Moore, 1999). Thus, in many cases the effects of intrasexual selection (i.e., male-male competition) and intersexual selection (i.e., mate choice) may result on the evolution of different secondary sexual traits.

Chemical signals play an important role in sexual selection of many vertebrates including lizards (Mason, 1992; Wyatt, 2003), but little is known on the origin and roles of specific chemicals as secondary sexual traits. In lizards, pheromonal detection is often based on femoral gland secretions of males (e.g., Mason, 1992; Alberts, 1993; Aragón et al., 2001a). Femoral glands can be considered as secondary sexual traits because their secretory activity is greater in males and during the breeding season, and is under direct androgenic control (Van Wyk, 1990; Alberts et al., 1992; Alberts, 1993). Femoral secretions are passively deposited on the substrate as male lizards move, and, therefore, they could convey information about residence, social status, or other characteristics of the signaler (Aragón et al., 2001b; López and Martín, 2002, 2005a; Olsson et al., 2003). However, the function and "message" conveyed by specific chemical compounds in secretions and, moreover, "for whom" they are delivered, remain largely unknown. We hypothesized that sexually dimorphic chemical traits may be advanced by different selection pressures. The secretion of some chemicals might have evolved to function in intrasexual male-male relationships (e.g., those signaling dominance), but they may be unimportant for females, which may "prefer" other chemical traits in their potential mates (e.g., those signaling genetic quality).

The Iberian rock lizard, *Iberolacerta cyreni* (formerly *Lacerta monticola cyreni*), is a small

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diurnal lacertid found in rocky mountains of the Central Iberian Peninsula (Pérez-Mellado, 1998). Males defend territories including several females, but overlap between home ranges is extensive, fights between males are frequent, and dominance hierarchies often emerge with larger males being more dominant (López et al., 2003; Aragón et al., 2004, 2006). Males can discriminate between self-produced scents and those of conspecific familiar and unfamiliar males (Aragón et al., 2000, 2001a,b). Scent marked substrates may inform on the body size-dependent dominance status of the signaler in *I. cyreni* lizards (Aragón et al., 2000, 2001b). However, male body size and dominance seem unimportant to females, which prefer scent marks of males with a presumably high heritable genetic quality (Martín and López, 2000, 2006a,b; López et al., 2002). Chemical analyses showed that the lipophilic fraction of femoral gland secretions of males is mainly a mixture of fatty acids and steroids (López and Martín, 2005b). Larger males have secretions with higher proportions of cholesterol (López et al., 2006), which might function as a body size-dependent fighting ability badge in agonistic interactions between males (Martín and López, 2007). On the other hand, males with a greater T-cell immune response had higher proportions of cholesta-5,7-dien-3-ol in their femoral secretions (López et al., 2006), which females prefer in substrate scent marks (Martín and López, 2006a,b). Thus, these two chemicals may be the relevant chemical traits eliciting chemosensory responses of either males or females to femoral secretions of other males.

In this paper, we tested whether chemosensory responses of male and female *I. cyreni* to femoral secretion of conspecific males were eliciting by different chemical traits. We used tongue-flick essays to test the predictions that, if males and females used different characteristics of males' scent (i.e., different chemicals) to decide their social behavior, 1) males and females might show similar chemosensory responses to the femoral secretions of males, but 2) males and females will differ in the magnitude of their chemosensory responses to the different chemicals found in secretions. Moreover, we predicted that, if males were able to assess body size of competitor males based on their scent alone, the responses to chemicals related to body size should depend on the own body size of the responding male, whereas female responses should not be body size-dependent.

We captured by noosing adult *I. cyreni* lizards with intact or fully regenerated tails (16 males and 16 females) at the beginning of May 2006, before the start of their mating season, in different places over a 5 km^2 area ("Puerto de Navacerrada", Guadarrama Mountains, Central Spain). We measured lizard snout-to-vent length (SVL) with a ruler, and housed lizards individually at "El Ventorrillo" Field Station (5 km from the capture site) in outdoor 80 × 50 cm PVC terraria containing, rocks for cover, and food (mealworms) and water *ad libitum*. We placed cages of males and females in different areas to avoid contact between them. All animals were healthy and we returned them to their capture sites at the end of trials.

Lizards respond to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper and Burghardt, 1990; Cooper, 1994). Tongue-flick (TF) rate can, therefore, be used as a quantitative bioassay of detection of chemical cues. Thus, we made comparisons of TF rate by male and female lizards in response to chemical stimuli arising from cotton applicators impregnated with femoral gland secretions of males or with deionized water (used as an odorless control to gauge baseline TF's rates in the experimental situation) (Cooper and Burghardt, 1990).

We prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) in deionized water. Femoral secretions of males consisted of a waxy substance, which was easily extracted by gently pressing around the femoral pores, and collected directly on cotton tips of applicators. We used a new swab in each trial. Every lizard was exposed in two different days to water and male's scent in a counterbalanced order. Trials were conducted in outdoor conditions at the middle of May, which coincided with the mating season of lizards (Aragón et al., 2004), and between 1100-1300 h (GMT) when lizards were fully active. To begin a trial, one experimenter slowly approached each lizard's home cage and slowly moved the cotton swab to a position 1 cm anterior to the lizards' snout. We recorded the time ('latency') between presentation of the swab and the first directed TF, and noted the number of TFs directed to the swab for 60 s beginning with the first TF.

To examine responses to specific chemical compounds by lizards, we compared latency to first TF and directed TF rate by male and female lizards in response to stimuli arising from cotton applicators bearing (1) dichloromethane (DCM; solvent control), (2) cholesterol, and (3) cholesta-5,7-dien-3-ol. We prepared chemical stimuli the same day of the tests by dissolving 24 mg of each compound (authentic standards, GC grade, from Sigma-Aldrich Chemicals) in

1 ml of DCM inside glass vials closed with Teflon-lined stoppers. Then, we mixed the solution with a vortex, and kept the vials in a refrigerator. Immediately before the trials, we prepared stimuli by dipping the cotton tip of a wooden applicator in vials containing chemicals. To human observers, the swabs prepared with all stimuli were visually similar. We used a new swab in each trial.

To examine differences in latency to first TF and number of directed TFs between conditions, we used two-way repeated measures analyses of variance (ANOVAs) with scent stimuli as a within factor, and sex of the responding individual as a between factor. Data were log-transformed to ensure normality and homogeneity of variances (tested with Levene's test) (Sokal and Rohlf, 1995). Pairwise comparisons used Tukey's honestly significant difference tests. We used Pearson's correlations to examine the influence of body size (logSVL) on chemosensory responses (log.-transformed TF rates and latencies) (Sokal and Rohlf, 1995).

In the tests of chemosensory responses to femoral secretions of males, all lizards responded to swabs by tongue flicking. Both males and females showed mean latencies to the first TF that were significantly shorter for femoral secretions of males than for water (repeated measures two-way ANOVA: $F_{1,30} = 174.68$, $P < 0.0001$), females tended to show shorter, but not significantly different, latencies than responding males ($F_{1,30} = 3.84$, $P = 0.059$), and the interaction was not significant ($F_{1,30} = 0.85$, $P = 0.36$) (fig. 1a). Both male and females elicited significantly more TFs directed to femoral secretions of males than to water (repeated measures two-way ANOVA: $F_{1,30} = 111.59$, $P < 0.0001$), there were not significant differences between sexes of responding lizards ($F_{1,30} = 0.21$, $P = 0.85$), and the interaction was not significant ($F_{1,30} = 2.52$, $P = 0.12$) (fig. 1b).

In the tests of responses to chemical compounds, all lizards responded to swabs by tongue flicking. Latency to the first TF differed significantly between chemical compounds stimuli (repeated measures two-way ANOVA: $F_{2,60} = 32.97$, $P < 0.0001$), females had significantly shorter latencies than males ($F_{1,30} = 35.15$, $P < 0.0001$), but the interaction was significant ($F_{2,60} = 13.87$, $P < 0.0001$) (fig. 2a). Thus, in males, there were significant differences between latencies to the three chemical compounds (Tukey's tests: $P < 0.0005$ in all cases), but latencies of females did not differ between stimuli ($P > 0.10$ in all cases). Males showed significantly longer latencies than females to DCM and cholesta-5,7-dien-3-ol ($P < 0.006$ in both cases) but latencies to cholesterol were equally short than those of females ($P = 0.95$).

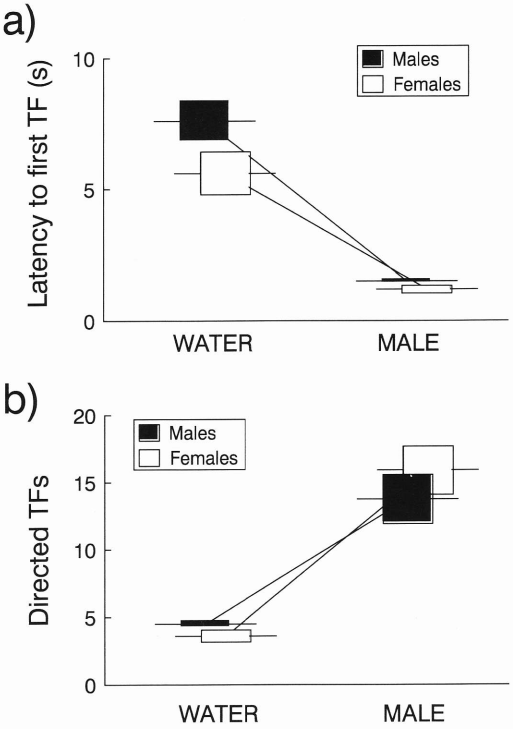

Figure 1. Mean (± SE) a) latency and b) number of tongue-flicks (TF) directed to swabs by male and female lizards in response to cotton-tipped applicators bearing deionized water or femoral gland secretions of conspecific males.

In males, latencies to cholesterol were significantly and positively correlated with the SVL of the responding male ($r = 0.55$, $F_{1,14} = 5.96$, $P = 0.028$), with smaller males showing shorter latencies to cholesterol. However, SVL did not significantly affect the latencies to DCM ($r = -0.19$, $F_{1,14} = 0.52$, $P = 0.48$) or to cholesta-5,7-dien-3-ol ($r = 0.16$, $F_{1,14} = 0.39$, $P = 0.54$). In contrast, in females SVL did not significantly influence their latencies to DCM ($r = -0.26$, $F_{1,14} = 1.00$, $P = 0.33$) or to cholesterol ($r = -0.37$, $F_{1,14} = 2.18$, $P = 0.16$), but larger females had significantly

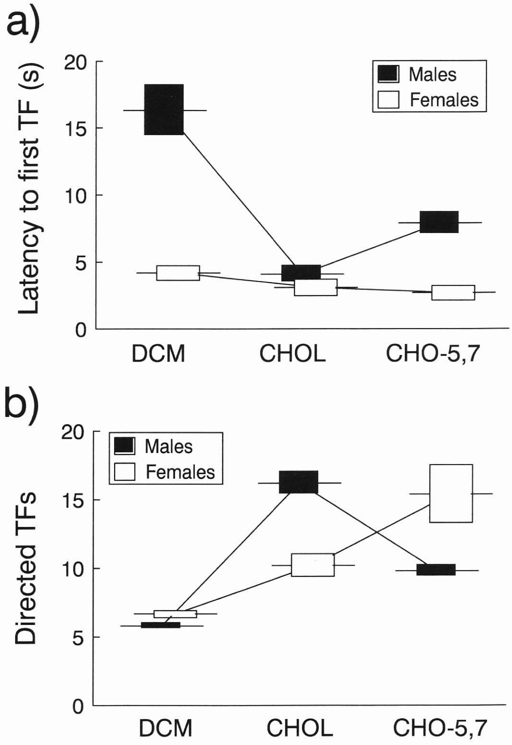

Figure 2. Mean (± SE) a) latency and b) number of tongue-flicks (TF) directed to swabs by male and female lizards in response to cotton-tipped applicators bearing dichloromethane (DCM; solvent control), cholesterol (CHOL) or cholesta-5,7-dien-3-ol (CHO-5,7), all dissolved in DCM.

shorter latencies to cholesta-5,7-dien-3-ol ($r = -0.71$, $F_{1,14} = 14.28$, $P = 0.002$).

The rate of TFs directed to swabs differed significantly among chemical compounds stimuli (repeated measures two-way ANOVA: $F_{2,60} = 116.44$, $P < 0.0001$). Overall TF rates did not differ significantly between sexes ($F_{1,30} = 0.18$, $P = 0.67$), but the interaction was significant ($F_{2,60} = 41.16$, $P < 0.0001$) (fig. 2b). Thus, both males and females elicited significantly different TF rates to the different chemicals (Tukey's tests: $P < 0.0001$ in all cases), but, although TF rates to DCM did not differ between sexes ($P = 0.89$), males elicited significantly higher TF rates to cholesterol ($P < 0.01$) and lower TF rates to cholesta-5,7-dien-3-ol ($P < 0.01$) than females.

In males, the TF rate to cholesterol was significantly and negatively correlated with the SVL of the responding male ($r = -0.54$, $F_{1,14} = 5.73$, $P = 0.03$), with smaller males responding more strongly to cholesterol. However, SVL did not significantly affect the responses to DCM ($r = -0.16$, $F_{1,14} = 0.37$, $P = 0.55$) or to cholesta-5,7-dien-3-ol ($r = -0.22$, $F_{1,14} = 0.18$, $P = 0.42$). In contrast, in females SVL did not significantly influence their TF rates to any stimuli (DCM: $r = 0.20$, $F_{1,14} = 0.57$, $P = 0.46$; cholesterol: $r = -0.17$, $F_{1,14} = 0.44$, $P = 0.52$; cholesta-5,7-dien-3-ol: $r = 0.03$, $F_{1,14} = 0.01$, $P = 0.98$).

The shorter latencies and greater directed TF rates in response to femoral secretions of conspecific males and the two steroids found in secretions indicated that both male and female *I. cyreni* were able to detect these secretions and these two chemicals. Because these lipids are also found in femoral gland secretion of males (López and Martín, 2005b), it is very likely that males and females can also detect their presence in scent marks of males. Males and females showed apparently similar chemosensory responses to femoral secretions. However, the intersexual differences in the responses to the different chemicals suggested that the magnitude of the responses to femoral secretions was elicited by different chemicals.

The higher TF rates of females to cholesta-5,7-dien-3-ol indicated that females discriminated this steroid from the chemically very similar cholesterol, an ability also found in female Iberian wall lizards *Podarcis hispanica* (Martín and López, 2006c). Our current results, together with the previous finding of female preference for scent marks of males with higher proportions of this steroid in their secretions (Martín and López, 2006a,b), support the idea that cholesta-5,7-dien-3-ol was a "key" chemical for females. This is probably due to the relationship between the proportion of this lipid in femoral secretions and the "quality" of the male (Martín and López, 2006b), a relationship also found in male *P. hispanica* (López and Martín, 2005a). This steroid is a precursor of vitamin D_3, which is essential in calcium metabolism (Fraser, 1995; Ferguson et al., 2005). Therefore, it is likely that only high quality males could divert vitamin D_3 from metabolism to allocate high amounts of cholesta-5,7-dien-3-ol to secretions. The chemical signal would, therefore, be costly and condition dependent, and may provide reliable information on the male's condition as suggested by a previous manipulative experiment (Martín and López, 2006a). Females might decide where to establish their home ranges based on the presence of high proportions of this steroid in scent marks left by territorial males (Martín and López, 2006b). The shorter latencies to this chemical by larger, older, females might suggest more experience in detecting it.

In contrast, cholesterol elicited the greatest chemosensory responses in males. Chemosensory discrimination of cholesterol from other lipids and water was also suggested in male lizards *Podarcis lilfordi* (Cooper et al., 2002). Moreover, our results showed that chemosensory responses of males to cholesterol, but not to other chemicals, depended on their own body size, further supporting the idea that cholesterol might be a chemical status badge in this lizard (Martín and López, 2007). This result agrees with previous experiments where the responses of male lizards to substrate scent marks of and unfamiliar male depended on the body size difference between both males (Aragón et al., 2000, 2001b), which indicated that males were able to assess body size of unknown males based on chemicals alone. Larger sized, presumably more dominant males (López et al., 2002), responded less strongly towards cholesterol. Considering that the presence of another male's chemical signal in a male's own home terrarium may represent a lower threat for dominant than for subordinate males, larger-dominant males may be expected to respond less strongly. This suggests that proportions of cholesterol in scent marks may be used as a signal of dominance or fighting ability between males, and not only during agonistic interactions (Martín and López, 2007). Similarly, other chemical traits alone may reveal dominance status in animals such as some invertebrates (Moore et al., 1997; Zulandt-Schneider et al., 2001), mammals (Hurst, 1983) and other lizards (Alberts et al., 1992; Martin et al., 2007).

Nonetheless, our results indicated that females also discriminate chemically cholesterol, although their responses were lower, which suggests that this steroid might, at least, convey information regarding the presence of a male. Since cholesterol is more abundant in secretions (López and Martín, 2005b) and may be "easier to find" in scent marks, it may release further chemosensory investigation of other, more relevant, chemical traits. Thus, it was suggested that cholesterol in gland secretions of *Liolaemus* lizards might constitute an unreactive apolar matrix that aids in the delivery of other truly semiochemicals (Escobar et al., 2003). The absence of a size-dependent response to cholesterol in females suggests that even if cholesterol proportions are related to male dominance (López et al., 2006), this message might be irrelevant for females.

We conclude that male and female *I. cyreni* are able to use their chemical senses to detect femoral secretions of males and some of the major lipids that they contain, but that different chemicals elicited different responses in males and females. These differential chemosensory responses, together with the previous finding of female preference for scent marks of males with higher proportions of cholesta-5,7-dien-3-ol (Martín and López, 2006a,b), the relationship between male body size and cholesterol in secretions (López et al., 2006), and the body size-dependent response of males to cholesterol, might support that femoral secretions convey different messages for male or female *I. cyreni* lizards.

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