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Links between male quality, male chemical signals, and female mate choice in Iberian Rock Lizards

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Summary

1. In spite of the fact that chemoreception plays an important part in sexual selection of many vertebrates, there is a lack of general knowledge on the role of specific chemical compounds as sexual signals, and on how the characteristics of chemical signals mediate behaviours such as mate choice.

2. We examined how female Iberian Rock Lizards use the chemical characteristics of femoral gland secretions of males to select potential mates.

3. Tongue-flick assays and choice of scent trials showed that females discriminated by chemosensory cues alone differences in chemical secretions of different males. Females showed stronger chemosensory responses and preferred the scent of males of presumably high quality (i.e. those more symmetric and with a greater immune response).

4. Chemical analyses by gas chromatography-mass spectrometry showed that these preferred males allocated relatively more cholesta-5,7-dien-3-ol and ergosterol to femoral secretions, which suggested that females used these chemicals to choose between males' scents.

5. Further experiments confirmed that females were able to discriminate cholesta-5,7-dien-3-ol, ergosterol, and changes in their concentrations, from similar steroids (i.e. cholesterol) also found in secretions of males. Moreover, females were more attracted to areas that were experimentally manipulated to increase the proportion of ergosterol in natural scent marks of males.

6. We suggest that femoral secretions with higher proportions of cholesta-5,7-dien-3-ol and ergosterol might be reliable advertisements of quality of a male, which females could use to select mates.

Key-words: chemoreception, fluctuating asymmetry, immune response, sexual selection, steroids

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Introduction

In spite of the fact that chemoreception plays an important role in intraspecific communication and sexual selection of many vertebrates, such as mammals or reptiles (Mason 1992; Penn & Potts 1998; Wyatt 2003), there is a lack of general knowledge on how the characteristics of chemical signals mediate these behaviours, and on the mechanisms that may maintain their use as sexual signals. In many lizard species, femoral glands, located on the ventral surface of the thigh, produce copious amounts of holocrine secretion, especially in males and during the mating season (Mason 1992; Alberts 1993). Secretions are composed of both proteins and lipids, but the latter are thought to be the main compounds involved in communication (e.g. Alberts 1990; Weldon *et al*. 1990; Alberts *et al*. 1992; Mason 1992; Escobar, Labra & Niemeyer 2001; López & Martín 2005a,b). Moreover, behavioural experiments suggested that these secretions might transmit chemical information about the characteristics of a male, which females may use to choose mates (Martín & López 2000a; López, Muñoz & Martín 2002; López, Aragón & Martín 2003; Olsson *et al*. 2003; López & Martín 2005a).

Theoretical models predicted that signals used in mate choice can only be evolutionarily stable if they are honest and condition dependent or costly to the signaller, and if the cost is correlated with the signaller's quality (Zahavi 1975; Pomiankowski 1988; Grafen 1990). However, in vertebrates, the role of specific chemical compounds as sexual signals, their relationships with male quality, and the mechanisms that

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confer honesty to these chemical signals remain largely unknown. Behavioural studies suggest that immunocompetence of males may affect the information content of chemical signals (Penn & Potts 1998; Rantala *et al*. 2002, 2003; López & Martín 2005a). Females might use this relationship to select males because immune function can be an indicator of health state (e.g. Sheldon & Verhulst 1996; Westneat & Birkhead 1998; Svensson, Sinervo & Comendant 2001). Low levels of asymmetry are also preferred by females in a potential mate in many species (Møller & Thornhill 1998; Swaddle 2003) because they presumably indicate the developmental stability of an individual and, thus, its ability to cope with genetic and environmental perturbations during development (Møller & Swaddle 1997). There is evidence that levels of symmetry of potential mates are sometimes also assessed by females based on chemical cues alone (Thornhill 1992; Rikowski & Grammer 1999; Martín & López 2000a). Therefore, the characteristics of chemical signals seem to be affected by the 'quality' of the male that produces them, and this might be the basis of mate choice in species that employ chemoreception in sexual selection processes. However, although there is indirect evidence through behavioural observations of a possible link between male quality, chemical signals, and female preferences, there is no direct evidence of which mechanisms are actually used by females to assess male characteristics by using chemical senses alone.

The Iberian Rock Lizard *Lacerta monticola*, is a small diurnal lacertid lizard found mainly in rocky mountain habitats of the Iberian Peninsula (Pérez-Mellado 1998). Scent marks of males seem to advertise residence and convey information about competitive ability (Aragón, López & Martín 2001). Femoral secretions might reveal some males' traits, such as the age, or the levels of asymmetry that may be used by female lizards in their mate choice process (Martín & López 2000a; López *et al*. 2002, 2003). Therefore, chemoreception may be especially important in sexual selection in this lizard species. Chemical analyses have shown that the lipophilic fraction of femoral secretions of males is mainly a mixture of fatty acids and steroids (López & Martín 2005b). Interestingly, males with a better health state (i.e. with a greater T-cell immune response) had higher proportions of cholesta-5,7-dien-3-ol and ergosterol in their femoral secretions (López, Amo & Martín 2006), which was also found in other lacertid lizard species (López & Martín 2005a). The data suggest that these steroids might be 'key' chemicals related to the quality of males, and that this relationship might be the basis of female mate choice based on chemical signals.

In this paper, we examined how female Iberian Rock Lizards use the chemical characteristics of femoral gland secretions of males to select potential mates. We analysed the chemical composition of femoral gland secretions of males by using gas chromatography-mass spectrometry (GC-MS), and examined the relationships between characteristics of chemical signals, males' traits related to their quality (i.e. symmetry levels and immune response), and the attractiveness of their scents to females. Based on previous correlational data (see López *et al*. 2006), we expected that if chemical signals were reliable signals of quality, males of presumably high quality (i.e. those more symmetric and with a greater immune response) would be able to allocate more cholesta-5,7-dien-3-ol and ergosterol to femoral secretions.

Then, we used tongue-flick (TF) assays and choice of scent trials to test the predictions that, if females used the chemical characteristics of males' scents to select mates, females should be first able: (1) to discriminate by chemosensory cues alone differences in chemical composition of secretions of different males, and (2) to discriminate cholesta-5,7-dien-3-ol, ergosterol, and changes in concentration of these chemicals, from similar steroids (i.e. cholesterol) also found in secretions of males. Also (3) females should be more attracted to areas scent marked by males with a more natural proportion of these steroids in their secretions. Furthermore, we did an experiment to test the prediction that (4) females should be more attracted to areas that were experimentally manipulated to increase the proportion of ergosterol in natural scent marks of males.

Materials and methods

STUDY ANIMALS

We captured study animals by noosing adult *L. monticola* lizards (16 males and 16 females) at the beginning of May 2005, before the start of their mating season, in different places over a 5 km^2 area ('Puerto de Navacerrada', Guadarrama Mountains, Central Spain). Only adult lizards of similar body size/age and with intact or fully regenerated tails were considered. Lizards were individually housed at 'El Ventorrillo' Field Station, 5 km from the capture site, in outdoor 80 × 50 cm PVC terraria containing rocks for cover. Food (mealworms) dusted with a multivitamin powder and water were provided *ad libitum*. Cages of males and females were in different places to avoid contact between them. All animals were healthy and were returned to their capture sites at the end of trials.

MEASUREMENTS OF SYMMETRY AND HEALTH OF MALES

We counted under a magnifying glass the number of femoral pores on the right and left hindlimbs of males (right limb: 19 ± 1 pores, range = 17–20; left limb: 18 ± 1 pores, range = 17–20; $n = 16$ males). Counts of femoral pores were highly repeatable, and the absolute value of asymmetry of the femoral pores, calculated as the unsigned right-minus-left number of pores, exhibited the properties of fluctuating asymmetry (Møller & Swaddle 1997; see Martín & López 2000a for statistical analyses of asymmetry in this lizard species). Individuals with symmetric femoral pores were also more symmetric in several other meristic characters (unpublished data).

To assess the T-cell-mediated immune (CMI) response of males we used a delayed-type hypersensitivity test; the phytohaemagglutinin (PHA) injection test. This is considered a reliable measure of T-cell-dependent immunocompetence *in vivo*, also in lizards (Svensson *et al*. 2001; Belliure, Smith & Sorci 2004). Tests were made the day after capture. We marked a point with permanent ink on the right foot pad, and measured the thickness at this point with a pressure sensitive spessimeter (to the nearest 0·01 mm) to standardize pressure during measurements. Then, we injected 0·02 mg of PHA dissolved in 0·01 mL of phosphate-buffered saline (PBS) water in the foot pad. Lizards were released in their terraria, and after 24 h we measured again the foot pad thickness at the marked point. The CMI response was calculated as the difference between pre- and postinjection measures (Smits, Bortolotti & Tella 1999; Belliure *et al*. 2004). The only appreciable effect of the PHA injection was a slight swelling of the skin, due to the immune response, which disappeared after 48 h. None of the lizards showed any signs of stress or pain during these tests.

We used nonparametric rank Spearman correlations and Kendall partial rank-order correlations (Siegel & Castellan 1988) to analyse the relationships between asymmetry in femoral pores and CMI response with TF rates or attractiveness indexes (see below) because of the particular half-normal distribution of unsigned absolute asymmetry data (see Swaddle, Witter & Cuthill 1994).

CHEMOSENSORY RESPONSES OF FEMALES TO SCENT OF MALES

Lizards react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper & Burghardt 1990; Cooper 1994). Thus, we can use TF rate as a quantitative bioassay of detection of chemical cues. We made comparisons of TF rate by female lizards in response to chemical stimuli arising from cotton applicators impregnated with femoral gland secretions of males, scent of females, or deionized water (odourless control). Water was used to gauge baseline TF's rates in the experimental situation (Cooper & Burghardt 1990).

We prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (100 cm) in deionized water. Femoral secretions of males consisted of a waxy substance, which was easily extracted by gently pressing with forceps around the femoral pores, and collected directly on cotton tips. We attempted to use approximately the same amount of femoral secretion in each stimulus (about 2 × 1 mm of solid secretion from each of three pores), thus, ensuring that any possible difference in TF rates was not due to differences in the amount of secretions presented to females, but to differences in presence or concentration of chemicals in secretions. Females had no apparent femoral secretions, but we rubbed the cotton swabs on the 'vestigial' femoral pores and the cloaca of females. A new swab was used in each trial. Cotton swabs with scent stimuli were used in the trials immediately after collection to avoid fading of the stimuli.

Every female was exposed to scents of two individual males and one female, and order of presentation was randomized. Thus, secretions of each of 16 individual males were presented to two different individual females. One trial was conducted per day for each animal. Trials were conducted in outdoor conditions between 21 and 24 May, which coincided with the mating season of lizards in their natural population (López *et al*. 2003), and between 11.00 and 13.00 hours (GMT) when lizards were fully active. Each individual was allowed to bask and attain an optimal body temperature for at least 2 h before trials (Martín & Salvador 1993). Thereafter, one of the experimenters slowly approached a lizard's cage and slowly moved the cotton swab to a position 2 cm anterior to the lizard's snout. Lizards allowed approach and testing without fleeing. We recorded TFs directed to the swab during 1 min, beginning with the first TF.

To examine differences in the number of directed TFs among conditions, we used one-way repeated measures analysis of variance (ANOVA) with scent stimuli as a within factor. Data were log-transformed to ensure normality (Shapiro–Wilk's test). Tests of homogeneity of variances (Levene's test) showed that in all cases, variances were not significantly heterogeneous after transformation. Pairwise comparisons used Tukey's honest significant difference (HSD) tests (Sokal & Rohlf 1995).

CHOICE OF SCENT EXPERIMENTS

At the beginning of the experiments, we had placed in males' cages several absorbent paper strips (35 × 10 cm) fixed to the floor, and left them there for 3 weeks to obtain the scents from males. Mate choice experiments were performed on 11 and 12 June, which coincided with the mating season of lizards (López *et al*. 2003).

Females' cages had two basking platforms (two identical flat tiles) placed symmetrically at each end of the cage, and rocks for cover in the centre. At the beginning of each test (07.00 hours GMT), when females were still inactive, we fixed with fresh gloves on one tile one paper strip from a male, and another from a different male on the other tile. Each female was tested over 2 days, once a day, with papers from two different pairs of unknown males. The males tested and the positions of papers were randomly determined, but for each female we excluded the two individual males that had been used as donor of scent for the previous TF trials with that female. Each trial lasted 6 h (from 09.00 hours GMT, shortly after females appeared from night refuges, and until 15.00 hours GMT, when females hid again), Females were monitored each 15 min (25 scans) from a hidden point. If a female was located on a tile with the paper strip, she was designated as having chosen temporarily that particular paper, whereas if she was located outside of the tiles she was designated as having made no choice (for a similar procedure see Martín & López 2000a: López *et al.* 2002, 2003; Olsson *et al.* 2003). To ensure that females were exposed to both males' tiles and were aware of both male's stimuli, at least two recordings in each male's section were considered necessary for a trial to be valid. This presumption was fulfilled in all tests. At the end of the trials the papers were removed and the cage was thoroughly rinsed with alcohol and clean water.

The paper on which a female spent greater than 60% of her time (excluding time in the no choice area) was designed as the preferred paper in that trial, and we gave a score of 1 to the preferred male and 0 to the other male. When there were not such differences in time between the two papers it was considered a tie, and we assigned a score of 0·5 to each male. Different papers from each male were used in four choice tests against the papers of other four males, with different females. We then calculated an 'attractiveness index' for each individual male from the sum of scores obtained in his four tests.

CHEMICAL ANALYSES OF FEMORAL SECRETIONS OF MALES

On the same day of capture, we extracted femoral pore secretion of males by gently pressing with forceps around the pores, and collected secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at –20 °C until analyses. Samples were analysed by GC-MS (Finnigan-ThermoQuest Trace 2000, ThermoQuest Corporation, Austin, Texas) equipped with a Supelco, Equity-5 column. The oven temperature programme was as follows: 50 °C isothermal for 10 min, then increased to 280 °C at 5 °C min^{-1}, and then isothermal (280 °C) for 30 min. Identification of compounds was done by comparison of mass spectra in the NIST/EPA/NIH 1998 library, and later confirmed with authentic standards (see López & Martín 2005b; López *et al.* 2006 for full details of chemicals in secretions).

The relative amount of each compound was determined as the per cent of the total ion current (TIC). Then, we selected the peaks that represented > 0·5% relative peak area (which altogether represent 98% of total TIC area). The relative areas of the selected peaks were restandardized to 100% and transformed following Aitchison's formula: $[Z_{ij} = \ln(Y_{ij}/g(Y_j))]$, where Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the peak area i for individual j, and $g(Y_j)$ is the geometric mean of all peaks for individual j (Aitchison 1986; Dietemann *et al.* 2003). The transformed areas were used as variables in a principal components analysis, which produced four components (PCs), which together accounted for 82·9% of the total variance (Table 1). To examine whether we could relate proportions of chemicals in secretions of a male with the average TF rate that his scent elicited in females or with his attractiveness index (both log-transformed), we used forward stepwise general regression models (GRM) with the four principal components (PC) extracted as potential predictors.

Table 1. Principal components analysis for relative proportion of main chemicals in femoral gland secretions of male lizards. Correlations in bold lettering are significant at $P < 0·0001$

Compound	PC-1	PC-2	PC-3	PC-4
Hexadecanoic acid	0·67	0·57	0·21	–0·13
Octadecanoic acid	**0·93**	0·13	0·04	–0·23
Octadecenoic acid	**0·84**	0·35	–0·05	–0·07
9,12-Octadecadienoic acid	0·59	0·48	–0·54	0·10
Cholesterol	**–0·90**	0·16	0·12	0·01
Cholesta-5,7-dien-3-ol	–0·13	**–0·84**	–0·29	–0·09
Ergosterol	–0·06	**–0·81**	0·17	0·14
Campesterol	–0·30	0·08	0·18	**0·83**
Ergosta-5,8-dien-3-ol	–0·20	0·27	**0·87**	0·03
Lanosterol	**–0·75**	0·47	–0·03	0·09
Sitosterol	0·02	–0·12	–0·08	**0·90**
4,4-Dimethyl-cholesta-5,7-dien-3β-ol	0·16	–0·10	**0·86**	0·05
Eigenvalue	4·26	2·36	1·84	1·48
% Variance	35·5	19·6	15·4	12·4

DETECTION OF CHEMICAL COMPOUNDS BY FEMALES

In these experiments made between 6 and 9 June, we used a methodology similar to the previous TF experiment (see above) to compare TF rate by female lizards in response to stimuli arising from cotton applicators bearing: (1) dichloromethane (DCM; pungency control); (2) cholesterol; (3) cholesta-5,7-dien-3-ol; and (4) ergosterol. DCM was used to gauge baseline TF rates in the experimental situation. We prepared chemical stimuli the same day of the tests by dissolving 15 mg of each compound (authentic standards, GC grade, from Sigma-Aldrich Chemicals, St. Louis, Missouri, USA) 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 between trials.

In a second test made between 13 and 17 June, we used a similar method to test whether females can discriminate differences in relative abundance of cholesta-5,7-dien-3-ol and ergosterol mixed with a majority of cholesterol. Thus, we aimed to simulate their natural occurrence in femoral secretions of males (i.e. average relative proportions of all lipids are 67% of cholesterol vs. 0·4% of cholesta-5,7-dien-3-ol and 0·8% of ergosterol; López & Martín 2005b). We measured the TF responses of females to two different concentrations of cholesta-5,7-dien-3-ol and ergosterol dissolved in DCM: 'low' (8 mg mL^{-1}), and 'high' (24 mg mL^{-1}), both mixed with cholesterol (100 mg mL^{-1}). We used DCM as the pungency control. Statistical analyses were conducted as in the previous TF experiment.

FEMALE CHOICE OF MANIPULATED MALES' SCENTS

We made two tests in two different days (4 and 5 June), using a similar procedure than in the choice of scent experiments (see above), but, before the beginning of the trials, we used Hamilton crystal syringes to spray over all the surface of each strip paper, previously scent marked by a male, with either 2 mL of DCM (control), or 2 mL of DCM that had dissolved ergosterol (24 mg mL^{-1}). DCM readily evaporated in a few minutes. With this procedure we added supplementary ergosterol to the natural scent marks already presented on the paper. For each female we excluded papers from the individual males that had been used as donor of scent for the previous trials with that female. Then, we compared the number of observations (squared root-transformed) of each female on each section of the terraria with a two-way repeated measures ANOVA with treatment (control vs. ergosterol vs. nonchoice) and day of the trial, both as within factors. Pairwise comparisons used Tukey's HSD tests (Sokal & Rohlf 1995).

Results

CHEMOSENSORY RESPONSES OF FEMALES TO SCENT OF MALES

All females responded to swabs by tongue flicking. The rate of TFs directed to swabs differed significantly between scent stimuli (repeated measures one-way ANOVA: $F_{3,45} = 37{\cdot}62$, $P < 0{\cdot}0001$) (Fig. 1). Females elicited significantly more TFs directed to males' scent than to any other stimuli (Tukey's tests: $P < 0{\cdot}001$ in all cases), and more to female scent than to water ($P < 0{\cdot}001$), but there were no significant differences between the first and the second male tested ($P = 0{\cdot}77$).

There was a significant negative relationship between the average TF rate of females to the scent of each individual male and the level of asymmetry in the femoral pores of this male (Spearman's rank correlation: $r_s = -0{\cdot}57$, $t = -2{\cdot}62$, $n = 16$, $P = 0{\cdot}02$) (Fig. 2a), with scent from more symmetric males eliciting higher average TF rates by females. Also, there was a significant positive relationship between the average TF rate of females to the scent of each individual male and the CMI response of this male (Spearman's rank correlation: $r_s = 0{\cdot}55$, $t = 2{\cdot}46$, $n = 16$, $P = 0{\cdot}027$) (Fig. 2b). Thus, males with a better health state, as indicated by their greater CMI response, elicited higher TF rates by females. Kendall partial rank-order correlations showed that both FA ($\tau = -0{\cdot}63$, $P < 0{\cdot}001$) and the CMI response ($\tau = 0{\cdot}61$, $P < 0{\cdot}001$) affected the average TF rate significantly and independently when the effects of the other were fixed.

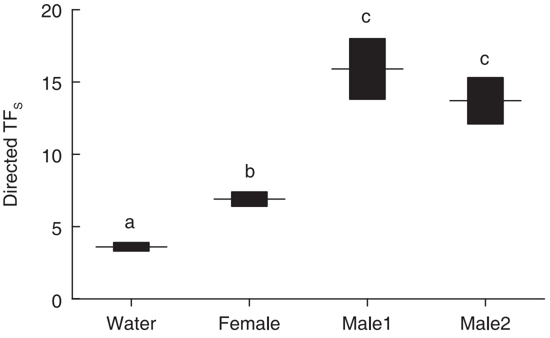

Fig. 1. Mean (± SE) number of tongue-flicks (TF) directed to swabs by female lizards in response to cotton-tipped applicators bearing deionized water, scent from females, or femoral secretions of two different individual males. The same letter above the bars denotes that means are not significantly different from each other.

Stepwise GRM showed that the average TF rate of females to a male scent presented on cotton applicators was significantly and negatively correlated with PC-2 scores, resulting from the PCA for relative proportions of major chemical compounds in femoral gland secretions of males ($\beta = -0{\cdot}56$, $t = -2{\cdot}54$, $P = 0{\cdot}02$; $R^2_{model} = 0{\cdot}31$, $F_{1,14} = 6{\cdot}44$, $P = 0{\cdot}02$) (Fig. 2c). All other PCs were not significantly related to the TF rate and were not included in the final model. Thus, femoral secretions of males that elicited higher TF rates by females have relatively higher proportions of ergosterol and cholesta-5,7-dien-3-ol.

CHOICE OF SCENT EXPERIMENTS

Scent of males that had elicited higher average TF rates by females had significantly higher 'attractiveness indexes' (Spearman's rank correlation: $r_s = 0{\cdot}69$, $t = 3{\cdot}58$, $n = 16$, $P = 0{\cdot}003$). Scent of males with higher 'attractiveness indexes' had significantly higher levels of symmetry in their femoral pores ($r_s = -0{\cdot}56$, $t = -2{\cdot}55$, $n = 16$, $P = 0{\cdot}023$) (Fig. 3a), and a greater CMI response ($r_s = 0{\cdot}57$, $t = 2{\cdot}61$, $n = 16$, $P = 0{\cdot}02$) (Fig. 3b), Kendall partial rank-order correlations showed that both FA ($\tau = -0{\cdot}62$, $P < 0{\cdot}001$) and the CMI response ($\tau = 0{\cdot}63$, $P < 0{\cdot}001$) affected the attractiveness indexes significantly and independently when the effects of the other were fixed.

Stepwise GRM showed that 'attractiveness indexes' of males in the mate choice trials were significantly and negatively correlated with PC-2, resulting from

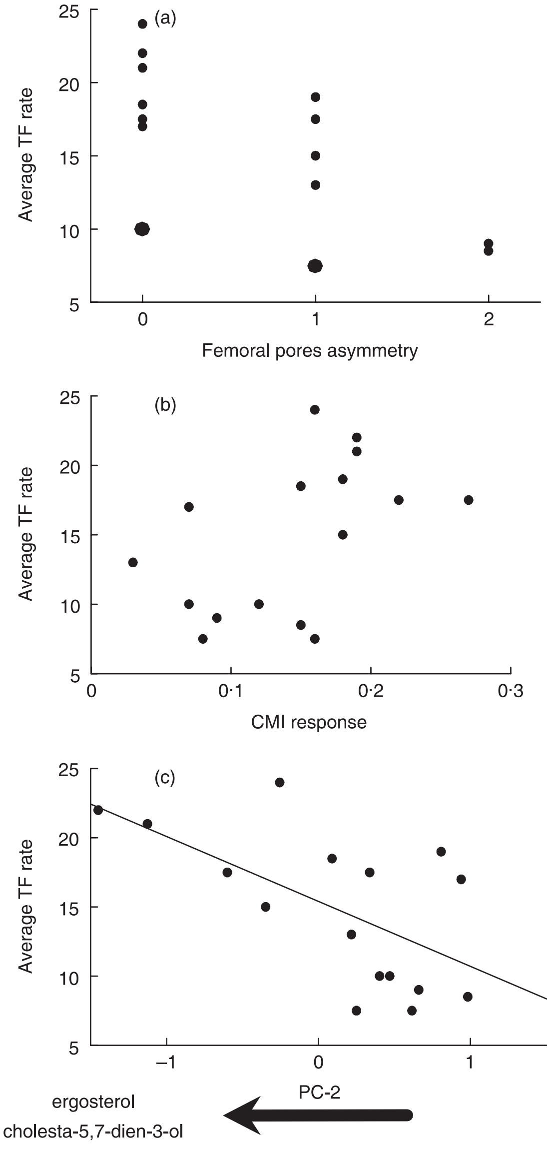

Fig. 2. Relationship between the average TF rate of females to the scent of each individual male and (a) the level of symmetry in the femoral pores, (b) the T-cell-mediated immune (CMI) response, or (c) the PC-2 scores, extracted in a principal components analysis for relative proportion of chemicals in femoral secretions of this individual male. Circles of increasing size represent one or two observations, respectively.

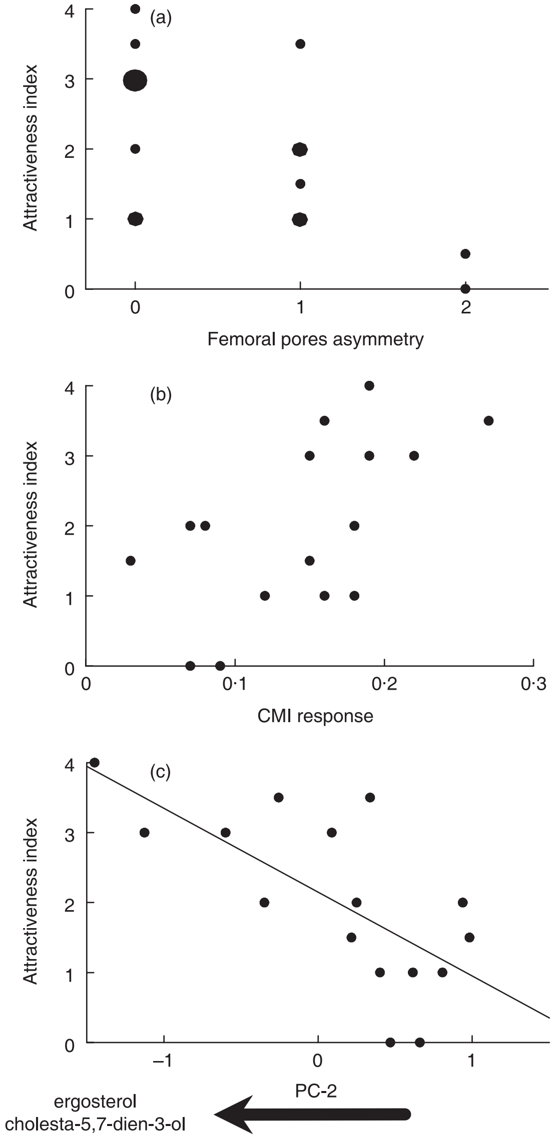

Fig. 3. Relationship between the index of attractiveness of each individual male's scent to females and (a) the level of symmetry in the femoral pores, (b) the T-cell-mediated immune (CMI) response, or (c) the PC-2 scores, extracted in a principal components analysis for relative proportion of chemicals in femoral gland secretions of this individual male. Circles of increasing size represent one, two or three observations, respectively.

the PCA for relative proportions of chemicals in femoral secretions ($\beta = -0{\cdot}65$, $t = -3{\cdot}21$, $P = 0{\cdot}006$; $R^2_{\text{model}} = 0{\cdot}42$, $F_{1,14} = 10{\cdot}32$, $P = 0{\cdot}006$) (Fig. 3c). Thus, femoral secretions of males more often selected by females have relatively higher proportions of ergosterol and cholesta-5,7-dien-3-ol.

DETECTION OF CHEMICAL COMPOUNDS BY FEMALES

The rate of TFs directed to swabs differed significantly between chemical compounds stimuli (repeated measures one-way ANOVA: $F_{3,45} = 59{\cdot}08$, $P < 0{\cdot}0001$) (Fig. 4a). Females elicited significantly less TFs directed to DCM than to other chemicals (Tukey's tests: $P < 0{\cdot}0002$ in all cases), and less TFs to cholesterol than to the other two steroids ($P < 0{\cdot}0002$ in both cases), but females elicited a similar TF rate to cholesta-5,7-dien-3-ol and ergosterol ($P = 0{\cdot}42$).

In the second test, the rate of TFs directed to swabs differed significantly between stimuli (repeated measures one-way ANOVA: $F_{4,60} = 133{\cdot}60$, $P < 0{\cdot}0001$) (Fig. 4b). All stimuli were significantly different (Tukey's tests: $P = 0{\cdot}001$ in all cases), with females eliciting significantly more TFs directed to high concentration of ergosterol, and in decreasing order, to low concentrations of ergosterol, high and low concentration of cholesta-5,7-dien-3-ol, and DCM.

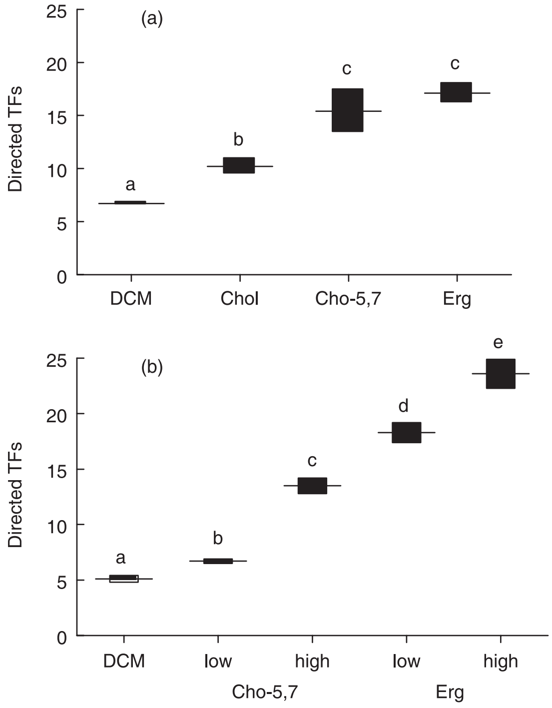

Fig. 4. Mean (± SE) number of tongue-flicks (TF) directed to swabs by female lizards in response to (a) cotton-tipped applicators bearing dichloromethane (DCM), cholesterol (Chol), cholesta-5,7-dien-3-ol (Cho-5,7), or ergosterol (Erg), all dissolved in DCM, or (b) applicators bearing two different concentrations of cholesta-5,7-dien-3-ol (Cho-5,7) or ergosterol (Erg), all dissolved in DCM and mixed with cholesterol. The same letter above the bars denotes that means are not significantly different from each other.

FEMALE CHOICE OF MANIPULATED MALES' SCENTS

In the tests with supplementation of ergosterol on papers scent marked by males, there were significant differences in the number of observations of females on a determined stimulus paper strip between treatments (repeated measures two-way ANOVA: $F_{2,30} = 15·20$, $P <$ 0·0001), and between days ($F_{1,15} = 4·79$, $P = 0·045$), and the interaction was not significant ($F_{2,30} = 1·02$, $P = 0·37$) (Fig. 5). Females spent more time on paper strips supplemented with ergosterol than on the nonchoice area (Tukey's test: $P = 0·002$) and than on paper strips with DCM alone ($P = 0·038$). Time spent on the non-choice area did not differ from time spent on papers with DCM alone ($P = 0·45$).

Discussion

The greater TF rates in response to femoral gland secretion of males presented on cotton swabs indicated that female *L. monticola* were able to detect males' scents from a pungency control, and to discriminate the scents of males and females. Moreover, the different TF rates to scents of different individual males, and the relationship of these TF rates with some male traits, suggested that females may base this discrimination on interindividual variations in chemical composition of femoral secretions of males. Interestingly, scent of males with a greater T-cell immune response and those more symmetric elicited higher TF rates in females. This suggested that these males of presumably high quality had 'scent signatures' different from those of other males, and that females may detect these differences. Similarly, variations in the responses of conspecifics to scents of different individuals suggest that parasitic infections and health state might affect the information conveyed by chemical signals in rodents (e.g. Kavaliers & Colwell 1995; Penn & Potts 1998; Zala, Potts & Penn 2004), beetles (Rantala *et al.* 2002, 2003), and other lizards (López & Martín 2005a).

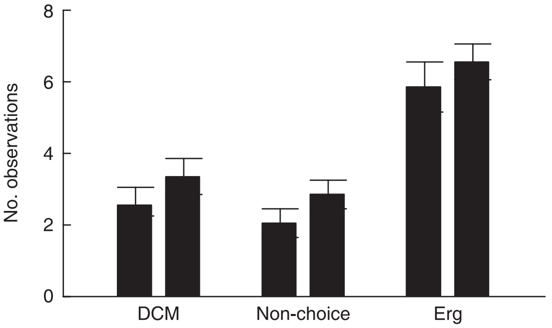

Fig. 5. Number of times (mean ± SE), during each of two scent's choice trials, that females were observed on paper strips scent marked by males, and on which we added dichloromethane (DCM) (control), or ergosterol (Erg) dissolved in DCM, or on a nonchoice area without males' scents.

Our results further showed that females spent more time on areas scent marked by previously unknown males, which scents had elicited higher TF rates by different individual females. Moreover, males with more attractive scents to females were those with a greater immune response and more symmetric. These results confirmed the previous findings of other experiments with this and other lizard species (Martín & López 2000a; López *et al.* 2002, 2003; López & Martín 2005a). Females seemed able to assess the males' traits by chemical cues alone, and decided where to establish themselves based on scent marks left by males, thus, increasing the probability of mating with males of high quality.

The chemical analyses indicated that femoral secretions of preferred males and those that elicited higher TF rates had higher proportions of cholesta-5,7-dien-3-ol and ergosterol. Thus, it is likely that females may assess interindividual variations in proportions of these chemicals, which would require females to discriminate these steroids, as well as to assess variations in their concentrations. This ability was confirmed in trials testing the responses of females to chemical standards. Thus, the higher TF rates to cholesta-5,7-dien-3-ol and ergosterol indicated that females discriminated these steroids from the chemically very similar cholesterol. However, similar concentrations of cholesta-5,7-dien-3-ol and ergosterol elicited similar TF rates, which initially suggested that females did not discriminate between these two compounds. A possible explanation is that females might not need to discriminate them because the proportions of these two compounds in natural secretions are strongly correlated (see the PCA for chemicals). Nevertheless, significant differences between these two chemicals arose when their concentrations changed.

Moreover, females seemed able to assess changes in concentration of these steroids, even if they were found in low relative proportion inside a matrix of cholesterol, which is similar to that naturally found in femoral secretions of males (López & Martín 2005b). Females responded more strongly (i.e. a higher TF rate) to higher concentrations of cholesta-5,7-dien-3-ol or ergosterol, which was similar to their stronger responses to femoral secretions of males that had natural higher proportions of these compounds in their secretions. Therefore, changes in concentration of these steroids in femoral secretion of males may explain the different chemosensory responses of females to different males. Furthermore, our results from the choice of scent experiments indicated that females showed a preference for areas scent marked by males with higher proportions of these chemicals in their secretions. Thus, it is likely that females selected the scents of males based on the concentrations of cholesta-5,7-dien-3-ol and ergosterol. This was supported by our experiment where we added supplementary ergosterol to the males' scent marks, which drove the choice of females toward areas with manipulated scent marks.

The question that arises is why females should base their mate choice in the proportions of these chemicals in males' secretions. We suggest that potential mechanisms linking mate choice with these chemicals might be inferred by analysing the origin and the function in metabolism of these specific compounds. These steroids, which males are continually wasting through the femoral pores during the mating season, are, however, important keys in many metabolism routes as precursors of signalling molecules with potent biological activity. Thus, cholesta-5,7-dien-3-ol is a precursor for vitamin D3, and is often found in the skin, where it will transform into vitamin after exposition to sun ultraviolet (UV) B irradiation (Fraser 1995; Ferguson *et al*. 2005). Vitamin D2 is similar but has to be acquired from the diet, as only fungus can convert it from the UV irradiation of ergosterol (Hay & Watson 1977). A primary function of vitamin D is the regulation of calcium and phosphorus homeostasis, and vitamin D is essential for the uptake of calcium from the gastrointestinal tract, as well as for adequate mineralization of bone. However, very often, the synthesis of vitamin D3 in the skin is not sufficient to meet physiological requirements, and lizards require dietary intake of vitamin D (Allen *et al*. 1994; Ferguson *et al*. 2005). Thus, when captive squamate reptiles are not exposed to UV light and do not receive vitamin D supplements in the diet, they may develop signs of calcium deficiency (Allen *et al*. 1994), which impairs bone growth (Laing & Fraser 1999). Under these conditions, vitamin D is an essential nutrient for lizards.

We hypothesize that by allocating cholesta-5,7-dien-3-ol (= provitamin D3) to femoral secretions male lizards will need to divert vitamin D3 from metabolism, whereas ergosterol in secretions might be directly related to the amount of vitamin D2 that a lizard has been able to acquire from the diet. Thus, signals based on variations in these chemicals might be reliable and costly to produce, especially for low-quality individuals (e.g. those with a worse health state or less symmetric) that would not be able to obtain from the diet enough vitamin D. In fact a previous manipulative experiment has shown that supplementation of vitamin D3 to male Iberian Rock Lizards resulted in an increase of cholesta-5,7-dien-3-ol in their femoral secretions (Martín & López 2006). Also, other studies showed that individual lizards with a worse health state were shyer and hid for longer in refuges after a predator approach (López *et al*. 2005), and that 'inferior' subordinate males were active for less time during the day (Martín & López 2000b). Therefore, these presumably low-quality individuals would have less time available for foraging and, thus, for acquiring vitamin D from the diet. These data would support the hypothesis that is costly to produce femoral secretions with high proportions of cholesta-5,7-dien-3-ol and ergosterol, and suggests the potential role of these secretions as condition dependent honest advertisements (Grafen 1990; Kotiaho 2001), which could then be used by females in sexual selection processes.

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