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ORIGINAL PAPER

Multimodal sexual signals in male ocellated lizards *Lacerta lepida*: vitamin E in scent and green coloration may signal male quality in different sensory channels

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Abstract The mechanisms that confer honesty to multiple sexual signals of vertebrates remain less known. We suggest that vitamin E in the scent (femoral secretions) and carotenoids in coloration of *Lacerta lepida* lizards might advertise quality because they are relevant in metabolism as major antioxidants and have a dietary origin. Results showed that there may be similar trade-offs between immunity and both chemical and visual signaling, as males with more vitamin E in secretions and those with more greenish, darker, and saturated carotenoid-dependent colorations had a higher immune response. This suggests that only high-quality males can divert from metabolism and allocate enough vitamin E to secretions and enough carotenoids to coloration. Therefore, information provided by visual and chemical signals is congruent. The use of multiple sensory channels may allow signaling a male quality under different circumstances or reinforce the reliability of the signal when both signals are perceived simultaneously. However, we also found that characteristics of carotenoid-dependent coloration mirror the amounts of vitamin E in secretions. This might support that carotenoids, which are not true antioxidants in the organism, would just reflect and "inform" on the actual contents of metabolic antioxidant vitamin E, which is part of the true system that prevent oxidation. We suggest that the metabolic roles of vitamins might be the actual basis of honesty of many sexual displays, and that carotenoid-based signals might be just the visible part of the whole system.

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Introduction

Chemical signals (pheromones) play an important role in intraspecific communication of many vertebrates such as mammals and reptiles (Mason 1992; Wyatt 2003; Müller-Schwarze 2006). In lizards, pheromonal detection, which is often based on femoral gland secretions, occurs in many species (e.g., Alberts 1993; Aragón et al. 2001). The presence and relative concentration of chemical components in femoral gland secretions may convey information on a male's characteristics and function in sexual selection processes (López et al. 2006; Martín and López, in press). Secretions could advertise residence or dominance status to other males (Aragón et al. 2001; Carazo et al. 2007; Martín et al. 2007) and be used by females to choose compatible or high-quality mates (Martín and López 2000, 2006a; Olsson et al. 2003).

In spite of the potential importance of chemical signals, only a few studies have described the composition of gland secretions in a few species of lizards (reviewed in Weldon et al. 2008). Secretions are composed of both proteins and lipids, which are thought to be the main compounds involved in communication (Mason 1992; Martín and López in press). The role of specific chemicals as sexual signals and the mechanisms that could confer reliability to these signals remain less understood. However, it is interesting that many of the lipids commonly found in femoral secretions of lizards, such as some fatty acids, tocopherol, or some steroids, function as signaling molecules or lipid mediators, show potent biological activity, and are important keys in many metabolic routes. Moreover, many of these compounds, considered as essential nutrients or vitamins, can only be obtained from the diet (Bender 2009).

Particularly, tocopherol (vitamin E) is found in abundance in femoral secretions of European lacertid “green lizards” such as *Lacerta schreiberi* (López and Martín 2006), *Lacerta viridis* (Kopena et al. 2009), or *Lacerta lepida* (this paper). Vitamin E consists of a group of isoprenoid compounds of plant origin (Brigelius-Flohe and Traber 1999), which were first described as essential micronutrients for normal fertility in rats (Evans and Bishop 1922). Later studies have established a wide range of functions for this vitamin family, especially the RRR-α-tocopherol stereoisomer, the most biologically active form (Burton and Traber 1990; Brigelius-Flohe and Traber 1999). Vitamin E is the main lipophilic antioxidant and radical scavenger involved in membrane defense, both in vitro and in vivo, (Winklhofer-Roob et al. 2003; Martínez et al. 2008). The lipophilic nature of α-tocopherol, together with its physiological relevance and the severe pathological consequences of its deficiency, such as neurological disorders or lung diseases, impose a major challenge to animals for sustaining an adequate supply of this vitamin to different tissues (Mardones and Rigotti 2004), particularly those highly sensitive to α-tocopherol deficiency such as the brain and gonads. In humans, α-tocopherol is a current candidate in preventing pathological states concerning cellular oxidative damage such as atherosclerosis, Alzheimer's disease, accelerated aging, and cancer (Brigelius-Flohe and Traber 1999). All these important metabolic functions, and the fact that tocopherol can only be obtained from the diet (Bender 2009), suggest that allocation of vitamin E to femoral secretions by lizards should be costly. Therefore, the function of vitamin E in secretions should be also of enough importance to induce lizards diverting it from metabolism and secreting it away from the body.

Tocopherol is a well-known lipophilic antioxidant (Brigelius-Flohe and Traber 1999; Martínez et al. 2008). Therefore, the first possible function of tocopherol in secretions of lizards might be to inhibit oxidation of other glandular lipid components, thus, increasing chemical stability of scent marks in wet environments (see Alberts 1992). However, we hypothesized that allocation of vitamin E to secretions is likely very costly, and, thus, it is possible that only males with an adequate supply of this vitamin could do it. Thus, the presence of high proportions of “costly” vitamins in secretions might be an honest condition-dependent signal of a male's quality (Martín and López 2006a, b). Nevertheless, the function of vitamin E in secretions of lizards remains to be analyzed properly.

The energetic expenditure of producing and maintaining components of the immune system may have a major effect on condition, thus creating a link between immune system and condition-dependent sexual advertisement (Wedekind 1992; Wedekind and Folstad 1994). A trade-off is expected such that resources are used up by both the sexual advertisement and the immune system (Kotiaho 2001). Therefore, only individuals in good condition can mount a strong immune defense and produce an extravagant sexual ornament (Sheldon and Verhulst 1996; Westneat and Birkhead 1998). For example, the experimental activation of the immune system of male lizards *Podarcis hispanica* affects negatively the expression of both nuptial coloration and chemicals (pro-vitamin D_3) in femoral secretions of males (López et al. 2009a, b). Vitamin E is an effective radical scavenger and has immunostimulatory activity (Brigelius-Flohe and Traber 1999; Ortuño et al. 2000). We hypothesized that the proportion of vitamin E in femoral secretions of male green lizards might be a condition-dependent trait related to the quality of the males' immune system.

On the other hand, many lizards have evolved colorful visual sexual ornaments that are also used in sexual selection (reviewed in Cooper and Greenberg 1992). For example, adult male “green lizards” have green brilliant dorsolateral colorations (Václav et al. 2007; Martín and López 2009). Characteristics of this coloration, which is often carotenoid-dependent, may reflect the health state of a male (Ressel and Schall 1989; Václav et al. 2007; Martín et al. 2008; Martín and López 2009) and might be potentially used by females to select mates. This relationship could be explained if allocating more carotenoids to sexual coloration was costly because there is a trade-off with the role of these pigments as antioxidants and in the maintenance of the immune function (Faivre et al. 2003; López et al. 2009a). However, Hartley and Kennedy (2004) suggested that the biological role of carotenoids might not be as antioxidants per se. Given that oxidation of carotenoids alters or destroys their color (i.e., bleaches them), preservation of their color intensity would indicate the possession of efficient means for their protection. Therefore, colorful carotenoids would just reflect the healthy functioning of the true systems that prevent oxidation, such as vitamins C and E, which are, however, not “visually informative” (Hartley and Kennedy 2004). Because vitamin E is part of the system that prevents oxidation in tissues (Martínez et al. 2008), we hypothesized that in green lizards, some characteristics of the carotenoid-dependent green coloration might be related to the immune response of males, but if carotenoids are reflected and “informed” on the actual contents of antioxidant vitamin E, characteristics of coloration related to health should mirror the amounts of vitamin E in secretions.

To examine these hypotheses, we first described here the chemical composition of femoral secretions and the characteristics of carotenoid-dependent “green” coloration of males of the ocellated lizard (*L. lepida*). Then, we analyzed the relationship between the quality of the immune system of males, as suggested by the T cell immune response, and (1) the proportions of different forms of vitamin E in their femoral secretions, and (2) the hue, brightness, and saturation of "green" coloration. Then, we examined the relationship between these chemical and visual signals and whether the potential information provided by these multimodal signals is similar and concordant.

Materials and methods

Study animals

The eyed or ocellated lizard *L. lepida* is the largest European lacertid (snout-to-vent length, SVL, and total length may reach 242 and 754 mm, respectively). This lizard inhabits Mediterranean forests and scrublands of the Iberian Peninsula, southern France, and northern Italy (Castilla and Bauwens 1992; Pérez-Mellado 1998). These lizards have a dorsolateral green ground color overlaid with black stippling and blue spots on the flanks (Arnold and Ovenden 2002; Font et al. 2009).

We captured by noosing and by hand 20 adult male *L. lepida* lizards from May–June, coinciding with the mating season of lizards in different places over a large area in the Guadarrama Mountains (Madrid province, Central Spain). Captured lizards were individually housed at "El Ventorrillo" Field Station (Navacerrada, Madrid province) close to the capture sites in outdoor opaque plastic cages (80× 50 cm) containing rocks for cover. Lizards were weighed (body mass $\overline{X} \pm \mathrm{SE} = 68.1 \pm 8.3$ g, range=11.7–111.8 g), and their SVL was measured ($\overline{X} \pm \mathrm{SE} = 151 \pm 6$ mm, range=89–181 mm). Male lizards had an average (±SE) of 12±1 (range=10–14) femoral pores on each leg.

Analyses of femoral gland secretions

In the field and immediately after capture, we extracted femoral pores secretion by gently pressing with forceps around the femoral pores and collected secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at −20°C until analyses (see below). We also used the same procedure on each sampling occasion but without collecting secretion to obtain blank control vials that were treated in the same manner to compare with the lizard samples.

Samples of femoral secretions were analyzed using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly(5% diphenyl/95% dimethylsiloxane) column (Supelco, Equity-5, 30 m length×0.25 mm ID, 0.25-µm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. The samples (2 µl of each sample dissolved in 250 µl of *n*-hexane, Sigma, capillary GC grade) were injected in the splitless mode. The gas chromatograph was programmed so that the oven temperature was kept at 50°C for 10 min, then increased to a final temperature of 280°C at a rate of 5°C/min, and kept at this temperature for 30 min. Impurities identified in the solvent and/or the control vial samples were not considered. Initial identification of secretion components was done by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass-spectral library or based on their characteristic mass spectra (see Kopena et al. 2009). When possible, identifications were later confirmed by comparison of spectra and retention times with those of authentic standards purchased from Aldrich Chemical Co.

The relative amount of each component was determined as the percent of the total ion current (TIC). For further statistical analyses, the relative areas of the peaks were 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. This is the recommended analysis for these compositional data generally used in chemical ecology (Aitchison 1986; Dietemann et al. 2003).

Color measurements

We measured reflectance of lizards' coloration from 350 to 700 nm using an Ocean Optics USB2000 spectroradiometer with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL). This range of wavelengths coincides with the spectral sensitivity measured for other lizard species, which includes visual perception in the UV range (e.g., Fleishman et al. 1997; Loew et al. 2002). To exclude ambient light and standardize measuring distance, a cylindrical metallic tube was mounted on the bifurcated fiber optic probe (Montgomerie 2006). The probe was held at a 90° angle to the skin, and reflectance was measured, always by the same person (PL). We measured coloration on two standardized spots on the flanks' background (i.e., the "green" coloration outside the black reticulated pattern) at each side of the body between the forelimbs and the hind limbs. We measured coloration at three different points at each spot, but because these three measurements as well as the measurements on both body sides were highly repeatable within each individual (intraclass correlation coefficients, $r>0.75$, $P<0.0001$ in all cases), we calculated the mean values of the three measures and used the mean values of both sides of the body.

Reflectance was calculated relative to a white standard (WS-1-SS) with the OOIBase32 software (Ocean Optics, Inc.). Then, to characterize color according to the tristimulus color variables, hue, saturation, and brightness, we transformed reflectance values following formulas described in Montgomerie (2006). The hue defines the color. Thus, for example, hue is negatively correlated to redness. Saturation (also called chroma) refers to the saturation or purity of the dominant frequency of the light. The farther away it is from zero, the more saturated the color is. Brightness (also called lightness) of a reflected light signal is a function of both the range of wavelengths reflected and the surface structure of the skin (Montgomerie 2006).

Assessment of the immune response

To assess T cell-mediated immune (CMI) response, we used a delayed-type hypersensivity test; the phytohemagglutinin injection (PHA) test. This is a reliable measure of T cell-dependent immunocompetence in vivo (Lochmiller et al. 1993; Tella et al. 2008), also in lizards (Svensson et al. 2001; Belliure et al. 2004). We marked a point with permanent ink on the footpad of both hind limbs of lizards. We then measured three times the thickness at this point with a pressure-sensitive spessimeter (to the nearest 0.01 mm). Immediately after, we injected 0.02 mg of PHA dissolved in 0.01 ml of phosphate-buffered saline (PBS) water in the right footpad and the same volume of PBS in the left footpad. Lizards were released in their terraria, and after 24 h, we measured again three times the footpad thickness at the marked points. Previous studies showed that repeatability of this measure is high (J Martín and P López, unpublished data). The CMI response was calculated as the difference between pre- and postinjection measures on the right footpad (PHA injected) minus the same difference on the left footpad (control, PBS injected) (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. No lizard showed any sign of stress or pain for these tests, and all looked healthy when they were thereafter released to their capture sites 3 days after being captured.

The CMI response was significantly related to body mass (r=0.68, $F_{1,18}$=15.55, P<0.001). Thus, we calculated the CMI residuals by regressing CMI against log-body mass. Thereafter, we calculated Pearson's correlations between CMI residuals and the geometrical transformed TIC areas (see above) of the forms of vitamin E found in secretions or the means of hue, saturation, and brightness of coloration. Analyses were performed with Statistica 6.0 software (StatSoft, Inc., Tulsa, OK).

Results

Chemical signals

A total of 59 lipophilic compounds were identified in femoral gland secretions of male *L. lepida* (Table 1). The main components were steroids (59.0% of TIC), four forms of tocopherol (27.9%), and carboxylic acids, and some of their ethyl esters ranged between C_9 and C_{22} (12.2%), but we also found five alcohols between C_{16} and C_{22} (0.7%), squalene (0.15%), two lactones (furanones) (0.15%), one ketone (0.02%), and one unidentified, but characteristic, compound (0.04%). Major compounds were detected in all individuals, although relative proportions of some chemicals show a high interindividual variability. On average, the five most abundant chemicals were D-α-tocopherol (25.4%), a probable methyl derivative of ergostanol (15.9%), campesterol (11.6%), cholesterol (10.8%), and ergostanol (6.3%).

We found four forms of tocopherol in secretions. The main one was D-α-tocopherol ($\overline{X} \pm S = 25.4 \pm 3.2\%$ of TIC, range=12.0–30.7%), followed by 6-*O*-methyl-α-tocopherol ($\overline{X} \pm S = 2.27 \pm 1.27\%$ of TIC, range=0.06–0.39%), and γ-tocopherol ($\overline{X} \pm S = 0.2 \pm 0.1\%$ of TIC, range=0.73–4.61%). Finally, we found much lesser amounts of an unidentified form of tocopherol ($\overline{X} \pm S = 0.09 \pm 0.04\%$, range=0.04–0.17%). Proportions of D-α-tocopherol were significantly related to proportions of 6-*O*-methyl-α-tocopherol (r=0.87, $F_{1,18}$=53.82, P<0.0001) and γ-tocopherol (r=0.54, $F_{1,18}$=7.39, P=0.014).

The CMI response of male lizards (residuals on body mass) was significantly related to the proportions of D-α-tocopherol (r=0.78, $F_{1,18}$=27.39, P<0.0001) and 6-*O*-methyl-α-tocopherol (r=0.47, $F_{1,18}$=5.20, P=0.035) (Fig. 1). Thus, males with a higher immune response had more D-α- and 6-*O*-methyl-α-tocopherol in their secretions. However, the CMI response was not significantly related to proportions of γ-tocopherol (r=0.14, $F_{1,18}$=0.35, P=0.56) or the unidentified form of tocopherol (r=0.21, $F_{1,18}$=0.81, P=0.38).

Visual signals

Male *L. lepida* lizards had flank colorations with hue values that ranged between 533 and 604 nm $\left(\overline{X} \pm \mathrm{SE} = 566 \pm 4\,\mathrm{nm}\right)$, saturation values between 14.6% and 20.0% $\left(\overline{X} \pm \mathrm{SE} = 17.0 \pm 0.4\%\right)$, and brightness values between 67.4 and 77.2 $\left(\overline{X} \pm \mathrm{SE} = 71.7 \pm 0.6\right)$.

The CMI response of male lizards was significantly and negatively correlated with hue (r=−0.64, $F_{1,18}$=12.51, P=0.0023) and brightness (r=−0.52, $F_{1,18}$=6.81, P=0.018), and positively correlated with saturation (r=0.55, $F_{1,18}$=7.99, P=0.011) of flank coloration (Fig. 2), Thus, males with a higher immune response had flanks that were more greenish, darker, and more saturated.

Table 1 Lipophilic compounds found in femoral secretions of male-eyed lizards, *L. lepida*

RT (min)	Compound	Mean ± *S*
25.3	Nonanoic acid	0.06±0.05
27.9	Decanoic acid	0.10±0.18
32.9	Dodecanoic acid	0.09±0.07
37.4	Tetradecanoic acid	0.09±0.06
39.4	Pentadecanoic acid	0.04±0.02
39.9	Hexadecanol	0.17±0.25
41.1	Hexadecenoic acid	0.24±0.11
41.5	Hexadecanoic acid	5.00±2.52
41.5	Hexadecanoic acid, ethyl ester	0.13±0.09
42.3	Heptadecanol	0.11±0.05
43.3	Heptadecanoic acid	0.11±0.08
43.8	Octadecanol	0.05±0.04
44.7	9.12-Octadecadienoic acid	0.17±0.06
44.8	Octadecenoic acid	2.56±0.80
45.2	Octadecanoic acid	2.74±0.82
45.8	Octadecanoic acid, ethyl ester	0.06±0.06
47.4	Eicosanol	0.20±0.13
47.8	2-Nonadecanone	0.02±0.01
48.3	Erucic acid	0.10±0.05
48.6	Eicosanoic acid	0.40±0.20
48.6	Eicosanoic acid, ethyl ester	0.04±0.04
47.4	Docosanol	0.13±0.11
51.3	4-Hydroxy-octadecanoic acid, γ-lactone	0.03±0.04
51.8	Docosanoic acid	0.05±0.04
51.8	Docosanoic acid, ethyl ester	0.02±0.03
53.8	Unidentified compound (179,291,403)	0.04±0.03
54.4	Unidentified lactone	0.12±0.02
55.7	Squalene	0.15±0.20
56.8	Cholesta-3.5-diene	0.10±0.06
57.6	unidentified form of tocopherol	0.09±0.04
59.2	6-*O*-methyl-α-tocopherol	2.27±1.27
59.4	γ-Tocopherol	0.18±0.14
60.8	Cholesterol	10.79±6.58
61.0	Cholestanol	12.30±1.54
61.2	D-α-Tocopherol	25.39±3.18
61.9	Cholestan-3-one	1.84±0.42
62.1	Ergost-22-en-3-ol	0.13±0.19
62.4	Unidentified steroid (367,385,401,430)	0.31±0.06
63.1	Ergosta-5,22-dien-3-ol	0.07±0.04
63.4	Campesterol	11.65±2.84
63.7	Ergostanol	6.30±3.40
64.0	Cholest-4-en-3-one	0.64±0.38
64.3	Stigmasterol	0.23±0.15
64.6	Unidentified steroid (271,299,314,355,401,445)	0.04±0.07
64.7	Cholestan-3-one, methyl derivative?	1.49±0.52
65.2	4,4-Dimethyl-cholesta-8,14-dien-3-ol	0.08±0.07
65.5	Stigmasta-5,22-dien-3-ol	0.39±0.45
65.8	Ergost-22-en-3-ol, methyl derivative?	0.54±0.63
65.9	γ-Sitosterol	5.12±2.35
66.3	Ergostanol, methyl derivative?	15.94±5.02
66.9	20-Methyl-pregn-20-en-3-ol	0.55±0.25
67.3	Cholest-5-en-3-one	0.35±0.25
67.6	Cholestan-3-one, methyl derivative?	1.37±0.73
68.8	Cholest-4-ene-3,6-dione	0.51±0.25
69.1	Unidentified steroid (229,244,369,384,405,429)	0.07±0.05
70.6	Stigmast-4-en-3-one	0.27±0.20
73.2	Unidentified steroid (137,252,261,287,384,414)	0.36±0.17
73.5	Unidentified steroid (229,245,383,398)	0.07±0.07
74.3	Unidentified steroid (255,273,301,331,401)	0.07±0.07

The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (±1S) for 20 individuals. Characteristic ions (*m/z*) are reported for unidentified compounds

RT Retention time

Relationships between chemical and visual signals

Lizards with higher proportions of D-α-tocopherol in secretions had flanks that were significantly more greenish (hue, $r=-0.48$, $F_{1,18}=5.40$, $P=0.032$) and more saturated (saturation, $r=0.50$, $F_{1,18}=5.99$, $P=0.025$), and that tended, although not significantly, to be darker (brightness, $r=-0.43$, $F_{1,18}=3.99$, $P=0.061$) (Fig. 3). Partial correlations showed that both proportions of D-α-tocopherol ($r=0.69$, $P=0.001$) and hue of flanks ($r=-0.49$, $P=0.001$) were significantly related to the immune response when controlling for the effect of the correlation between these variables.

Relationships between the three color variables and proportions of 6-*O*-methyl-α-tocopherol, γ-tocopherol, or the unidentified form of tocopherol were all nonsignificant ($-0.28<r<0.17$, $F_{1,18}<1.57$, $P>0.22$ in all cases).

Discussion

Our results show that male lizards *L. lepida* that have higher proportions of α-tocopherol (vitamin E) in their femoral secretions, but also those with more greenish saturated colorations, have a higher immune response. This suggests that only males of higher quality, as indicated by their higher T cell immune response, are able to allocate enough vitamin E to secretions and enough carotenoids to coloration. Previous experiments with other lacertid lizard species have shown that the experimental activation of the immune system affects negatively the expression of both coloration and chemicals in femoral secretions of males

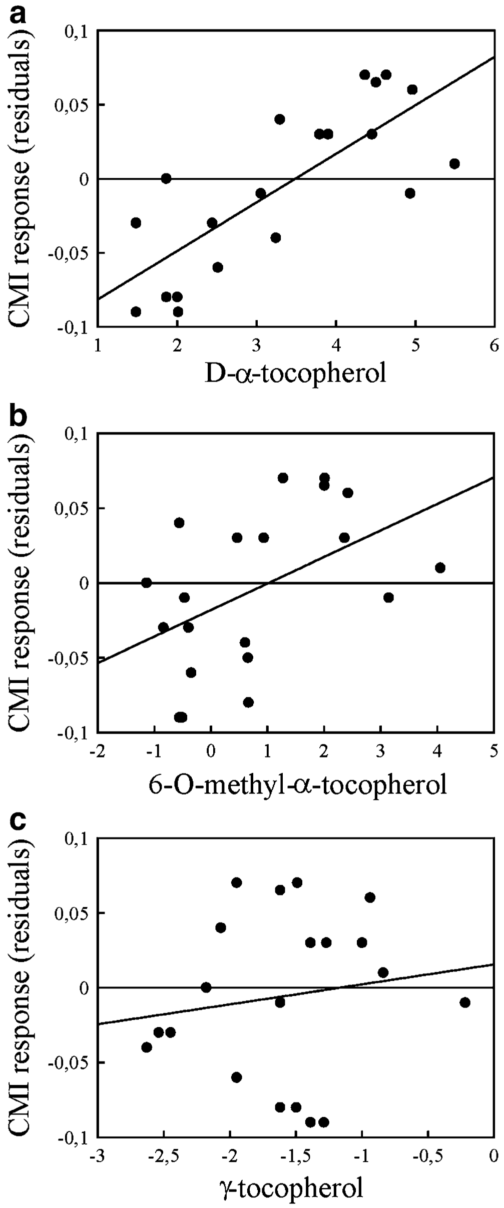

Fig. 1 Relationships between the cell-mediated immune (CMI) response of male *L. lepida* lizards (residuals on body mass) and the relative proportions (transformed TIC areas) of different forms of tocopherol (vitamin E) in their femoral secretions

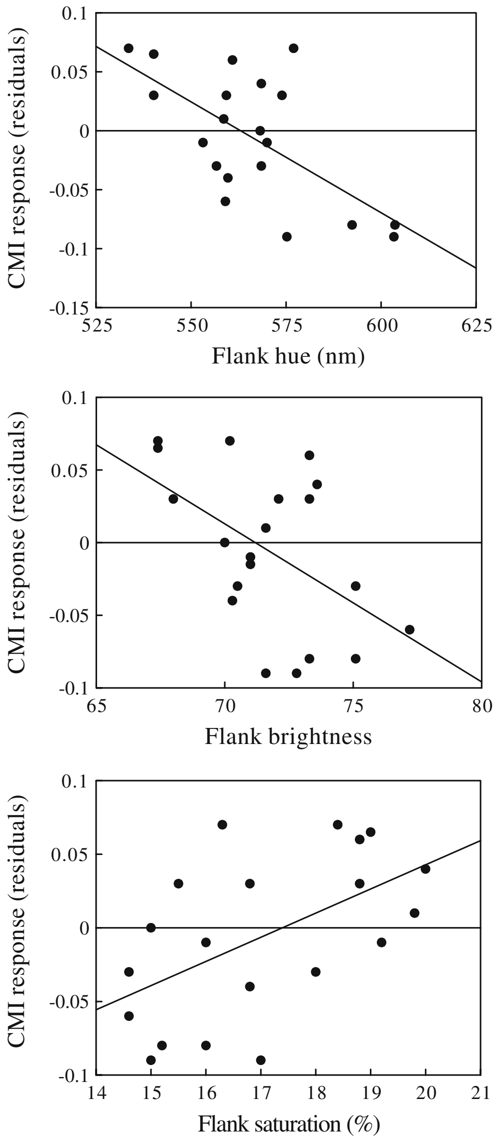

Fig. 2 Relationships between the cell-mediated immune (CMI) response of male *L. lepida* lizards (residuals on body mass) and the hue, brightness, or saturation of their flank colorations

(López et al. 2009a, b). These results would support the hypothesis that it is costly to produce both chemical and visual signals and suggests their potential role as condition-dependent honest advertisements, which could be used by females to choose mates (Grafen 1990; Kotiaho 2001).

Carotenoid-based sexual visual signals have been considered good examples of honest signaling of male quality (Olson and Owens 1998). This is because carotenoids might be scarce, and therefore might indicate the ability of males to gather high-quality food and because they are involved in important physiological functions (Olson and Owens 1998). Moreover, males that can afford to produce more elaborate carotenoid-dependent displays seem to be healthier individuals with superior immunocompetence (Blount et al. 2003; Faivre et al. 2003). For example, in other green lizard species, male *L. schreiberi* with a higher immune response had darker dorsal green coloration and more saturated yellowish throats (Martín and López 2009). Similarly, less-parasitized male *Podarcis muralis* lizards had brighter and more yellowish ventral colorations, and males with a greater immune response had more saturated coloration (Martín et al. 2008). We have also found similar results in this study with male *L. lepida* with a higher immune response having flanks that are more greenish, darker, and more saturated.

There seem to be similar trade-offs for specific chemical compounds allocated to chemical signals. In other lacertid lizards, a precursor of vitamin D_3 in femoral secretions has

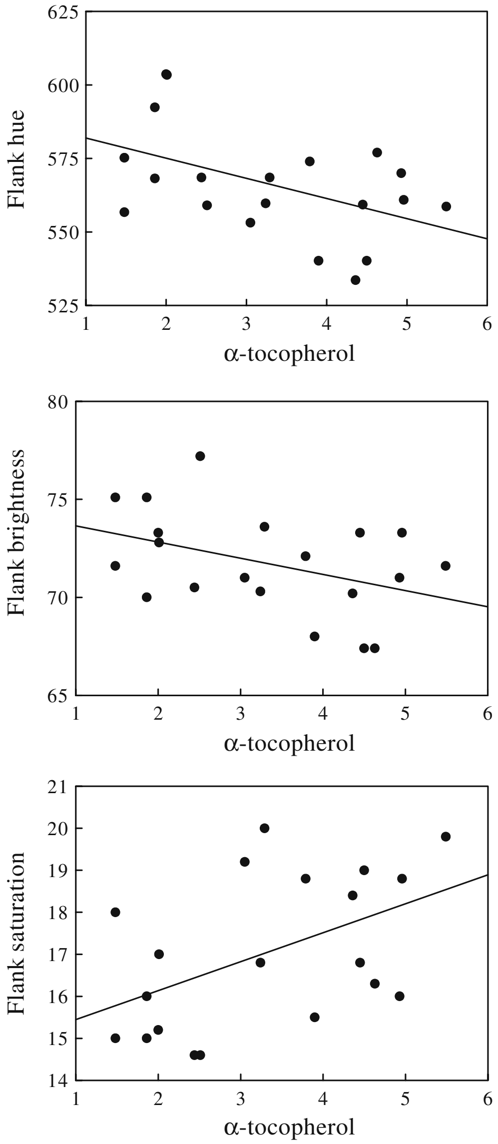

Fig. 3 Relationships between the relative proportions (transformed TIC areas) of α-tocopherol (vitamin E) in the femoral secretions of male *L. lepida* lizards and the hue, brightness, or saturation of their flank colorations

been considered as a potentially costly compound that would ensure reliability and honesty of chemical signals; males of two lizard species that allocate more pro-vitamin D_3 to secretions have higher immune responses (López and Martín 2005; Martín and López 2006a), and challenging the immune system results in a decrease in proportions of pro-vitamin D secreted (López et al. 2009b). Moreover, male lizards with a diet experimentally supplemented with vitamin D may increase proportion of pro-vitamin D in secretions (Martín and López 2006b), and females prefer scent marks of males with higher proportions of this compound in secretions (Martín and López 2006a, b). This relationship could be explained because vitamin D has important physiological functions in calcium metabolism and as a potent immunomodulator (Fraser 1995; Hayes et al. 2003), such that its allocation to femoral secretions would be differentially costly or beneficial for males of different quality.

Our current study suggests that vitamin E could have a similar role in secretions of green lizards. The primary function of vitamin E in secretions could be as a potent antioxidant that would increase duration of scent marks, and that even might just avoid the oxidation of other chemicals in secretions that were the true semiochemicals (Alberts 1992; Martínez et al. 2008). This function would be especially useful in the wet grassy environments usually occupied by green lizards. However, the allocation of vitamin E to secretions has to be costly. First, because this vitamin has a dietary origin (Bender 2009), such that only lizards with a high-quality diet were able to obtain enough amounts of vitamin E for metabolism functions and elaboration of chemical signals. Thus, male *L. viridis* lizards that received a dietary supplement of vitamin E had femoral secretions with higher proportions of vitamin E (R Kopena, G Herczeg, P López, and J Martín, unpublished data). Second, because among these males, only those with a good health condition would have enough vitamin E that does not need to be used in the maintenance of the immune system and can be available for secretions. Therefore, the cost of allocating vitamin E to secretions, which should be differentially costly for different individual males of different quality, may be a mechanism that confers reliability to scent marks of green lizards and allows their evolution as sexual signals. Either by assessing directly the contents of vitamin E, or thanks to the greater protection provided to other semiochemicals, females might estimate the quality of a male or of his territory.

The relationship between the cell-mediated immune (CMI) response and proportions of vitamin E in secretions was valid for DL-α-tocopherol and its methyl derivative, which are the most abundant forms of vitamin E in secretions, but not for γ-tocopherol. This may be explained because α-tocopherol functions as an oxygen radical scavenging, whereas γ-tocopherol has a different function as a powerful nucleophile that traps electrophilic mutagens, such as peroxynitrite in lipophilic compartments (Christen et al. 1997). Thus, γ-tocopherol may protect lipids, DNA, and proteins from peroxynitrite-dependent damage. It seems that our measurement of CMI response was not related to availability of γ-tocopherol, but high proportions of this component in secretions might still signal a good ability to avoid peroxynitrite-dependent damage.

Interestingly, we have found that information provided by visual and chemical signals is congruent. Males with a better immune response were those with more vitamin E and with more greenish coloration. Similarly, less-parasitized male *P. muralis* lizards had more yellowish ventral colorations and also femoral secretions with higher proportions of two esters of octadecenoic acid (Martín et al. 2008). In addition, *P. muralis* lizards with a greater immune response had more saturated coloration and secretions with higher proportions of octadecenoic acid methyl ester (Martín et al. 2008). This consistence between chemical and visual signals would allow signaling the same characteristics of a male in different contexts. For example, colorful visual signals might be more important in long-distance communication, whereas chemical signals may be more important when two individuals are close together and also provide information when the signaler has left the area (e.g., López et al. 2002, 2003). Also, environmental conditions (light levels, humidity, etc.) may render some type of signals more effective than others in different microhabitats (Alberts 1992; Endler 1992). Therefore, our results support that in animals that use different multiple sensory systems, such as lizards, different types of traits may have evolved to signal similar characteristics of a male quality using different sensory channels. The use of multiple sensory channels may allow signaling a male quality under different circumstances, but also may reinforce the reliability of the signal when both types of traits may be perceived simultaneously. If both types of signals are not congruent, a cheating male may be easily detected (López et al. 2003; Whiting et al. 2009).

We have also found that characteristics of coloration produced by carotenoids mirror the amounts of vitamin E in secretions. This might support the hypothesis of Hartley and Kennedy (2004) that the biological role of carotenoids might not be as antioxidants, but that colorful carotenoids would just reflect and "inform" on the actual contents of antioxidant vitamin E in the organism, which is part of the true system that prevents oxidation. This may be explained because oxidation of carotenoids alters or destroys their color, and preservation of their color intensity would indicate the possession of enough efficient antioxidants, such as vitamin E, for their protection (Hartley and Kennedy 2004).

We suggest that the metabolic roles of vitamins might be the actual basis of honesty of many sexual displays, and that carotenoid-based signals might be just the visible part of the whole system informing in the visual channel on the presence of "invisible" costly chemicals. Because lizards also use their chemosensory system, they might directly use vitamins to extend the signal to an additional sensory channel. Nevertheless, we also found that both proportions of vitamin E and hue of flanks were related to the immune response when controlling statistically for the effect of the correlation between these variables. This suggests that although carotenoids in coloration might mainly reflect the functioning of the antioxidant system in which vitamin E is important, carotenoids might also signal per se some other additional characteristics of the immune response in which they participate (Hartley and Kennedy 2004). However, to properly test these hypotheses, we would need to manipulate the available contents of carotenoids and vitamin E in the diet, and to examine whether visual and chemical signals are expressed independently, or whether coloration characteristics only mirror characteristics of femoral secretions.

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