Short Notes

Chemical stimuli mediate species recognition in *Podarcis* wall lizards

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The mechanisms whereby closely related lineages, often very similar in morphology, achieve reproductive isolation in sympatry have attracted considerable attention (Andersson, 1994; Tregenza et al., 2000). Results from several taxa suggest that behaviour plays a major role in preventing interspecific matings. Differences in mating signals between species are often much more marked than morphological differences and provide a major barrier to gene exchange, which may lead to speciation (Uzendoski and Verrell, 1993; Butlin and Ritchie, 1994). In animals with well-developed chemosensory systems, detection of species-specific chemicals allows individuals to recognize each other as potential mates and promotes assortative mating (Dawley, 1987; Uzendoski and Verrell, 1993; Verrell, 2003; Linn and Roelofs, 1989; Shine et al., 2002; reviewed in Wyatt, 2003). Squamates are arguably among the most chemosensory of vertebrates (Burghardt, 1980; Halpern, 1992; Font, 1996) and chemicals released at the time of reproduction may provide the basis for species recognition and avoidance of interspecific matings among closely related sympatric species (Cooper and Vitt, 1985, 1986a, 1987; Mason and Gutzke, 1990; Mason, 1992; Labra et al., 2001).

In recent years, molecular techniques, combined with morphological studies, have clarified the phylogenetic relationships within the genus *Podarcis* (Harris et al., 1998; Harris and Arnold, 1999; Oliverio et al., 2000). However, in the Iberian Peninsula, *Podarcis* taxonomy has been controversial and unstable, with the identification of several cryptic forms (Sá-Sousa, 2001a; Harris and Sá-Sousa, 2001, 2002; Harris et al., 2002; Pinho et al., 2003). Initially, the limited morphological variation between forms led to the belief that they were morphotypes, but recent molecular studies have revealed large genetic distances suggesting that some of these forms are probably distinct species (Harris and Sá-Sousa, 2002).

Few studies of chemoreception in *Podarcis* have concerned themselves with the issue of species recognition. Gómez et al. (1993) provided the first evidence of chemically-mediated species recognition in a lacertid: male *Podarcis hispanica* are capable of detecting and discriminating conspecific from non-conspecific (*Psammodromus algirus*) chemicals. However, the two species used in this study were not even congeneric. More recently, Cooper and Pérez-Mellado (2002), working also with *P. hispanica*, reported that males of this species have the ability to discriminate between sympatric con-

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geners based on chemical cues alone. These findings point to a possible role of chemoreception as an ethological isolating mechanism in this complex genus.

In this study, we set out to determine whether chemical stimuli might contribute to reproductive isolation between *Podarcis bocagei* (Seoane, 1884) and *Podarcis carbonelli* (Pérez-Mellado, 1981). Both species are small ground-dwelling lizards endemic from the western Iberian Peninsula that were considered conspecific until very recently (e.g. Barbadillo et al., 1999; Salvador and Pleguezuelos, 2002). *P. carbonelli* was described as a subspecies of *P. bocagei* following the revalidation of the latter as a species (Pérez-Mellado, 1981a, b). However, morphological and genetic studies not only support the specific status of *P. carbonelli* (Sá-Sousa et al., 2000; Sá-Sousa, 2001a; Sá-Sousa and Harris, 2002), but also suggest that *P. carbonelli* and *P. bocagei* are not even sister taxa (Harris and Sá-Sousa, 2001, 2002). The two species are similar in their ecology, occupy the same habitats, reproduce at the same time of the year, and may even occur in sympatry (Sá-Sousa, 2001b; Carretero et al., 2002). The fact that no morphologically intermediate individuals between *P. carbonelli* and *P. bocagei* have been reported (but see Galán, 2002) raises the question of how these two species achieve reproductive isolation. As no extrinsic barriers to hybridization have been described, a likely possibility is that interbreeding is prevented by behavioural differences between the two species. Here, we especifically test the ability of males of *P. carbonelli* and *P. bocagei* to discriminate between substrates labelled by females of their own or of the other species.

During May of 2003, lizards of both species were collected in coastal sandy areas of northern Portugal (near Porto). The lizards were transported to the laboratory in Valencia in individual plastic terraria (20 × 12 × 16 cm) with a substrate of sand obtained at the capture site. In the laboratory, lizards were kept in the same terraria, with a permanent supply of water and a rock for basking and shelter. Light and heat were provided by incandescent (40 W) bulbs, placed above each terrarium. Lights were scheduled to provide a 14-10 hour L:D cycle. Terraria were kept in a temperature-controlled room (20-30°C) at ambient humidity (31-74%). Lizards were fed daily with *Tenebrio monitor* larvae dusted with vitamins, supplemented occasionally with other insects, such as small crickets (*Acheta domestica*).

The experimental trials were conducted during June 2003. Trials consisted of gently picking up a male and transferring it to a test terrarium (40 × 20 × 25 cm) for a 10-minute observation period. The test terrarium was kept in the same room where lizards were housed and had a 40W incandescent bulb suspended ca. 20 cm above the floor of the terraria as a source of heat and light. The floor of the test terrarium was covered with a paper substrate. The stimulus conditions were prepared by placing an odour donor inside the test terrarium in the evening preceding an experimental trial and allowing it to remain there until 20 minutes before the trial. Shed skin, faeces and other obvious visual stimuli left by the donor were removed prior to the trial. For control trials, the test terrarium was fitted with a clean paper substrate. Eleven females of both species (five *P. bocagei* and six *P. carbonelli*) acted as donors of chemical stimuli. Males were tested three times, once in a clean test terrarium (control), once in a test terrarium bearing chemical stimuli from a conspecific female, and once in a test terrarium bearing chemical stimuli from a heterospecific female. Each lizard was tested only once per day with an inter-trial interval of two days. Ten *P. carbonelli* and nine *P. bocagei* males were tested. The order of stimulus presentation was partially counterbalanced to avoid sequence effects. At the end of each trial, the paper substrate was discarded and the terrarium was washed thoroughly with water and alcohol to eliminate residual chemical traces. Trials were conducted between 12:00 h and 15:00 h (official local time) when the lizards were fully active. Room temperature at the time of testing was maintained between 26 and 29°C in order to minimize variability arising from thermal dependence of tongue-flick rates (Cooper and Vitt, 1986b).

Each trial was video-recorded and the lizards' behaviour was later analysed with the aid of a portable computer equipped with JWatcher event-recording software (Blumstein et al., 2000). As we were interested in the lizards' chemosensory responses, we focused our behavioural observations on tongue-flicks, which in squamates function to acquire chemicals for analysis by the vomeronasal organ located in the roof of the mouth (Graves and Halpern, 1989; Halpern, 1992). We scored the latency to the first tongue-flick and three types of tongue-flicks: tongue-flicks directed at the substrate, tongue-flicks directed at the terrarium walls, and tongue-flicks directed at the air.

We fitted a robust partly nested ANOVA model to rank-transformed data using SPSS 11.5. We used a group by trials repeated measures design with responses to each of the three stimulus conditions as the repeated measure and with individual males (random factor) nested within species (Quinn and Keough, 2002). The sphericity assumption was tested with Mauchly's test. In those cases in which significant departures from sphericity were detected, we tested the significance of treatment effects and of the treatment by species interaction using adjusted univariate F-ratios and multivariate ANOVA statistics (Pillai Trace). Following detection of

Table 1. Mean (range) number of tongue-flicks and latency (s) shown by the lizards in each treatment.

Behavior	Treatment		
	Control	Conspecific female	Heterospecific female
Podarcis carbonelli (**$n = 9$**)			
Tongue-flicks (substrate)	53.2 (2-128)	138.3 (38-274)	93.1 (2-194)
Tongue-flicks (walls)	94.0 (7-194)	108.9 (51-206)	79.2 (23-146)
Tongue-flicks (air)	23.7 (3-58)	25.8 (7-83)	22.9 (4-89)
Latency to the first tongue-flick (s)	36.96 (0.99-124.62)	38.93 (1.15-169.28)	22.39 (2.03-74.54)
Podarcis bocagei (**$n = 8$**)			
Tongue-flicks (substrate)	77.5 (13-148)	145.9 (0-249)	97.5 (3-214)
Tongue-flicks (walls)	139.4 (79-199)	126.7 (1-263)	135.5 (16-287)
Tongue-flicks (air)	26.1 (3-40)	30.4 (3-50)	29.9 (5-55)
Latency to the first tongue-flick (s)	15.08 (0.55-67.78)	34.56 (0.49-156.59)	20.88 (2.39-69.31)

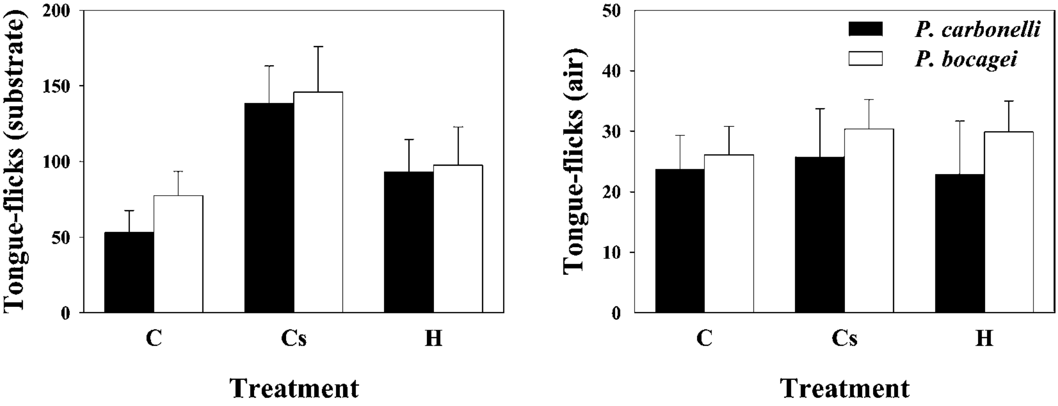

Figure 1. Mean (standard error) number of tongue-flicks performed by the lizards in each treatment (C — control; Cs — conspecific female; H — heterospecific female).

significant treatment effects, we conducted planned pairwise comparisons between conspecific stimuli and the other two stimulus conditions. Significance level for the rejection of the null hypothesis was set at $\alpha = 0.05$.

The responses of the two species to the different treatments are summarized in table 1 and in fig. 1. Two *P. carbonelli* and one *P. bocagei* males remained motionless or exhibited persistent escape attempts during some of the trials and their data were discarded from the analysis.

Of the three types of tongue-flicks recorded, there was a significant treatment effect only for tongue-flicks directed at the substrate. Table 2 shows F-ratios adjusted by means of two commonly used types of correction (Quinn and Keough, 2002). As we could not assume sphericity (Mauchly's test: $W = 0.61$, $df = 2$, $p = 0.03$), we tested for treatment and treatment by species interaction effects on the number of tongue-flicks directed at the substrate using adjusted univariate and multivariate statistics. Both tests indicated a highly significant treatment effect. Lizards in both species directed more tongue flicks at substrates bearing chemical stimuli from conspecific females than to clean (control) substrates ($F = 27.94$, $df = 1$, $p < 0.001$) or substrates labelled by heterospecific females ($F = 9.51$, $df = 1$, $p = 0.008$). In no case were the species and treatment by species interaction terms significant. Latencies to the first tongue-flick were highly variable and showed no obvious trends between treatments or species.

Our results indicate that *P. carbonelli* and *P. bocagei* males respond differently to chemical cues of conspecific and heterospecific

Table 2. ANOVA table for the number of tongue-flicks directed at the substrate (after rank transformation). Note that the conclusions of a significant treatment effect and lack of species and treatment by species interaction effects are unchanged irrespective of whether adjusted univariate or multivariate results are used.

Source of variation	*F*	*df*	*p*
Between subjects			
Species	0.226	1	0.641
Within subjects			
Treatment			
Greenhouse-Geiser	18.212	1.434	<0.001
Huyn-Feldt	18.212	1.65	<0.001
Pillai Trace	16.783	2	<0.001
Treatment *x* Species			
Greenhouse-Geiser	0.566	1.434	0.520
Huyn-Feldt	0.566	1.65	0.543
Pillai Trace	1.338	2	0.294

females, exhibiting more chemosensory behaviours (i.e. tongue-flicks) to conspecifics. This clearly indicates their ability to discriminate conspecific females from females of a closely related species using only substrate-borne chemical cues. Thus, differences in female chemical cues may underlie mating preferences in these species. The lack of significant species or treatment by species interaction effects further indicates that males of the two species respond similarly to chemicals of conspecific and heterospecific females. To our knowledge, this is the first demonstration of symmetrical chemically-mediated species discrimination in lizards.

Recently, Cooper and Pérez-Mellado (2002) showed that *P. hispanica* males were capable of discriminating between chemicals of conspecific females presented on cotton swabs and those of sympatric *P. carbonelli*. However, these authors did not test the ability of *P. carbonelli* males to discriminate between chemical cues of their own and those of the other species. Moreover, the use of cotton swabs in chemical discrimination studies is controversial, since the cotton swab adds an unwanted visual stimulus that may complicate interpretation of the results (Cooper, 1998; Desfilis et al., 2003). Our experimental design using labelled substrates, on the other hand, provides a more naturalistic approach and may thus have more ethological validity (Greenberg, 1994).

Our knowledge of the specific chemicals that mediate chemosensory responses in lizards is very poor. The compounds involved may be present in femoral (Alberts, 1990, 1991; Alberts et al., 1992) or cloacal gland secretions (Trauth et al., 1987; Cooper and Trauth, 1992), skin secretions (Weldon and Bagnall, 1987; Mason and Gutzke, 1990), or in faeces (Carpenter and Duvall, 1995; Bull et al., 1999; Labra et al., 2002). Although our study did not specifically address the source of chemicals involved in species recognition, the fact that a differential response was obtained for tongue-flicks directed at the substrate, but not for those directed at the terrarium walls or at the air, suggests that discrimination is based on non-volatile chemicals detected by vomerolfaction (Burghardt, 1980; Font and Desfilis, 2002). Since we did not observe any behaviour suggesting active marking by females, we assume that chemical marking by females is a passive process: females probably label paper substrates as they move around dragging their ventral surface, as has been described for juvenile *P. hispanica* lizards (Font and Desfilis, 2002). Field observations suggest that, particularly in sand dunes such as those where these animals were collected, passive marking by females would probably be limited to vegetation or objects of anthropogenic origin, since sand is a moving substrate that presents a major challenge for chemical marking (Alberts, 1992).

Chemical species recognition has been reported in other squamates, especially in complex groups such as *Liolaemus* (Escobar et al., 2001; Labra et al., 2001) and *Eumeces* (Cooper and Vitt, 1985, 1986a, 1987). In closely related sympatric species, an ability to distinguish between conspecifics and heterospecifics may be very important for reducing energy costs of finding potential mates (Cooper and Pérez-Mellado, 2002) or sexual rivals (Cooper and Garstka, 1987).

Vision and chemoreception are important for courtship and mating in lacertids (Verbeek, 1972; López and Martín, 2001; López et al., 2003), although the relative importance of these sensory modalities may vary among species. Females of *P. carbonelli* and *P. bocagei* are morphologically very similar (Sá-Sousa, 2001a), which suggests that mechanisms other than visual recognition may be at work when a male is searching for a potential mate. Our results demonstrate that chemical cues may be one of the mechanisms involved in mate recognition and in preventing interspecific matings, since males discriminate in favour of substrates labelled by females of their own species (Labra et al., 2001; Shine et al., 2002). However, chemically-mediated discrimination may not constitute a complete reproductive barrier and males could occasionally recognize heterospecific females as appropriate targets for courtship, since hybrids between *P. carbonelli* males and *P. bocagei* females have been obtained in the laboratory (Galán, 2002).

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