J Chem Ecol (2006) 32: 1587–1598
DOI 10.1007/s10886-006-9072-5

Chemically Mediated Species Recognition in Closely Related *Podarcis* Wall Lizards

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Received: 19 December 2005 / Revised: 30 January 2006 /
Accepted: 17 February 2006 / Published online: 23 May 2006

Abstract In many animals, chemical signals play an important role in species recognition and may contribute to reproductive isolation and speciation. The Iberian lizards of the genus *Podarcis*, with up to nine currently recognized lineages that are often sympatric, are highly chemosensory and provide an excellent model for the study of chemically mediated species recognition in closely related taxa. In this study, we tested the ability of male and female lizards of two sister species with widely overlapping distribution ranges (*Podarcis bocagei* and *P. hispanica* type 1) to discriminate between conspecific and heterospecific mates by using only substrate-borne chemical cues. We scored the number of tongue flicks directed at the paper substrate by each individual in a terrarium previously occupied by a conspecific or a heterospecific lizard of the opposite sex. Results show that males of *P. bocagei* and *P. hispanica* type 1 are capable of discriminating chemically between conspecifics and heterospecifics of the opposite sex, but females are not. These results suggest that differences in female, but not male, chemical cues may underlie species recognition and contribute to reproductive isolation in these species. The apparent inability of females to discriminate conspecific from heterospecific males, which is not because of reduced baseline exploration rates, is discussed in the context of sexual selection theory and species discrimination.

Keywords Ethological isolation mechanisms · Cryptic species · Chemoreception · Species recognition · *Podarcis* · Lizards · Reptiles

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Introduction

The evolution of prezygotic reproductive isolation remains a central issue in the study of speciation and one of the least well-understood questions in evolutionary biology (Eady, 2001). Results from several taxa suggest that behavior plays a major role in preventing interspecific mating (so-called "ethological isolation mechanisms"): differences in mating signals between species or populations are often much more pronounced than morphological differences and provide an important barrier to gene exchange (e.g., Butlin and Ritchie, 1994; Ptacek, 2000; Shine et al., 2002). Furthermore, behavioral characters are increasingly recognized as useful tools for phylogenetic reconstruction (de Queiroz and Wimberger, 1993; Gittleman and Decker, 1994). Thus, behavioral assays of species recognition may provide important clues to the elucidation of the taxonomic status in complex groups.

In many animals, chemical signals play an important role in species recognition and speciation: the detection of species-specific chemical cues allows individuals to recognize each other as potential mates and promotes assortative mating (reviewed in Wyatt, 2003). This phenomenon has been widely studied, although with a strong taxonomic bias toward invertebrates, mainly moths (e.g., Phelan and Baker, 1987), and also flies (e.g., Mas and Jallon, 2005), beetles (Symonds and Elgar, 2004), spiders (e.g., Roberts and Uetz, 2004), and polychaetes (Sutton et al., 2005). Squamate reptiles have well-developed chemosensory systems and are among the most chemosensory of vertebrates: for many lizards and snakes, chemical stimuli are important sources of information about several aspects of the environment crucial to fitness (Halpern, 1992; Schwenk, 1995). Chemicals released at the time of reproduction may provide the basis for species recognition and avoidance of interspecific mating among closely related sympatric species (Cooper and Vitt, 1987; Mason, 1992; Labra et al., 2001) or between different populations of the same species (LeMaster and Mason, 2003). Among lizards, the discrimination of conspecifics from heterospecifics based on chemical cues alone has been reported in a few scincid (Cooper and Vitt, 1987), iguanian (Labra et al., 2001), and lacertid species (Gómez et al., 1993; Cooper and Pérez-Mellado, 2002; Barbosa et al., 2005).

The lizards of the genus *Podarcis* are the predominant lacertid group in southern Europe, and their taxonomy has remained unstable and controversial until recently. In the Iberian Peninsula, the combination of nuclear and mitochondrial molecular markers has allowed the identification of several cryptic forms, probably with specific status, confirmed also by morphological studies (Harris and Sá-Sousa, 2002; Sá-Sousa, 2001; Sá-Sousa et al., 2002; Pinho et al., 2003, 2006; Kaliontzopoulou et al., 2005).

Although the chemosensory abilities of *Podarcis* lizards have been previously studied (e.g., López and Martín, 2001; Font and Desfilis, 2002; Desfilis et al., 2003), only a few studies have dealt 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* (*sensu lato*) were capable of detecting and discriminating conspecific from heterospecific (*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* (*sensu lato*), reported that males of this species have the ability to discriminate among conspecifics and sympatric female congeners (*Podarcis carbonelli*) based on chemical cues alone. These findings point to a possible role of chemoreception as an ethological isolating mechanism in this complex genus.

In a previous study (Barbosa et al., 2005), we tested the ability of males of two closely related species, *P. bocagei* and *P. carbonelli*, to discriminate between substrates labeled by conspecific and heterospecific females. Our results demonstrated the existence of reciprocal chemical discrimination between these two species. In the present study, we focused on *P. hispanica* type 1 and *P. bocagei*, a monophyletic and closely related species pair (in fact, sister taxa) within this genus, (Pinho et al., 2006), and tested the ability of individuals of either sex to discriminate between conspecifics and heterospecifics of the opposite sex. Because they are sister species, have largely overlapping distributions that includes contact even at a microscale (personal observation), no introgression events have been detected so far, and no extrinsic barriers to hybridization have been described, this species pair presents an interesting case study for research on the role of chemical cues in species discrimination and reproductive isolation.

Methods and Materials

Study Species

Podarcis hispanica type 1 and *P. bocagei* are small-sized lizards, which have a widely overlapping distribution in the NW Iberian Peninsula, living in sympatry over large areas. *P. hispanica* type 1 is a rock-dwelling lizard found in Galicia (Spain), northern Portugal, and the western Castilian plateau and Central System (Spain). *P. bocagei* is a ground-dwelling lizard occurring in western Asturias, Cantabria, Galicia (Spain), and northwestern Portugal (Galán, 1986; Sá-Sousa, 2000). Although very closely related, the two species can be easily identified by their morphological characteristics (for details, see Sá-Sousa, 2001; Sá-Sousa et al., 2002).

Animal Capture and Maintenance

Lizards were collected at the beginning of the reproductive season (February 2005) in the coastal area of Moledo do Minho (northern Portugal), where both species occur syntopically. They were transported to and housed at the laboratory in Valencia, Spain. Lizards were transported in individual plastic terraria (20 × 12 × 16 cm) with damp paper to avoid dehydration and then housed in glass terraria (40 × 20 × 25 cm for females and 35 × 20 × 20 cm for males), with a permanent supply of water and a rock for basking and shelter. Incandescent (40-W) bulbs placed above each terrarium provided light and heat. Lights were scheduled to provide a 12-hr light/12-hr dark photoperiod cycle. Terraria were kept in a temperature-controlled room (temperature cycle of 24–6 hr: 19°C; 6–11 hr: 23°C; 11–20 hr: 28°C; 20–22 hr: 23°C; 22–24 hr: 22°C) at ambient humidity (2–45%). Lizards were fed daily with mealworm (*Tenebrio molitor*) larvae dusted with vitamins.

Experimental Design

The experimental trials were conducted during March and April 2005. Trials consisted of gently picking up a lizard and transferring it to a test terrarium (40 × 20 × 25 cm) for a 10-min observation period. The test terrarium was kept in the same room where lizards were housed and had a 40-W incandescent bulb suspended ca. 20 cm above the floor of the terrarium 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 odor donor inside the test terrarium in the evening preceding an experimental trial and allowing it to remain there until 15 min before the trial. Shed skin, feces, 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. 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 11:00 and 13:30 hr GMT, when the lizards were fully active. Room temperature at the time of testing was maintained between 26 and 29°C to minimize variability arising from thermal dependence of tongue flick rates (Cooper and Vitt, 1986a).

Each trial was video-recorded, and the lizards' behavior was later analyzed 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 behavioral observations on tongue flicks. Tongue flicking functions to acquire chemicals for analysis by the vomeronasal organ and provides an observable index of the chemosensory investigation of a stimulus (Cooper and Burghardt, 1990; Halpern, 1992). We scored the number of tongue flicks directed at the substrate, which is indicative of chemosensory investigation of traces left by other individuals, and the number of tongue flicks directed at the air (air licks). Although results were similar for both variables, based on our previous work (Font and Desfilis, 2002; Barbosa et al., 2005), we restricted further analyses to the number of tongue flicks directed at the substrate in each experimental condition. Moreover, it has been suggested that air licks may be more influenced by stress than specific stimuli, at least in other lizards (Greenberg, 1985).

In the first set of experiments, we tested the ability of *P. hispanica* type 1 and *P. bocagei* males to chemically discriminate conspecific from heterospecific females. Thirteen females (five *P. hispanica* type 1 and eight *P. bocagei*) acted as donors of chemical stimuli for the trials (no female was used more than five times as a donor). 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 individual was tested only once per day with an intertrial interval of 2 d. The order of stimulus presentation was partially counterbalanced to avoid sequence effects. Eleven *P. hispanica* type 1 and 11 *P. bocagei* males were tested.

In the second set of experiments, we tested the ability of females of both species to discriminate chemical stimuli of conspecific and heterospecific males. The procedure was identical to that described for the first experimental set, except for the sex of donors and experimental lizards. Thirteen males (six *P. hispanica* type 1 and seven *P. bocagei*) acted as donors of chemical stimuli for the trials (no male was used more than five times as a donor). Twelve *P. hispanica* type 1 and 11 *P. bocagei* females were tested.

Statistical Analyses

We fitted a robust partly nested analysis of variance (ANOVA) model to rank-transformed data using SPSS 11.5. We used a group × trials repeated-measures design with responses to each of the three stimulus conditions as the repeated measure and with individual males/females (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 × species interaction using adjusted univariate *F* ratios and multivariate ANOVA statistics (Pillai Trace). Following detection of significant treatment effects, we conducted pairwise comparisons between the different stimulus conditions. Power analyses were performed using *R*.

To compare the rate of tongue flicks of males and females in control conditions (which indicates the level of baseline chemosensory investigation by each sex), we used a Mann-Whitney nonparametric test for two independent samples (Siegel and Castellan, 1988).

Significance level for rejection of the null hypothesis was set at 0.05, and all tests were two-tailed. Data are presented as mean ± SEM.

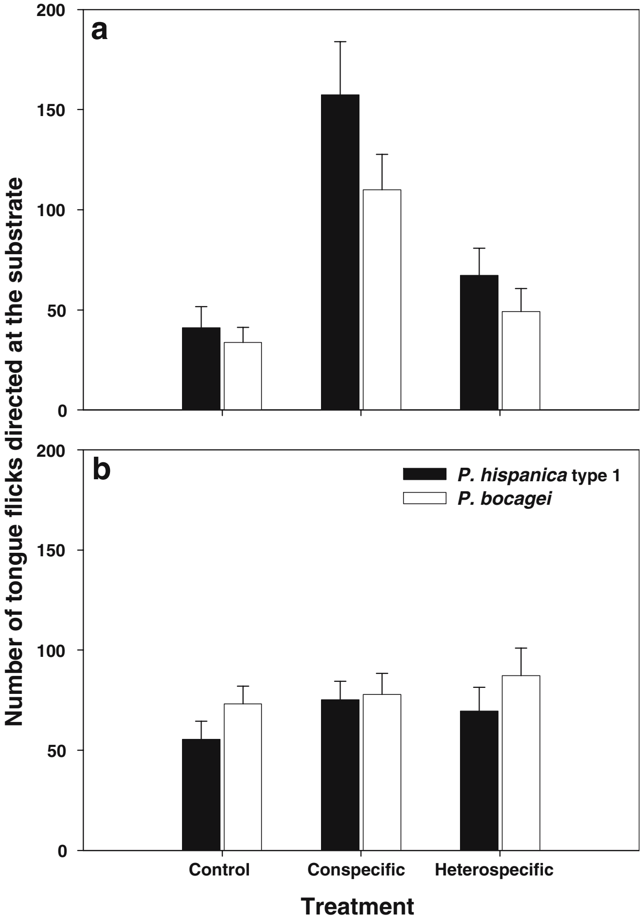

Fig. 1 Number (mean ± SEM) of tongue flicks of *P. hispanica* type 1 and *P. bocagei* males (a) and females (b) directed at the clean substrate (control) or at the substrate labeled by a conspecific or a heterospecific of the opposite sex

Results

Five *P. bocagei* (two males and three females) exhibited persistent escape attempts during the trials and were discarded from the analyses.

The males' responses to the treatments are illustrated in Fig. 1a. Table 1 shows *F* ratios adjusted by means of two commonly used types of correction (Quinn and Keough, 2002), although sphericity could be assumed (Mauchly's test: $W = 0.939$; $df = 2$; $P = 0.587$). All tests indicate a highly significant treatment effect. Lizards of both species directed more tongue flicks at substrates bearing chemical stimuli from conspecific females than to clean (control) substrates ($F = 65.68$, $df = 1$, $P < 0.001$) or substrates labeled by heterospecific females ($F = 36.706$, $df = 1$, $P < 0.001$). Moreover, lizards directed more tongue flicks to substrates labeled by heterospecifics than to clean substrates ($F = 5.079$, $df = 1$, $P = 0.037$). In no case was the species and treatment × species interaction term significant.

The females' responses to the treatments are depicted in Fig. 1b. Table 1 shows adjusted *F* ratios (as in the case of males). As we could not assume sphericity (Mauchly's test: $W = 0.507$; $df = 2$; $P = 0.003$), we tested for treatment and treatment × species interaction effects on the number of tongue flicks directed at the substrate using adjusted univariate and multivariate statistics. There were no significant species, treatment, or treatment × species interaction effects. This test had sufficient power (0.883) to detect a difference of the same magnitude as that found in the males' experimental set.

There was a statistically significant difference in the number of tongue flicks performed in control conditions between sexes in *P. bocagei* (females perform more tongue flicks than males: Mann-Whitney's test, $U = 8.5$, $P = 0.008$); in the case of *P. hispanica* type 1, no differences were detected between sexes (Mann-Whitney's test, $U = 44.5$, $P = 0.185$).

Table 1 ANOVA table for the number of tongue flicks directed at the substrate (after rank transformation) by *P. hispanica* type 1 and *P. bocagei*

Sex		Males			Females		
Source of variations		*F*	*df*	*P*	*F*	*df*	*P*
Between subjects							
Species		0.819	1	0.378	0.957	1	0.341
Within subjects							
Treatment	Sphericity assumed	33.024	2	<0.001	–	–	–
	Greenhouse-Geiser	33.024	1.885	<0.001	1.846	1.339	0.187
	Huyn-Feldt	33.024	2	<0.001	1.846	1.488	0.184
	Pillai Trace	37.685	2	<0.001	5.63	2	0.013[a]
Treatment × Species	Sphericity assumed	0.144	2	0.866	–	–	–
	Greenhouse-Geiser	0.144	1.885	0.855	0.683	1.339	0.458
	Huyn-Feldt	0.144	2	0.866	0.683	1.488	0.472
	Pillai Trace	0.109	2	0.897	1.827	2	0.191

[a] Note that the multivariate adjustment (Pillai Trace) indicates a significant treatment effect. However, because there is a disagreement between the univariate and multivariate adjusted results, we conclude, following Quinn and Keough (2002), that there is a lack of treatment effect.

Discussion

The results show that *P. hispanica* type 1 and *P. bocagei* males respond differentially to chemical cues of conspecific and heterospecific females, exhibiting more chemosensory behaviors in response to conspecifics. These results clearly indicate the males' ability to discriminate between conspecific and congeneric females of a sympatric, closely related species using only substrate-borne chemical cues. Thus, differences in female chemical cues may underlie specific recognition in these species. The lack of significant species or treatment × species interaction effects further indicates that males of the two species show consistent responses to the paper substrates. Moreover, the difference between the number of tongue flicks directed at substrates labeled by heterospecific females and controls indicates that the males also respond to heterospecific cues, which may suggest their detection as a biologically relevant stimulus. These findings are in agreement with previous results of reciprocal chemical discrimination in *P. carbonelli* and *P. bocagei* (Barbosa et al., 2005) and underscore the importance of chemical cues in species discrimination by *Podarcis* males, even in closely related lineages. These results also suggest that the chemicals deposited by females on the paper substrate vary in composition. These differences may be related to the genetic differences between the two species, as has been reported in mammals (Bininda-Emonds et al., 2001; Heth et al., 2001), although this hypothesis is still to be tested in lacertids.

Cooper and Pérez-Mellado (2002) showed that *P. hispanica* (*sensu lato*) males are capable of discriminating between chemicals of conspecific females and those of sympatric *P. carbonelli* presented on cotton swabs. However, these authors did not test the ability of *P. carbonelli* males to discriminate between chemical cues of their own females and those of the other species. In closely related sympatric species, an ability to distinguish between conspecifics and heterospecifics may be important for reducing energy costs of finding potential mates (Labra et al., 2001; Cooper and Pérez-Mellado, 2002) or sexual rivals (Cooper and Garstka, 1987) and for preventing interspecific courtship or fighting. Although vision and chemoreception have been recognized as important sensory modalities in courtship and mating in lacertids (Verbeek, 1972; López and Martín, 2001), the fact that *Podarcis* females belonging to different species are generally more similar in morphology and coloration than males (Sá-Sousa, 2001; Sá-Sousa et al., 2002) suggests that there may be a selective pressure for chemical recognition mechanisms to take precedence over visual recognition when a male is searching for a potential mate.

Chemical species recognition has been reported in other squamates, particularly in complex groups such as tropidurids of the genus *Liolaemus* and scincids of the genus *Eumeces*. *Liolaemus jamesi* and *L. bellii* are capable of discriminating conspecifics from sympatric heterospecifics (*L. alticolor* and *L. nigroviridis*, respectively) based on chemical cues, manifested by higher chemical exploratory behavior in enclosures previously occupied by conspecifics (Labra et al., 2001). In the species of the *Fasciatus* group of the genus *Eumeces*, lizards exhibit higher tongue flick rates to conspecifics than to members of closely related species (Cooper and Vitt, 1987). In addition, the sympatric sibling species of sea snakes *Laticauda colubrina* and *La. frontalis*, so similar in morphology that they were considered conspecific until recently, are reproductively isolated because of species-specific chemical cues (female skin lipids acting as pheromones) that elicit courtship by males of their own species (Shine et al., 2002).

Contrary to males, females of *P. hispanica* type 1 and *P. bocagei* did not respond differentially to conspecific and heterospecific male chemical cues. The contrasting discrimination by males and apparent lack thereof by females require an explanation. It could be argued that, compared to males, females have reduced chemosensory exploration rates when placed in a novel environment. However, this hypothesis is not supported by our results because a comparison of the basal rate of tongue flicks (number of tongue flicks performed in control conditions) of males and females revealed that females explore as much or even more than males.

Tongue flicks provide a convenient assay of the ability to respond differentially to biologically relevant stimuli and have been extensively used in the study of chemoreception in squamates (Cooper and Burghardt, 1990). Whereas significant results are usually taken as a *bona fide* evidence for discrimination, the interpretation of nonsignificant results in experiments where tongue flick rates are the dependent variable is not straightforward. In a thoughtful review of methodological issues relating to the study of squamate chemoreception, Cooper (1998) argued that, although differential tongue flick rates may indicate discrimination, their absence does not necessarily indicate a lack of discriminatory ability. On the other hand, several authors have suggested that the discrimination of individual characteristics of conspecifics, such as sex (Mason, 1993), body size and shape (Shine et al., 2003), or recent reproductive history (Shine et al., 2000), may require only a few tongue flicks. According to this interpretation, the differential tongue flicking rates would not indicate semiochemicals' discrimination but rather an interest in pursuing a scent trail and locating its source, as suggested by Cooper and Vitt (1986b). Thus, females may detect differences through vomerolfaction, but not respond by differential tongue flicking.

An alternative explanation is that species discrimination in females is based on sensory modalities other than vomerolfaction. Lacertids have well-developed olfactory systems (Gabe and Saint-Girons, 1976), and it is conceivable that olfaction alone may be sufficient to make adaptively significant discriminations (Halpern, 1992). This type of discrimination would not be detectable through varying tongue flicking rates, although other behaviors, such as buccal pulsing, could serve as observable indices indicating olfactory discrimination (Dial and Schwenk, 1996; Desfilis et al., 2003). The role of visual stimuli may also be important and cannot be discarded, especially considering that *Podarcis* males are, in general, more colorful and display much more interspecific variation in coloration patterns than females (Sá-Sousa, 2001; Sá-Sousa et al., 2002). Because males and females undergo different selective pressures to optimize different aspects of reproduction, it is conceivable and even likely that they use different criteria to assess potential mates and, thus, use different sensory modalities in mate choice/recognition (for an example in spiders, see Rypstra et al., 2003).

The apparent inability of females to discriminate substrate-borne chemical cues of males of their own and of a closely related species could be due to a lack of species specificity of semiochemicals released by males. According to this hypothesis, male-produced semiochemicals would have not diverged sufficiently to allow females to discriminate conspecific from congeneric males by using substrate-borne chemicals alone. This hypothesis seems unlikely for two reasons: (1) males are capable of species chemical discrimination of congeneric females (which suggests that female chemicals do have diverged), and (2) several studies with lizards have revealed the presence of variability in the chemical composition of male-produced semiochemicals, even among closely related species (Alberts, 1991; Labra et al., 2001).

Differences in discriminatory abilities of males and females have been reported in other studies. For example, Verrell (2003) found that males of the salamander *Desmognathus ocoee* discriminate conspecific from heterospecific and heterotypic females based on chemical cues, but females cannot make a similar discrimination. Similarly, studies of sex recognition in lizards and snakes have reported differences in the discriminating abilities of males and females. Females of *Eumeces laticeps* (Cooper and Vitt, 1984) and *Eublepharis macularius* (Steele and Cooper, 1997) do not discriminate sexes by differential tongue flicking, while males of these species do. In garter snakes, *Thamnophis sirtalis*, males discriminate and follow female trails during the breeding season, whereas females, in general, do not trail either sex (Mason, 1992; LeMaster et al., 2001). These findings may reflect the lack of a selective pressure for females to locate mates by scent tracking (Cooper and Vitt, 1986b). Likewise, it is conceivable that in the species studied here, there has not been a selection pressure for the evolution of female chemical discrimination.

Although a naive interpretation of sexual selection theory would lead to the expectation of female mate choice and, thus, discrimination, the apparent lack of chemically based species recognition shown by female *Podarcis* would seem to agree with reports that female lizards are generally indiscriminate and lack mate choice based on male quantitative traits (Olsson and Madsen, 1998; Tokarz, 1995; LeBas and Marshall, 2001). However, the evidence regarding female mate choice in lizards is controversial (see, for example, Hamilton and Sullivan, 2005), and studies of the mating systems of autarchoglossans, and lacertids in particular, are scarce.

Although chemically mediated species discrimination may not function as a complete reproductive barrier, our study demonstrates the contribution of chemical stimuli in species discrimination and reproductive isolation in a complex and diverse genus. The relative roles of chemoreception and of other sensory modalities in species discrimination by males and, especially, females are still not clear. Further studies are clearly necessary to clarify the role of different sensory systems in specific recognition between *Podarcis* species leading to the maintenance of reproductive isolation between lineages.

Acknowledgments This work was financially supported by project POCTI/BSE/45664/2002 of Fundação para a Ciência e a Tecnologia (FCT, Portugal). D.B. was funded by a Ph.D. grant (SFRH/BD/14084/2003) and M.A.C. by a postdoctoral grant (SFRH/BPD/3596/2000), both also from FCT. We thank two anonymous reviewers for helpful advice and comments and R. Ribeiro, R. Vasconcelos, and D. J. Harris for helping in fieldwork. Animals were collected under permits provided by the Instituto da Conservação da Natureza (ICN, Portugal) and were maintained and treated in accordance with Spanish Law (Real Decreto 223/1988) and Animal Behavior Society Guidelines for the Treatment of Animals in Behavioral Research and Teaching.

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