Chemosensory species recognition may reduce the frequency of hybridization between native and introduced lizards

M. Gabirot, A.M. Castilla, P. López, and J. Martín

Abstract: The introduction of alien species to islands by human activity can cause catastrophic consequences for small populations of island endemics. Hybridization between the endangered and endemic insular lizard *Podarcis atrata* (Boscá, 1916) from the Columbretes Islands (Mediterranean, Spain) and the common mainland lizard *Podarcis hispanica* (Steindachner, 1870) could potentially occur because mainland haplotypes have already been detected in the islands, the two species are closely genetically related, and the frequency of visitors to these islands is increasing. However, reproductive decisions of lizards are often mediated by species recognition mechanisms based on chemical cues. On the basis of this observation, even if some mainland *P. hispanica* lizards were introduced to the islands, interspecific recognition might make rare an eventual hybridization with the insular *P. atrata*. We examined interspecific chemical recognition between the insular *P. atrata* and the mainland *P. hispanica*. Our results showed that lizards of both sexes responded more strongly (i.e., directed a significantly higher number of tongue flicks) to scents of conspecific individuals than to scents of heterospecifics. Chemical recognition of conspecifics by endemic island *P. atrata* lizards may reduce the occurrence of hybridization with introduced mainland *P. hispanica* lizards and protect the insular gene pool.

Résumé : L'introduction d'espèces étrangères dans les îles par l'activité humaine peut avoir des conséquences catastrophiques sur les petites populations insulaires endémiques. L'hybridation entre *Podarcis atrata* (Boscá, 1916), lézard endémique et menacé des îles Columbretes (Méditerranée, Espagne), et le lézard commun de la péninsule ibérique *Podarcis hispanica* (Steindachner, 1870) pourrait potentiellement se produire du fait que certains haplotypes continentaux ont déjà été décelés sur l'île, que ces deux espèces sont relativement proches génétiquement et que la fréquence des visites humaines a augmenté sur l'île. Cependant, les lézards utilisent souvent des signaux chimiques pour la reconnaissance entre espèces lors des prises de décisions de reproduction. À cause de cette observation, même si quelques *P. hispanica* lézards continentaux sont introduits dans les îles, la reconnaissance interspécifique par les signaux chimiques raréfierait la possibilité éventuelle d'hybridation. Nous avons examiné la reconnaissance chimique interspécifique entre l'insulaire *P. atrata* et le continental *P. hispanica*. Les résultats indiquent que les lézards des deux sexes montrent plus d'intérêt (c.-à-d., un nombre plus grand de coups de langue) en direction d'odeurs d'individus de leur propre espèce que de l'autre espèce. La reconnaissance chimique des individus de même espèce par les lézards endémique *P. atrata* peut réduire la possibilité d'hybridation avec les *P. hispanica* introduits du continent et protéger le pool génique insulaire.

Introduction

Small isolated islands are ideal environments where evolutionary processes may lead to genetic divergence of local populations of animals and plants and the appearance of new endemic species (Thorpe and Malhotra 1996; Coyne and Orr 2004). However, the introduction of alien species to islands by human activity can cause catastrophic consequences for the often endangered small populations of endemics (Fritts and Rodda 1998). Island endemics are often closely related to common mainland species, which can be easily introduced and hybridize with the island species, thus reversing the island speciation processes (Seehausen et al. 2008). Many endangered species are considered to be seriously threatened by crossbreeding with closely related species as a result of introduction by human activities (Gunnell et al. 2008; Oliveira et al. 2008; reviewed in Rhymer and Simberloff 1996; Allendorf et al. 2001).

The Columbretes wall lizard, *Podarcis atrata* (Boscá, 1916), is an endangered lizard endemic to the Columbretes Islands (Mediterranean, Spain) (Castilla et al. 1998*a*, 1998*b*). This is the European lizard with the smallest (19 ha) distribution area, and it has a small population size (total of approx. 12 000 individuals) (Castilla and Bauwens 1991). *Podarcis atrata* is closely related to the mainland Iberian wall lizard, *Podarcis hispanica* (Steindachner, 1870) (Pinho et al. 2006, 2007), which is very common and widely distributed in the Iberian Peninsula (Barbadillo et al. 1999).

The Columbretes archipelago is a natural park that receives fishermen, visitors, and divers from the Spanish

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mainland and other European countries. In addition, the number of boat arrivals has increased considerably during recent years (Columbretes Natural Park, unpublished data). The lighthouse, the housing for gamekeepers and researchers, and the paths connecting to the disembarkation sites are all being rebuilt. These activities bear a high risk of accidental transportation of lizards from the mainland to the islands. In fact, haplotypes from mainland lizards have already been found in these islands (Castilla et al. 1998*b*). It is unclear whether those haplotypes were retained or are due to recent introgression, but if they are due to recent introductions from mainland lizards, then they may represent a threat (e.g., hybridization, competition) and a consequent loss of genetic isolation for insular lizards (Castilla et al. 1998*b*).

The occurrence of gene flow and hybridization between related species of lizards, and even between clearly distinct species of the genus *Podarcis* Wagler, 1830, has been observed in the laboratory (e.g., *Podarcis bocagei* (Seoane, 1884) × *Podarcis carbonelli* Pérez-Mellado, 1981; Galán 2002) and inferred from genetic analyses of field populations (*Podarcis sicula* (Rafinesque, 1810) × *Podarcis tiliguerta* (Gmelin, 1789), Capula 2002; *P. hispanica* of various genetically distinct types, Pinho et al. 2007).

Human-mediated secondary contact, hybridization, and interbreeding between different taxa are challenges of increasing relevancy in conservation biology (Rhymer and Simberloff 1996; Allendorf et al. 2001). Some studies have reported complete replacement of haplotypes in natural populations owing to hybridization (Gyllensten and Wilson 1987; Dowling and Hoeh 1991; Bernatched et al. 1995). Hybridization between species commonly results in outbreeding depression (Wang et al. 2007) and is involved in the risk of extinction of many species. For this reason, hybridization is of major concern for the appropriate implementation of species conservation plans (Sutherland et al. 2006; Gunnell et al. 2008; Oliveira et al. 2008; Vincenzi et al. 2008). Some management actions include the removal of non-native species to prevent hybridization (Copp et al. 2008).

Interestingly, however, there are some cases where related species can live together while showing a low percentage of hybrid genotypes, suggesting that there is a mechanism for species recognition that acts as a barrier to hybridization (Shine et al. 2002; Kingston and Gwilliam 2007). Even if individuals of different populations or species share the same habitats, successful reproduction might not always occur between them. For example, two subspecies of the mouse *Peromyscus maniculatus* (Wagner, 1845) do not hybridize even though their ranges overlap (Campbell 1995). Therefore, some species might have mechanisms to avoid hybridization, and behavioral studies may contribute to clarifying the actual relevance of the conservation problem of hybridization (Caro 1998).

It is well known that sexual selection is an important mechanism that may facilitate reproductive isolation (Boughman 2001; Panhuis et al. 2001). Understanding the pattern of mating and the cues that individuals use to select mates can help to identify the potential for hybridization between species (Grant and Grant 1997). Species recognition may prevent interspecific mating between sympatric related taxa (Cooper and Vitt 1986; Shine et al. 2002). Thus, if reproductive isolation is mediated by species recognition, the risk of interspecific hybridization should be low even if heterospecifics are common.

In many species of lizards, including *P. hispanica*, the chemical senses play important roles in intraspecific communication, mate choice, and species recognition (Cooper and Vitt 1986; Barbosa et al. 2005, 2006; López and Martín 2005; Martín and López 2006*a*, 2006*b*; see review in Mason 1992). Chemosensory recognition is well developed in *P. hispanica*, and chemicals in femoral gland secretions of males vary between populations (Martín and López 2006*a*, 2006*b*). Also, sex identification of females is based mainly on chemical cues (Gómez et al. 1993; López and Martín 2001; López et al. 2002), and female pheromones are necessary to elicit male courtship (López and Martín 2001; Cooper and Pèrez-Mellado 2002), as in other lizards (Cooper et al. 1986). Chemical cues of males are also important in male–male interactions (López and Martín 2002; Carazo et al. 2007) and it seems that female mate choice decisions are, at least partially, based on characteristics of chemical signals of males (López and Martín 2005; Martín and López 2006*c*; Martín et al. 2007).

In this paper, we examined whether interspecific chemical recognition exists between the insular *P. atrata* and the mainland *P. hispanica*, which could prevent mating and hybridization between these two species. We expected that if chemical recognition exists, chemosensory responses to scent of conspecific lizards should be different than responses to scent of heterospecifics. The existence of interpecific recognition may have important consequences for conservation strategies for the threatened *P. atrata*, because if *P. atrata* can maintain its reproductive isolation by recognition of conspecifics over heterospecifics, then the threat of hybridization with introduced lizards may be reduced.

Material and methods

Study area and species

The Columbretes wall lizard, *P. atrata*, is a small (mean adult snout–vent length of 50–70 mm) lizard endemic to the Columbretes archipelago (39°55′N, 0°40′E, Castellón, Spain) that inhabits three small islands characterized by high aridity and vegetation dominated by perennial shrubs, herbs, and grasses (Castilla and Bauwens 1991, 2000; Castilla 2000). We collected 15 males and 15 females from the main island, Columbrete Grande (13 ha), during the reproductive season in April 2008.

The Iberian wall lizard, *P. hispanica*, is a small lizard (mean adult snout–vent length of 49–61 mm) with a broad distribution in Spain and the southeast coast of France (Barbadillo et al. 1999). We collected lizards in three different populations of central Spain (Madrid province) during the reproductive season in March 2008. In the first area (Fuenfria, 40°47′N, 04°03′W), we captured 20 males and 21 females (Ph1); in the second (Golondrina, 40°44′N, 04°02′W), 17 males and 34 females (Ph2); and in the third (Aranjuez, 40°02′N, 03°37′W), 12 males and 11 females (Ph3). The three zones are characterized by having a large cover of rocks, inside pine or oak forest or Mediterranean shrubland.

We chose these populations for logistical reasons, but also because it is very difficult to predict the mainland origin of a putative colonizing population. Apart from accidental introductions from coastal zones, we should also consider intentional introductions from very distant areas. Recently, the lizard *Podarcis pityusensis* (Boscá, 1883) from the Balearic Islands (Mediterranean) was introduced to islands of the Basque Country (Atlantic, North Spain) (Sanz-Azkue et al. 2005). In another study that examined hybridization in islands due to human activities, recent artificial introductions were assumed to be from different localities and not from those closest to the island (Gantenbein and Largiadèr 2002).

We captured lizards with a noose and immediately transported them to the Field Station “El Ventorrillo” (Cercedilla, Madrid). Lizards were kept individually in indoor 60 cm × 40 cm PVC terraria containing sand substratum and rocks for cover. Terraria with lizards of different populations and sexes were housed separately to avoid visual or chemical contact among them outside experimentation. Every day, lizards were fed mealworm larvae (*Tenebrio molitor* L., 1758) dusted with multivitamin powder for reptiles, and fruit and tomatoes (for *P. atrata* only). Water was provided ad libitum. All terraria were heated with 40 W spotlights for 6 h/day, and received overhead light (36 W full-spectrum daylight tubes) on a 10 h light : 14 h dark cycle.

Lizards were held in captivity for at least 1 week before the tests were conducted to allow their acclimation to the laboratory conditions. All lizards were healthy during the trials, did not show behavioral or physiological changes due to possible stress of experiments, and maintained or increased their original body mass. All lizards were returned to their exact capture sites at the end of experiments (middle of May 2008). Captures and experiments were performed under license from the Consejería del Medio Ambiente de la Comunidad de Madrid (Environmental Agency of the Community of Madrid), Spain, and the Generalitat Valenciana.

Scent recognition experiments

Lizards have been shown to react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper and Burghardt 1990). Tongue flick (TF) rate can, therefore, be used as a quantitative bioassay of detection of chemical cues and of discrimination of conspecific and heterospecific lizards (e.g., Cooper and Pèrez-Mellado 2002; Barbosa et al. 2006). To test for differential responses to scents, we made comparisons of TF rate of lizards (males and females) in response to chemical stimuli arising from cotton applicators impregnated with scent of male or female *P. hispanica* of the three mainland populations, scent of male or female *P. atrata*, or deionized water (odorless control) (Cooper and Burghardt 1990). Water was used to gauge baseline TF rates in the experimental situation. We did not use a pungency control because in many previous studies, including those with wall lizards (e.g., Cooper and Pèrez-Mellado 2002), it has already been well established that these lizards can discriminate scent of conspecific lizards from biologically irrelevant scents.

We tested lizard scents from the femoral pores of males or from the cloacal area of females because these are the bodily areas most frequently and intensely investigated by tongue-flicking during social encounters (López and Martín 2001, 2002; López et al. 2002). After first dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) in deionized water, we rolled the tip over those bodily areas (one population and sex per applicator). Individuals that donated the scent were the same individuals used as responding individuals in other trials on different days. A new swab from a different donor individual was used in each trial.

Individual *P. atrata* were confronted randomly with scents of males and females of their own species and of three mainland populations of *P. hispanica* (Ph1, Ph2, and Ph3). Thus, we analyzed the responses of island *P. atrata* lizards to a simulated introduced *P. hispanica* lizard from the mainland in comparison with their responses to conspecifics. Also, we randomly presented to individuals of *P. hispanica* scents from conspecific males and females of their own population, and scents from male and female *P. atrata*. Thus, we compared the responses of mainland *P. hispanica* lizards when finding island *P. atrata* lizards with their responses to conspecifics of their own mainland population. We were interested in those social interactions that may lead to reproductive interactions and possible hybridization. These include male–male recognition, which is needed to prevent intrasexual competition, and male–female recognition, which is required for matings. Female–female recognition is not important in these lizards because there is no competition between females for access to mates. Therefore, we did not consider female–female chemical recognition because it is irrelevant to potential hybridization.

Every lizard was exposed to each stimulus and the order of presentation was randomized. One scent presentation was conducted per day for each animal. Trials were conducted during April, which coincided with the mating season of lizards in their original natural populations (Castilla and Bauwens 1991, 2000; P. López and J. Martín, unpublished data), and between 1100 and 1300 (GMT), when lizards were fully active. Because temperature strongly influences TF rates of lizards (Van Damme et al. 1990), lizards were maintained at their preferred body temperature (i.e., *P. hispanica*, 35.5 °C; *P. atrata*, 35.7 °C; see Bauwens et al. 1995) inside their terrarium for at least 1 h before and during the experiments. To begin a trial, the experimenter slowly approached the terrarium and slowly moved the cotton swab to a position 1 cm anterior to the lizard’s snout. Lizards usually did not flee from the swab, but explored it repeatedly by tongue-flicking or ignored it after the first TFs. The number of TFs directed at the swab was recorded for 60 s beginning with the first TF. Only data from individuals that responded to the swab without fleeing were included in the analyses.

Statistical analyses were done separately for responding males and females, and for responses to male and female scents. To examine differences in the number of TFs directed at the swab among treatments, we used repeated measures one- or two-way ANOVAs examining the effects of the different scent stimuli presented to the same individual (within factor) and, in the case of *P. hispanica* responses, the population of the responding lizard (between factor; Ph1 vs. Ph2 vs. Ph3). We included the interaction in the models to analyze whether responses to the different scents differed as a function of the population of the responding lizard. Data were log-transformed to ensure normality. Tests of homogeneity of variances (Levene's test) showed that in all cases variances were not significantly heterogeneous after transformation. Pairwise comparisons were planned using Tukey's honestly significant difference (HSD) tests (Sokal and Rohlf 1995). All analyses were performed using the *STATISTICA* (StatSoft, Inc., 2001) data analysis software system, version 6 (www.statsoft.com).

Results

We found significant differences in the number of TFs directed by lizards to the different scent stimuli presented (one-way repeated measures ANOVA, $p < 0.0001$ in all cases) (Figs. 1, 2). In all cases, male and female lizards from different populations directed significantly more TFs to scents of other lizards than to water (Tukey's tests, $p = 0.00013$ in all cases), indicating detection of lizard scents in all cases.

Responses of island lizards

Insular male *P. atrata* directed significantly more TFs to scents of conspecific males than to scents of males from any of the three mainland *P. hispanica* populations (one-way repeated measures ANOVA, $F_{[4,56]} = 82.25$, $p < 0.0001$; Tukey's tests, $p = 0.00013$ in all cases). Responses of male *P. atrata* to all the different stimuli from male *P. hispanica* were not significantly different ($p > 0.97$ in all cases) (Fig. 1*a*).

Similar results were obtained when male *P. atrata* were confronted with scents of female *P. hispanica* ($F_{[4,56]} = 72.67$, $p < 0.0001$; Tukey's tests, $p = 0.00025$ in all cases). Responses of male *P. atrata* to all the different stimuli from female *P. hispanica* were not significantly different ($p > 0.30$ in all cases) (Fig. 1*a*).

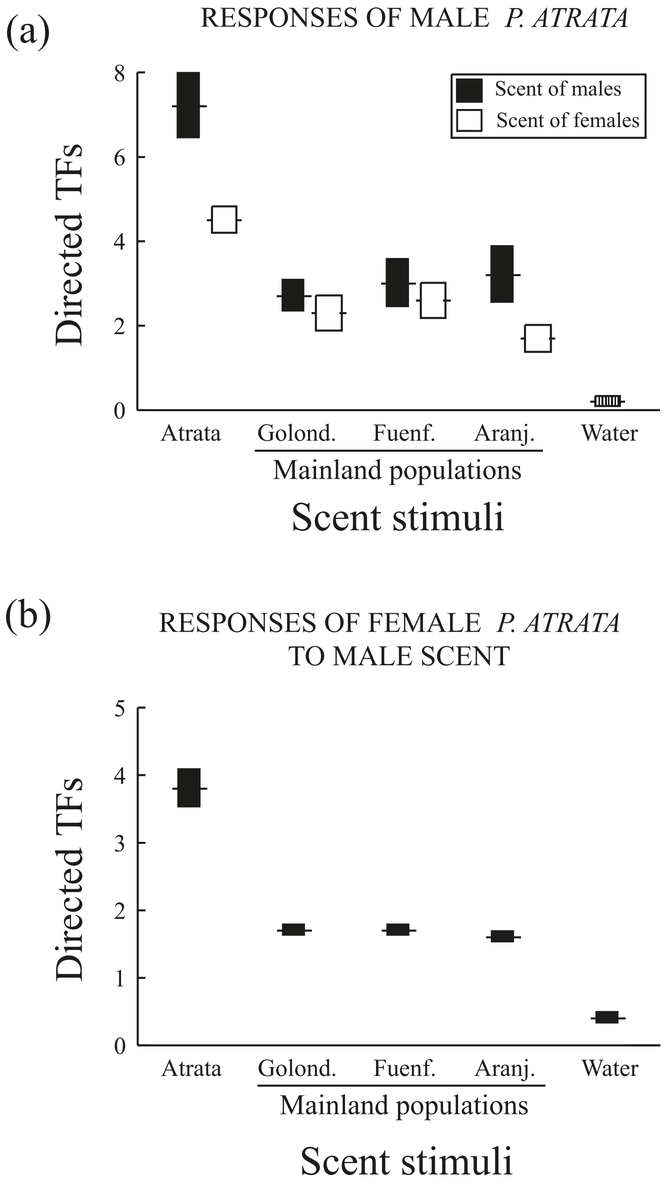
Female *P. atrata* directed significantly more TFs to scents of conspecific males than to scents of male *P. hispanica* from any of the three mainland populations ($F_{[4,56]} = 74.16$, $p < 0.0001$; Tukey's tests, $p = 0.00013$ in all cases) (Fig. 1*b*). Responses to all the different stimuli from male *P. hispanica* were not significantly different ($p > 0.95$ in all cases).

Responses of mainland lizards

Male *P. hispanica* of the three mainland populations directed significantly fewer TFs to scents of insular male *P. atrata* than to scents of conspecific males from their own population (two-way repeated measures ANOVA, $F_{[2,92]} = 319.02$, $p < 0.0001$; Tukey's tests, $p < 0.001$ in all cases) (Fig. 2*a*). The population of origin of responding male *P. hispanica* significantly affected their response to conspecific males from their own population ($F_{[2,46]} = 10.87$, $p = 0.00014$). This was because males from Fuenfria directed more TFs than males from Aranjuez to scents of males from their own population (Tukey's tests, Ph1 vs. Ph2, $p = 0.21$; Ph1 vs. Ph3, $p = 0.0.92$; Ph2 vs. Ph3, $p = 0.02$), but male *P. hispanica* from all populations directed a lower number of TFs to male *P. atrata* ($p > 0.97$ in all comparisons between populations) than to all conspecific males.

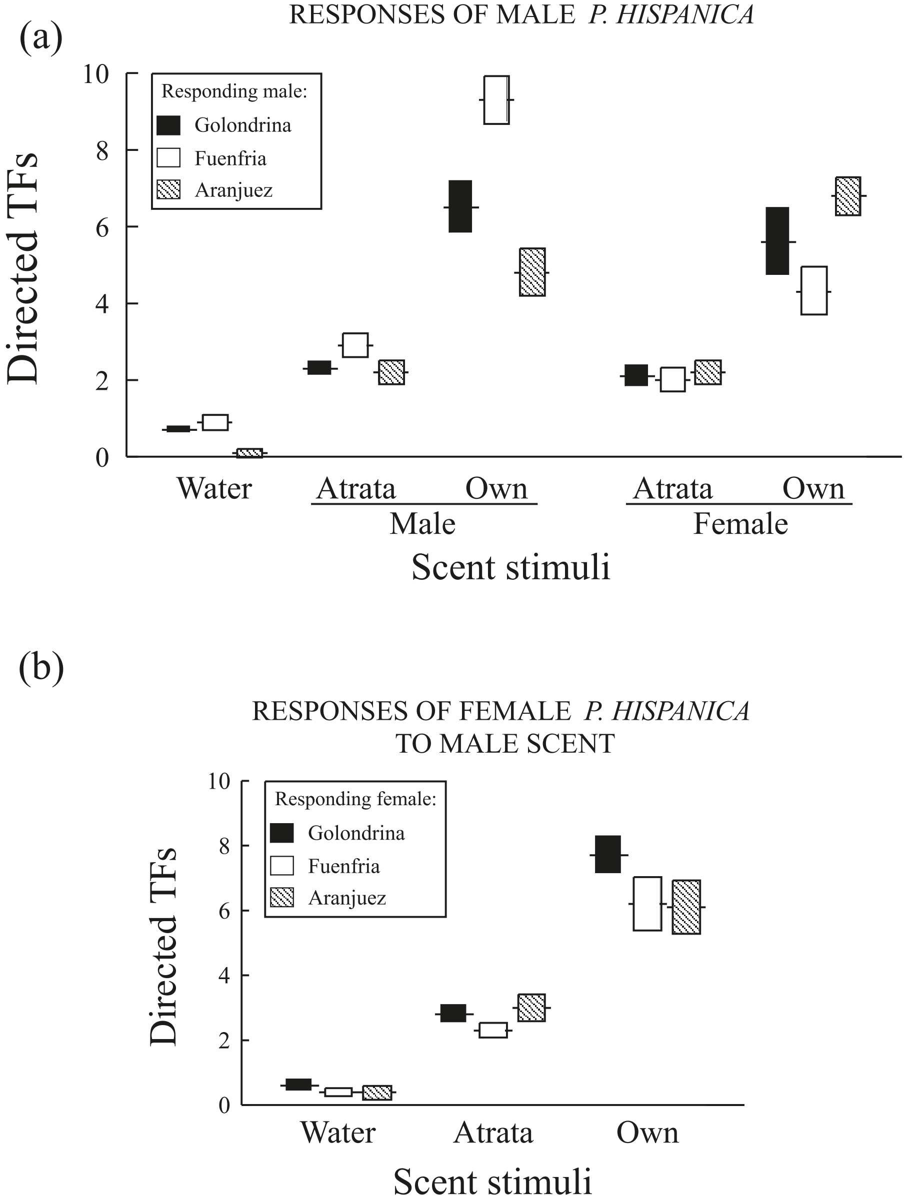
Male *P. hispanica* directed significantly fewer TFs to scents of insular female *P. atrata* than to scents of conspecific females from their own population ($F_{[2,84]} = 198.28$, $p < 0.0001$; Tukey's tests, $p = 0.0001$ in all cases) (Fig. 2*a*). The population of origin of responding male *P. hispanica* significantly affected their response to conspecific females from their own population ($F_{[2,42]} = 3.48$, $p = 0.04$). This was because males from Aranjuez directed more TFs than males from Fuenfria to scents of females from their own population (Tukey's tests, Ph1 vs. Ph2, $p = 0.68$; Ph2 vs. Ph3, $p = 0.035$; Ph1 vs. Ph3, $p = 0.20$), but male *P. hispanica* from all populations directed a lower number of TFs to female *P. atrata* ($p > 0.95$ in all comparisons between populations) than to conspecific females.

Fig. 1. Mean (±1 SE) number of tongue flicks directed to the swab by island male (*a*) or female (*b*) *Podarcis atrata* in response to control deionized water or scents from males (solid boxes) and females (open boxes) from three mainland populations of *Podarcis hispanica* (Golondrina, Fuenfria, and Aranjuez).



Female *P. hispanica* directed significantly fewer TFs to scents of insular male *P. atrata* than to scents of conspecific males from their own population ($F_{[2,122]} = 405.99$, $p < 0.0001$; Tukey's tests, $p < 0.0001$ in all cases) (Fig. 2*b*), in-

Fig. 2. Mean (±1 SE) number of tongue flicks directed to the swab by male (*a*) or female (*b*) *Podarcis hispanica* from three mainland populations (solid boxes, Golondrina; open boxes, Fuenfria; hatched boxes, Aranjuez) in response to control deionized water or scents from island male or female *Podarcis atrata* and male or female *P. hispanica* from their own mainland population.



dependently of the population of origin ($F_{[2,61]} = 0.68$, $p = 0.51$).

Discussion

We initiated this study with the supposition that hybridization between the endangered island species *P. atrata* and the common mainland species *P. hispanica* could occur because (*i*) mainland *P. hispanica* haplotypes have already been detected in the Columbretes Islands (Castilla et al. 1998*b*), (*ii*) there is close genetic relatedness between these species (Pinho et al. 2006, 2007), (*iii*) gene flow and hybridization occur between *Podarcis* species (Galán 2002; Capula 2002; Pinho et al. 2007), and (*iv*) there is a strong risk of *P. hispanica* incursions from mainland sites during frequent boat contact (Columbretes Natural Park, unpublished data). However, there is no evidence that actual hybridization between the two lizard species currently occurs.

The results of our study suggest that a breakdown in mechanisms allowing species discrimination via chemical cues would be necessary to allow reproductive interactions between *P. hispanica* and *P. atrata*. Interspecific reproductive interactions might occasionally occur between other wall lizard species as a consequence of weak species recognition mechanisms (Cooper and Pèrez-Mellado 2002; Barbosa et al. 2005, 2006; Martín and López 2006*a*, 2006*b*).

In our experiment, the different TF rates of lizards in response to different scents indicated that *P. hispanica* and *P. atrata* detected and discriminated between scents based on chemical cues alone. In all cases the rate of TFs directed to scent of lizards was higher than to the control baseline odor (water), indicating that detection of the lizard scent occurred in all cases.

Our results clearly demonstrated that lizards of both species and sexes responded more strongly to scents of individuals of their own species than to scents of the other species. This suggests that lizards of both sexes and species have more “interest” in exploring the scent of conspecific lizards than the scent of the other species. This indicates the occurrence of a clear interspecific chemosensory discrimination between these two species.

These results suggest that the chemicals of *P. hispanica* and *P. atrata* used in intraspecific communication might be different in composition and (or) proportions, which might be related to genetic differences between the two species. Preliminary chemical analyses appear to support this prediction (J. Martín et al., unpublished data). For example, cholesta-5,7-dien-3-ol is a major steroid found in femoral secretions of mainland male *P. hispanica* (López and Martín 2005; Martín and López 2006*a*) but is absent in secretions of male *P. atrata* (J. Martín et al., unpublished data). That compound is apparently selected by female *P. hispanica* when selecting scent marks of conspecific males (López and Martín 2005; Martín and López 2006*c*). Similarly, in a species complex of rodents, it has been shown that a greater genetic relatedness between two individuals is associated with more similarity in the qualities of their individual scents (Heth et al. 2001).

Our results on chemosensory abilities of *P. atrata* suggest a similar role of chemical cues in social behavior and reproduction in this species too. Therefore, reproductive decisions may be linked to a previous chemosensory recognition of chemicals that identify each species. Therefore, our results could be a preliminary indication of the high improbability of reproduction between *P. hispanica* and *P. atrata*. If individuals do not recognize or discriminate between species, interspecific matings could occur. However, further experiments of mate choice and staged encounters should be done to ensure that premating reproductive isolation actually prevents hybridization between island and mainland lizards.

Implications for conservation

Considering the results of our study, the risk of hybridization between *P. atrata* and *P. hispanica* may not be as high as expected (owing to the high degree of connectivity with the mainland) because of the interspecific differences in chemical recognition. Therefore, the maintenance of the insular genetic pool appears to be protected. However, even if we suggest that introduced lizards may not hybridizate with endemic island lizards based on chemical discrimination, we recommend governmental managers to interpret our results with caution, since we have examined only one of the potential effects of species introductions. We should be aware that many other dangers are associated with species introductions, such as competition for food, habitat, and nesting sites, or transmission of parasites or new diseases (Lockwood et al. 2007).

Behavioral studies of species interactions can contribute to a greater understanding of conservation problems such as the actual risk of hybridization (Caro 1998). Thus, even in small islands with a high degree of connectivity with the mainland, the risk of hybridization should not be considered high if behavioral experiments indicate that reproductive isolation is guaranteed. There are many other endemic lizards inhabiting other small islands in the Mediterranean where the risk of hybridization with common species is a conservation issue. Further research involving visual and chemical reproductive interactions between species would provide valuable insight into the mechanisms governing possible hybridization and species recognition in mate choice (e.g., Grant and Grant 1997; Kingston and Gwilliam 2007).

In any case, irrespective of our results, efforts should be directed to prevent the introduction of mainland lizards to the islands. The introduction of alien competitors or predators can create novel ecological contexts in which the adaptive antipredatory responses of native prey may not be successful, and this can lead to extinctions of island species (Shea and Chesson 2002; Courchamp et al. 2003; Blackburn et al. 2004).

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