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Pre-mating mechanisms favouring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*

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ABSTRACT

Question: Do chemical recognition mechanisms and sexual selection processes contribute to reproductive isolation between closely related taxa?

Organism: The lacertid Iberian wall lizard (*Podarcis hispanica*).

Background: Recent molecular studies have suggested that this lizard forms a species complex with ongoing speciation processes. Two of the monophyletic types of this lizard are mainly allopatric. However, they live close together in the Guadarrama Mountains (Central Spain) and are not geographically isolated.

Methods: We measured tongue-flick rates to chemical stimuli from males and females, and staged intra-sexual agonistic encounters and inter-sexual courtships between lizards of both types.

Conclusions: Males discriminated between males of different types based on chemical cues alone, and there were differences in the aggressiveness and outcome of agonistic encounters depending on the types of interacting males. However, males did not discriminate chemically between female types, and females did not discriminate between male types. Inter-sexual courtships suggested that matings may occur between lizards of the two types. Therefore, in spite of differences between male types, behaviourally mediated reproductive isolation may not be entirely effective at this moment.

Keywords: chemoreception, lizards, pre-mating isolation, sexual selection, speciation.

INTRODUCTION

Sexual selection may facilitate speciation if male mating signals and female preferences diverge (Boughman, 2001; Panhuis *et al.*, 2001; Kirkpatrick and Revigné, 2002) and, thereafter, any of several species recognition mechanisms prevent interspecific matings (e.g. Couldbridge and Alexander, 2002;

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Shaw and Parsons, 2002; Shine *et al.*, 2002). Many studies have documented various species' ability to discriminate between conspecific and heterospecific cues (for a review, see Ptacek, 2000). But only a few studies have analysed this ability in very closely related taxa that are not yet differentiated as 'well-defined' species (i.e. a species complex). Examples include Hawaiian *Drosophila* (Kaneshiro and Boake, 1987), some rodents (Heth and Todrank, 2000; Heth *et al.*, 2001), and sticklebacks (McKinnon and Rundle, 2002).

Recently, molecular studies have highlighted relationships and genetic distances between populations of several taxa. The results suggest the existence of ongoing speciation processes within taxa previously considered to be conspecific. For example, molecular and morphological studies suggest that the Iberian wall lizard, *Podarcis hispanica*, is paraphyletic, and forms a species complex with at least five monophyletic lineages (Guillaume, 1987; Harris and Sá-Sousa, 2001, 2002).

Two allopatric morphotypes exist in western and central Iberia:

- *P. hispanica* type 1 occurs in northwestern Iberia, mainly in highlands and where Atlantic environmental conditions prevail.
- *P. hispanica* type 2 occurs in central and southern Iberia, where Mediterranean conditions are typical (Sá-Sousa, 2000; Sá-Sousa *et al.*, 2002).

Mitochondrial DNA sequences from the two types form monophyletic units (Harris and Sá-Sousa, 2002). The populations of the two types are mainly allopatric. However, in the Guadarrama Mountains (Central Spain), they live close together in similar microhabitats and without apparent geographical isolation, but at different altitudinal ranges (type 1, 1700–1800 m; type 2, below 1500 m). There, individuals of both types are able to locate each other easily (Mellado and Olmedo, 1981; García-Paris *et al.*, 1989). No stable *P. hispanica* population is found between 1500 and 1700 m even though suitable habitat (i.e. rocky outcrops) is available and populations of other similar lacertid lizard species are present (Martín-Vallejo *et al.*, 1995). Thus, in this particular area, they provide an excellent system in which to examine inter-population recognition or isolation mechanisms that might one day lead to their speciation.

Do sexual selection processes contribute to the reproductive isolation of the two lizard types? Most studies of sexual selection-mediated speciation have focused on the role of female choice in the evolution of species recognition and isolation (Ryan, 1990; Ptacek, 2000). Female lizard rejection behaviours are often easy to identify. One can use them to assess the relative importance of heterospecific mate rejection and choice on conspecific traits for the evolution of interspecific mating barriers. However, in reptiles, fights over mates are a more important selective process than mate choice (Olsson and Madsen, 1998).

Male–male contests for females occur in most lizards (Olsson and Madsen, 1998). But the role, if any, of intra-sexual interactions in speciation is unclear. Some studies and theoretical models suggest that social selection and certain types of intraspecific competition can lead to pre-zygotic reproductive isolation (West-Eberhard, 1983; Wolf *et al.*, 1999; Hochberg *et al.*, 2003).

We hypothesized that if males of different but closely related taxa do not recognize each other as rivals, they may co-exist and obtain easy access to females of their sister taxon. If they do not avoid such females and these females do not prefer males of their own taxa, the absence of inter-taxon rivalry might preclude speciation. Alternatively, if males of each taxon can exclude their sister taxon's males from preferred habitats (see, for example, Diamond, 1978; Case *et al.*, 1994), and prevent inter-taxon matings more successfully than they prevent

intra-taxon matings, then the outcome of fights between males may well contribute to speciation.

Many taxa use sex pheromones for species recognition. Like other signals favoured by sexual selection, pheromones may also have played important roles in speciation (Phelan and Baker, 1987; McLennan and Ryan, 1999; Wyatt, 2003). In reptiles, the chemical senses play important roles in intraspecific communication (Halpern, 1992; Mason, 1992), and chemical recognition may be especially important in speciation. Some examples are as follows:

- Two sympatric sea snakes species might be reproductively isolated due to differences in skin scents (Shine *et al.*, 2002).
- Variation in female sexual attractiveness pheromones results in some sexual isolation among populations of red-garter snakes (LeMaster and Mason, 2003).
- Chemical cues also prevent heterospecific matings between closely related species of skinks of the genus *Eumeces* (Cooper and Vitt, 1987a).

Chemosensory abilities are well-developed in *P. hispanica*. This lizard can discriminate between conspecifics and heterospecifics, and between sexes, by chemical cues alone (Gómez *et al.*, 1993; López and Martín, 2001b; Cooper and Pérez Mellado, 2002; López *et al.*, 2002a). Moreover, the aggressive response of male wall lizards to intruding individuals depends on pheromonally mediated sex recognition. Both males and females impregnated experimentally with the scent of males were attacked by other males, whereas males impregnated with the scent of females were not (López and Martín, 2001a; López *et al.*, 2002a).

Chemical cues also play a role in the recognition of rivals during agonistic interactions between males (López and Martín, 2002). Similarly, males of the skink *Eumeces laticeps* behave aggressively towards conspecific males, whereas they ignore heterospecific males of the same genus (after chemosensory examination), although both appear quite similar to the human eye (Cooper and Vitt, 1987a, 1987b). Also, chemical cues of female *P. hispanica*, in conjunction with coloration, elicit courtship by males (López and Martín, 2001a). As in other rock-dwelling lacertid lizards (Martín and López, 2000; López *et al.*, 2002b), chemical cues might also be involved in mate choice by female *P. hispanica* (López and Martín, 2005).

In rodents at least, greater genetic similarity between two individuals is associated with greater similarity of their scents (Heth and Todrank, 2000; Heth *et al.*, 2001; Talley *et al.*, 2001). Thus we hypothesized that the genetic differences between *P. hispanica* types (Harris and Sá-Sousa, 2001, 2002) are associated with differences in their pheromonal secretions, and that these differences affect chemical recognition and promote sexual isolation between them. So we performed a laboratory study of chemical recognition between these two types of Iberian wall lizard in the geographical area (Guadarrama Mountains) where they co-occur. We searched for evidence of sexual selection. We also studied their chemical recognition abilities and sexual behaviour (intra-sexual agonistic encounters and inter-sexual courtships). We analysed the latter to determine whether differences between the lizard types might lead eventually to their reproductive isolation and evolutionary divergence, or, in contrast, whether a lack of chemical discrimination between types might preclude speciation for the foreseeable future.

MATERIALS AND METHODS

Study animals

During April 2002, we captured *P. hispanica* with nooses at two localities near Cercedilla (Guadarrama Mountains, Madrid Province, Spain). We captured individuals of type 1 from a small population occupying rocky cliffs at the edge of a pine forest in the upper part of Valle de la Fuenfría (40°47′N, 4°03′W; 1750 m altitude). We captured individuals of type 2 on rocky outcrops in an oak forest near Cercedilla village (40°44′N, 4°02′W; 1250 m altitude). The two study populations are 6 km apart, but we have observed a few isolated type 2 individuals of *P. hispanica* as close as 2 km from the type 1 population (P. López and J. Martín, unpublished data). These occurrences strongly suggest that contacts between individuals of both populations should not be rare.

We identified the types based on their distinctive morphology and coloration. Type 1 lizards have a flattened head and body, a reticulated or striped dark dorsal pattern, and a pearly-white belly. Type 2 lizards have a moderately robust head and body, a light brown pattern, and an orange belly (for details, see Pérez-Mellado and Galindo, 1986; Guillaume, 1987; Sá-Sousa, 1995, 2000; Sá-Sousa *et al.*, 2002).

Lizards were individually housed about 5 km from the capture sites at 'El Ventorrillo' Field Station. We kept them in outdoor 60×40 cm PVC terraria containing a sand substratum and rocks for cover. Every day, we fed them mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles. We also provided water *ad libitum*. We did not regulate the photoperiod or ambient temperature.

To allow acclimation to laboratory conditions, we held all lizards in captivity at least one week before we tested them. To prevent lizards from having contact with the scent and visual stimulus of the other taxon before they were tested, we housed terraria with lizards from the two populations separately.

All animals appeared healthy during the trials. All individuals maintained or increased their original body mass. Despite the possible stress of the experiments, they did not show any behavioural or physiological changes. We returned them to their exact capture sites at the end of the experiments.

Detection of chemical cues from conspecifics

Reptiles react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper and Burghardt, 1990). Therefore, we used tongue-flick rate as a quantitative bioassay of detection of conspecific chemical cues (see Cooper and Pérez-Mellado, 2002; Shine *et al.*, 2002).

To test lizards for differential responses to scents, we used cotton applicators impregnated with scents of *P. hispanica* lizards of both sexes from populations of the two types. We also used an odourless control of cotton applicators wetted with de-ionized water (Cooper and Burghardt, 1990).

We wished to test lizard scents from the femoral pores and cloacal area of lizards because these are the bodily areas most frequently and intensely investigated by tongue-flicking during social encounters (López and Martín, 2001b, 2002; López *et al.*, 2002a). Therefore, after first dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (100 cm) in de-ionized water, we rolled the tip over those bodily areas (of one type and sex per applicator). We used a new applicator in each trial.

Every lizard was exposed to each stimulus and we varied their order of presentation to lizards. We conducted one trial per day for each animal. Trials were performed outdoors during April (the natural mating season of these lizards) (P. López and J. Martín, unpublished data). Trials occurred between 11.00 and 13.00 h (GMT); this is the time of day when lizards are most active.

To begin a trial, the experimenter carefully approached the terrarium and slowly moved the applicator to a position 1 cm anterior to the lizard's snout. Lizards usually did not flee from it, but ignored it or explored it by tongue-flicking. The numbers of tongue-flicks were recorded for 60 s beginning with the first tongue-flick.

Staged agonistic encounters between males

We staged agonistic encounters between pairs of males in the home-cage of one of the males, where he had been maintained and fed for at least 10 days prior to encounters. Thus, as resident he competed to maintain his status of owner (López and Martín, 2001a), whereas the other male acted as an unfamiliar intruder. With this design we tried to mimic natural field conditions, whereby one of the males was resident and found another male in his home range.

We staged encounters between males of the same type (M1 × M1 and M2 × M2), and between males of different types, where males of each type were resident in one set of trials and intruders in the other in a random order (M1 × M2 and M2 × M1; the first listed indicates the resident male). Thus, in the case of encounters between males of different type, we simulated a dispersing male of one type that arrives at a resident population of males of the other type. To avoid the effects of previous experience between individuals (López and Martín, 2001a, 2002), in each test the two individual lizards had never encountered each other before the trials. Males participated in only one encounter per day to avoid stress. We also spaced staged encounters sufficiently (at least one day apart) so that potential fatigue resulting from one test did not affect performance on subsequent tests. All tests were conducted outdoors in sunny conditions between 09.00 and 12.00 h (GMT) when lizards were fully active.

To begin a trial, we gently took the intruder lizard from its cage and placed it in the resident male's cage, which was temporally divided into two equal compartments by a plywood partition. Males were placed in separate compartments and given 10 min to habituate to the new environment. Then, we removed the partition, and recorded lizard behaviour from behind a blind. We considered an interaction to have occurred when two lizards approached to within 5 cm of each other and then when they moved further than 5 cm apart. Multiple interactions between the two males could occur during each trial.

Each interaction was scored according to the intensity of the aggressive behavioural response observed on a ranked scale representing increasing escalation:

- Score 0, 'neutral'. Individuals were close (less than 5 cm) but no response or non-aggressive interaction was observed.
- Score 1, 'retreat'. One male approached its opponent without an aggressive display or contact and the other male ran away.
- Score 2, 'approach'. One male approached the other with an aggressive display but without physical contact. The other male ran away. Approaching males employed threatening postures; they strutted towards their opponent on raised, stiff forelegs with neck arched and snout pointing slightly down.

- Score 3, 'touch'. One male approached the other with an aggressive display, touching him on the tail or flanks. The other male ran away.
- Score 4, 'bite'. One male approached the other and bit him, especially on the snout or head. The other male ran away.
- Score 5, 'physical combat'. Both males simultaneously interlocked jaws by reciprocal biting and clasping. One of them eventually fled.

For further analyses, we also classified these types of agonistic interactions into two categories:

- 'ritualized' ('retreat' and 'approach'), and
- 'with physical contact' ('touch', 'bite', and 'physical combat').

We also noted which of the males won. The criterion for establishment of a loser was observed avoidance behaviour in one of the contestants (e.g. a rapid retreat or running away). We calculated for each trial an unweighted sum of the number of interactions that each male won. We defined the male with the highest positive sum as the dominant individual (Martín and Salvador, 1993; López *et al.*, 2002b). Typically, winners repeatedly dominated their opponents over a series of interactions in an encounter.

For each staged encounter, we calculated two aggression indexes, one for each male, as the sum of the number of interactions in each category won by that male multiplied by the corresponding aggression score. We also calculated an average aggression index per interaction within each trial for each male, as the aggression index of each male divided by the total number of interactions observed during the trial (Downes and Bauwens, 2002).

A trial was ended after 15 min. We also stopped encounters if persistent attacks or desperate attempts to escape were recorded. This was unnecessary, however, as interactions mostly consisted of threatened displays and short chases, and only very rarely escalated to single quick bites that did not cause observable injury. Although agonistic interactions between lizards were not very aggressive, we used a minimal number of animals over the minimum time necessary to test our hypothesis.

Staged inter-sexual encounters

We staged 15 min encounters between a male and a female in the home-cage of the responding male, and thus the responding male acted as the owner. With this design we tried to mimic natural field conditions in which a resident male finds a female in his home range. Males should be neutral or attempt to court a newcomer female (López and Martín, 2001b). We considered a 'neutral response' when the two individuals were close (less than 5 cm) but no response was observed, or 'courtship behaviour' if the male approached the female slowly and began to lick her tail or the surrounding substrate. He then gripped and shook the female's tail with a gentle bite, which did not result in any discernible wounds. If a female is receptive, she allows the male to mount her (Verbeek, 1972; López and Martín, 2001b). We noted the number of courtships observed, but we stopped observations before actual matings occurred. Each time that the two lizards were together, we noted whether males explored by tongue-flicking the body of the females or the surrounding substrate. We noted the frequency of such tongue-flicking events, each exploration consisting of several consecutive tongue-flicks emitted during sequences of variable duration.

For each male's courtship we noted whether the female was receptive or tried to flee from the approaching male, even by biting him when he tried to mount her. Also, females often showed foot shaking, each event consisting of several continuous and quick movements of the legs during sequences of variable duration in response to a male courtship, while she moved slowly away avoiding the male. These behaviours seemed to deter the courtship of a male, and thus they could indicate rejection behaviour of the male. Females sometimes showed rejection behaviour to an approaching male, even before he started courtship. We noted the response of females (receptive vs. rejection) during each interaction with a male. We also calculated the rate of courtships and the rate of rejections in relationship to the number of interactions.

Data analyses

To identify differences in number of tongue-flicks among conditions, we used two-way repeated measures analyses of variance to examine the effects of scent stimuli (within factor) and type of the responding lizard (between factor; type 1 vs. type 2) (Sokal and Rohlf, 1995). We included the interaction in the model to determine whether the response to the different scents differed as a function of the type of the responding lizard.

To compare aggression indexes between males across treatments, we used two-way repeated measures analyses of variance, examining the effects of types of interacting males (between factor) and residence condition within the pair of males (within factor) (Sokal and Rohlf, 1995).

To compare the number or the rate of courtships, number of tongue-flick explorations of females by males, and number of rejections by females observed in each trial across treatments, we performed one-way analyses of variance (Sokal and Rohlf, 1995).

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. Pairwise comparisons were performed using Tukey's honestly significant difference (HSD) tests. Statistical significance was set at $P < 0.05$ and all tests were two-tailed.

RESULTS

Detection of chemical cues from conspecifics

Differential tongue-flick rates showed that males discriminated between different scents, with lower responses to water than to male scents (two-way repeated measures ANOVA: $F_{2,36} = 21.77$, $P < 0.0001$; Fig. 1A). There were no differences between the overall response of the two types of males ($F_{1,18} = 1.12$, $P = 0.30$), but males of the two types responded in different ways to the different stimuli (interaction: $F_{2,36} = 20.28$, $P < 0.0001$). Thus, all males discriminated between scents of the two types of males, and responded more strongly to scents of males of their own type (Tukey tests: type 1, $P = 0.03$; type 2, $P = 0.0001$). In fact, responses to water did not differ from responses to the scent of males of the other type (type 1, $P = 0.32$; type 2, $P = 0.81$).

Males were more aggressive towards scent stimuli from males of their own type; 14 males (type 1: $n = 6$; type 2: $n = 8$) bit the applicators bearing scent of males of their own type, whereas only 7 (type 1: $n = 3$; type 2: $n = 4$) bit them in response to scents of males of a different type (binomial test, $P = 0.0016$; based on the null hypothesis that the likelihood of

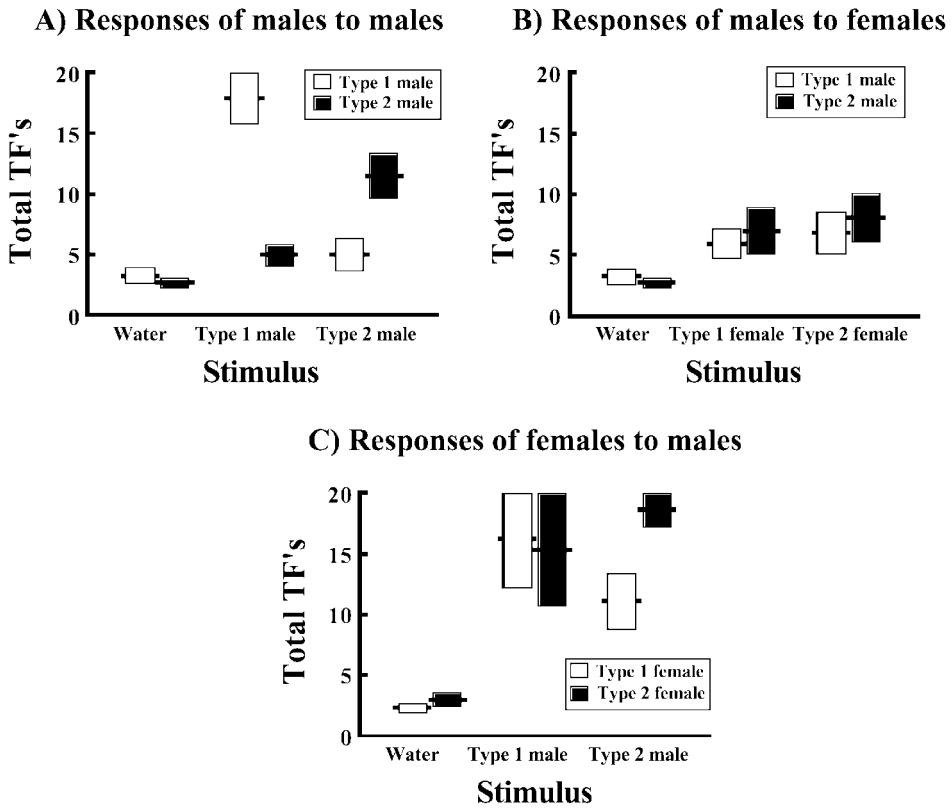

Fig. 1. Total number of tongue-flicks (TFs) (mean ± standard error) of two types of male and female *P. hispanica* (□, type 1; ■, type 2) in response to de-ionized water (control) or the scents of two types of conspecific males or females, presented for 60 s on cotton-tipped applicators.

biting was equal in all three conditions). Males did not bite the applicators bearing water stimuli.

Males discriminated the scent of females from water (two-way repeated measures ANOVA: $F_{2,36} = 6.32$, $P = 0.004$; Fig. 1B), with fewer tongue-flick responses to water than to female scents (Tukey tests: $P < 0.02$ in both cases); the responses to the two types of female were similar ($P = 0.86$). There were no differences between male types ($F_{1,18} = 0.08$, $P = 0.78$), or in the way that they responded to different stimuli (interaction: $F_{2,36} = 0.51$, $P = 0.61$).

Females discriminated the scent of males from water (two-way repeated measures ANOVA: $F_{2,36} = 67.78$, $P < 0.0001$; Fig. 1C), with fewer tongue-flick responses to water than to the scent of males (Tukey tests: $P < 0.001$ in both cases); females did not discriminate between male types ($P = 0.82$). There were no differences in the responses of either female type ($F_{1,18} = 1.98$, $P = 0.18$), who responded in a similar way to the different stimuli (interaction: $F_{2,36} = 3.24$, $P = 0.05$).

Staged agonistic interactions between males

The aggression index in each staged encounter and the average aggression index of each interaction varied significantly depending on the types of interacting males (Fig. 2; Table 1), with lower overall aggression indexes in the M1 × M2 interactions (Tukey tests: $P < 0.01$ in all cases). Residence condition also affected aggression (Fig. 2; Table 1). In general, residents were more aggressive than intruders (Tukey tests: $P < 0.05$ in all cases), except in the M2 × M1 interactions when both residents and intruders were similarly aggressive ($P = 0.99$).

These differences in aggressiveness affected the outcome of interactions. Thus, resident males won significantly more encounters than intruders in the M1 × M1 (15 out of 19, binomial test: $P = 0.019$), M2 × M2 (15 out of 19, $P = 0.019$), and M1 × M2 treatments (17 out of 19, $P = 0.0007$), but not in the M2 × M1 treatment (9 out of 19, $P = 1.00$).

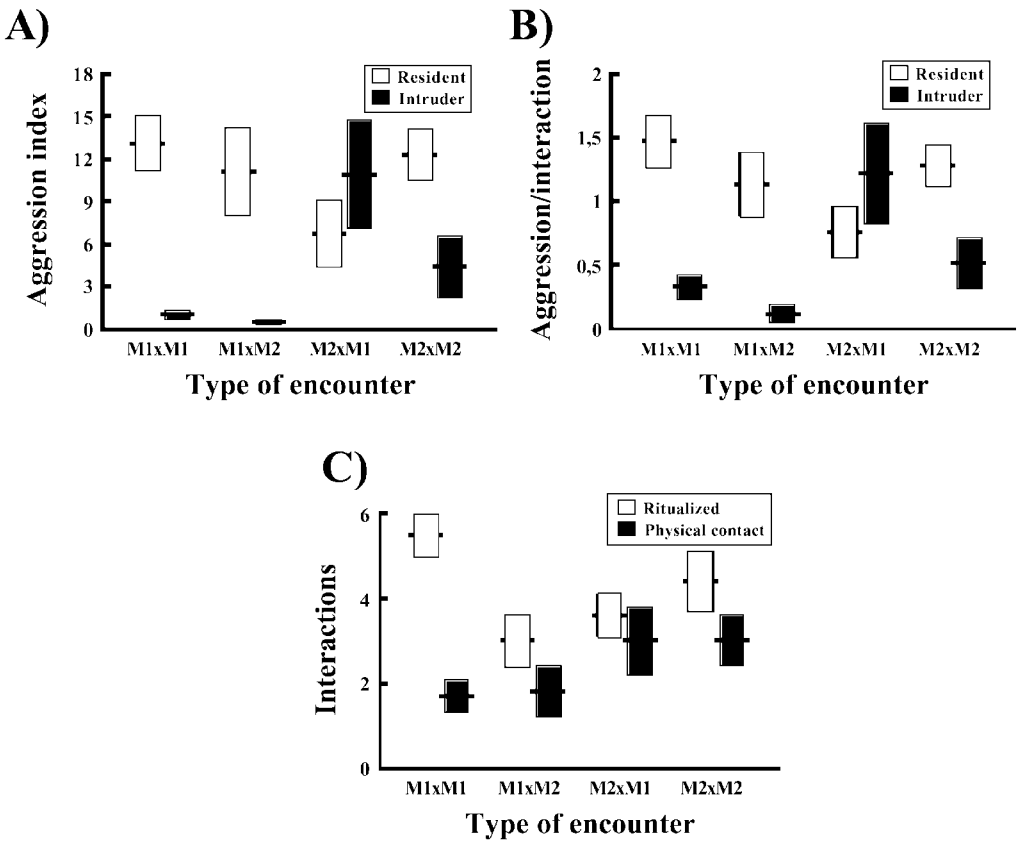

Fig. 2. (A) Aggression index (i.e. the sum of the number of interactions in each category won by the resident (□) or the intruder (■) male multiplied by the corresponding aggression score), (B) average aggression index per interaction (i.e. the aggression index of each male divided by the total number of interactions), and (C) types of agonistic interactions in 15 min staged inter-sexual agonistic encounters between male *P. hispanica* of the same or different type (see Methods). Results are the mean ± standard error.

Table 1. Intra-sexual staged encounters: results of two-way repeated measures analyses of variance of the effects of types of interacting males ('treatment', between factor) and residence condition within the pair of males ('residence', within factor) on aggression index in each encounter (i.e. the sum of the number of interactions in each category won by the resident or the intruder male multiplied by the corresponding aggression score) and average aggression index per interaction (i.e. the aggression index of each male divided by the total number of interactions)

Effect	Treatment	Residence	Treatment × residence
Aggression index in each encounter	$F_{3,72} = 6.51$, $P = 0.0006$	$F_{1,72} = 27.67$, $P < 0.0001$	$F_{3,72} = 3.77$, $P = 0.01$
Aggression index per interaction	$F_{3,72} = 3.77$, $P = 0.014$	$F_{1,72} = 17.34$, $P < 0.0001$	$F_{3,72} = 3.13$, $P = 0.03$

With respect to the characteristics of the interactions, ritualized interactions were more frequent than interactions with physical contact (two-way repeated measures ANOVA: $F_{1,72} = 29.61$, $P < 0.0001$), there were significant differences between treatments ($F_{3,72} = 3.31$, $P = 0.025$), and the magnitude of the difference between types of interactions varied between treatments ($F_{3,72} = 2.92$, $P = 0.04$) (Fig. 2C). *Post-hoc* tests indicated that ritualized interactions were significantly more frequent than those with physical contact in the M1 × M1 encounters (Tukey test: $P < 0.001$), but not in the other treatments ($P > 0.40$ in all cases). This suggests that contests between type 1 males were based mainly on ritualized behaviours without physical contact, whereas type 2 males used both categories of interactions.

Staged inter-sexual encounters

Males did not differ significantly in the number or rate of courtships, or in the number of tongue-flick explorations of females of different types (Table 2). However, females differed significantly between treatments in the number and rate of rejections of males, with type 2 females showing rejection behaviours more often than type 1 females (Tukey tests: $P < 0.002$ in all cases), but these responses were independent of the type of male that approached them ($P > 0.90$ in both cases) (Table 2).

DISCUSSION

Detection of chemical cues from conspecifics

The different tongue-flick rates of male *P. hispanica* in response to different scents indicated that males detected and discriminated between males of different types based on chemical cues alone. This result strongly suggests that chemicals of males of different types vary in composition and/or proportions, and that these differences might be related to genetic differences between types. Examples of similar geographic variation in the pheromone system include some rodents (Heth and Todrank, 2000; Heth *et al.*, 2001), salamanders (Rollman *et al.*, 2000), moths (McElfresh and Millar, 2001), and snakes (LeMaster and Mason, 2003).

Chemosensory recognition mechanisms between conspecific males are important because they modulate the intensity of agonistic encounters (López and Martín, 2002; López *et al.*, 2002a).

Table 2. Behaviour of *P. hispanica* lizards during 15 min staged intra-sexual encounters between males (M) and females (F) of the same or different type (see Methods), and results of one-way analyses of variance comparing behaviour across treatments (mean ± standard error)

	Type of encounter				
	M1 × F1	M1 × F2	M2 × F1	M2 × F2	One-way ANOVA
Male behaviour					
Courtships	4.5 ± 0.8	3.5 ± 1.2	4.2 ± 0.6	4.8 ± 1.1	$F_{3,76} = 1.62$, $P = 0.19$
Courtship rate	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	$F_{3,76} = 1.92$, $P = 0.13$
Tongue-flick explorations	3.8 ± 0.7	3.1 ± 1.1	3.8 ± 0.6	4.4 ± 1.1	$F_{3,76} = 1.26$, $P = 0.29$
Female behaviour					
Rejections	0.7 ± 0.4	4.2 ± 1.4	0.6 ± 0.2	4.1 ± 0.9	$F_{3,76} = 11.13$, $P < 0.0001$
Rejection rate	0.1 ± 0.1	1.4 ± 0.8	0.1 ± 0.1	1.4 ± 0.5	$F_{3,76} = 9.68$, $P < 0.0001$

Male lizards reacted aggressively (i.e. by increasing exploration rate and bites) when they found scents of conspecific males of the same type (for other lizard species, see Aragón *et al.*, 2001). In contrast, the scent of males of the other type elicited lower responses, which suggests that they were not considered as conspecific rival males.

Moreover, the low response to males of the other type cannot be explained by the lack of experience with unknown scents, because, in other experiments, previous known males elicited lower tongue-flick rates and were the recipients of less aggression (López and Martín, 2001a, 2002). Also, in another lacertid lizard, *Lacerta monticola*, the tongue-flick rate to other males' scent decreased with the degree of overlap (i.e. degree of familiarity) between their home ranges, which might reflect the need for more information or a lesser ability to discriminate the scent of infrequently encountered individuals than those often encountered (Aragón *et al.*, 2001).

In contrast, although males detected females using chemical cues, they did not discriminate between the scents of females of different types. In *P. hispanica*, sex identification of females is based mainly on chemical cues, and female pheromones are necessary to elicit male courtship (López and Martín, 2001b; Cooper and Pérez Mellado, 2002). Pheromones of female lizards are known to signal sex and reproductive condition (e.g. Cooper *et al.*, 1986; Cooper and Pérez Mellado, 2002). However, female pheromones required to elicit courtship might not differ between populations [but see LeMaster and Mason (2003) for inter-population variations in sexual attractiveness pheromones of female garter-snakes].

Females detect the scent of males but did not seem to discriminate between male types based on chemical cues. Thus, it is likely that males' chemicals that presumably differ between types are not used by females to identify males. Nevertheless, females might still use the individual characteristics of a male's scent to estimate his quality in mate choice decisions (López and Martín, 2005). Therefore, it seems that chemosensory recognition between male and female *P. hispanica* lizards is not contributing to reproductive isolation between types.

Staged agonistic encounters between males

There were differences in aggressiveness and outcome of agonistic interactions between males depending on the types of interacting males. Aggressiveness was high in encounters between males of the same type. The less aggression seen in the M1 × M2 interactions suggests that resident type 1 males did not consider an intruding type 2 male as a great threat. This is in line with the results of chemosensory recognition experiments. However, type 1 males still became dominant and maintained ownership of territories.

In contrast, in the M2 × M1 interactions, although resident type 2 males were also less aggressive, intruder type 1 males behaved more aggressively and won more interactions than expected for an intruder. This high aggressiveness cannot be easily explained, considering the behaviour of type 1 males when they were residents or in encounters with males of their own type. Nevertheless, it remains possible that, even if males of different types recognized each other to belong to different taxa, as the chemosensory responses strongly suggest, there might be some interspecific aggression as a result of competitive exclusion by interference competition (Ortiz and Jenssen, 1982; Nishikawa, 1987; Hess and Losos, 1991; Case *et al.*, 1994; Downes and Bauwens, 2002).

Staged inter-sexual encounters

The results of inter-sexual courtships suggest that there are no current behavioural pre-mating reproductive barriers at this stage. Matings may occur between lizards of different types if they are encountered, provided that there is no interference from other males. This also is in line with the lack of chemical discrimination between types of the opposite sex by males and females.

It remains to be determined whether these inter-taxa matings lead to fertile or equally viable offspring, although this is likely given the phylogenetic proximity between types (Harris and Sá-Sousa, 2001, 2002). In fact, artificial hybridization is possible between other closely related species of *Podarcis* (Galán, 2002). Thus, apparently, the possibility of successful matings between lizards of different types may be precluding a quicker speciation in this system.

Females of different types showed a differential rate of acceptance of matings, which probably reflects different intensities of sexual selection. However, this response was independent of the type of male that courted them. This might suggest that a successful inter-taxa mating with a type 2 female would be more difficult than with a type 1 female.

Although at this moment there is limited data to explain these differences, they might be related to different population sizes of the two types of lizards. In the Guadarrama Mountains, type 2 populations are larger (P. López and J. Martín, unpublished data) and females might be more selective when choosing between many available males (e.g. López and Martín, 2005). In contrast, type 1 populations are smaller, and females might have to accept every mating opportunity to avoid sperm limitation and to ensure being fertilized (Uller and Olsson, 2005). These differences in the intensity of sexual selection could have some consequences for speciation processes (see below).

Is there behaviourally mediated reproductive isolation between *Podarcis* types?

Although further studies are clearly needed, we could hypothesize from our results the existence of a complex scenario in potential encounters between males of different types. If

a type 2 male encounterd a type 1 population, he would not be considered a great threat by local resident males, possibly due to differences in pheromone chemicals. However, resident males would win most interactions anyway, thus excluding type 2 males from favourable microhabitats, and probably precluding them from easy access to type 1 females.

In contrast, if a type 1 male encountered a type 2 population, he also would receive less aggression from local males. But type 1 males are more aggressive and might easily win interactions, gain a territory, and thus access to type 2 females. However, if type 2 females are more selective when selecting males, the probability that a successful mating occurs could be low. Also, given the different population sizes, the probability of a dispersing type 1 male reaching a type 2 population is lower than the converse.

Therefore, multiple factors might be acting either against or in favour of speciation in occasional encounters between these two lizard types. Behaviourally mediated reproductive isolation may not be entirely effective at this moment. However, micro-geographic differences could be leading to differences between male types in pheromones, chemical recognition, and outcome of fights, thus contributing to isolation.

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