Abstract

Sex recognition is based on colour signals in many species of lizards. However, olfactory stimuli are also clearly involved, and many species might rely more on chemoreception. We aimed to examine whether colour pattern or odours, or both, are used in sex recognition and which cues elicit courtship of females by males of the lizard *Podarcis hispanica*. We experimentally manipulated the coloration and odour of female *P. hispanica*, thereby creating groups with all combinations between coloration and odour of males and females. Using data from staged encounters, we compared the responses of resident males to manipulated and unmanipulated individuals (males and females). Responding males reacted significantly more aggressively to female intruders with male odours, independently of their coloration. Nevertheless, coloration seemed to be important in long-distance sex recognition since, in the first minutes, females painted as females received a lower number of aggressive responses. Both colour and odour were important in eliciting male courtship. However, females painted as females and with female odours were preferentially courted. Comparisons with unmanipulated male and female intruders agreed with these expectations. Therefore, at close range, odoriferous cues seem to be more important than colour patterns in sex recognition, but female coloration is also useful at long range to deter the aggressive response of males and to elicit courtship in conjunction with odours.
Introduction

Many species of vertebrates use body coloration to convey information about the sender such as fighting ability, sex recognition or reproductive status (Andersson 1994; Cooper & Greenberg 1992). Sex recognition is based on colour signals in many species of lizards, especially in those with sexual dichromatism where one sex, often the male, displays brighter colours than the female (Cooper & Burns 1987; see Cooper & Greenberg 1992 for a review). In species lacking bright female secondary sexual colours, dull female coloration presumably allows sexual recognition by conspecific males.

The artificial manipulation of body colours by painting experiments has provided strong evidence that sex determination relies on visual cues in many lizards. Thus, in experiments where *Lacerta agilis* lizards were artificially painted to resemble the body colours of the opposite sex, males attacked rather than courted females painted to resemble males, and males courted other males painted with the female-like pattern (Kitzler 1941; Kramer 1937). However, olfactory stimuli were also clearly involved in sex recognition because male *L. agilis* that had responded aggressively to females painted with male-like colours stopped behaving aggressively after tongue-flicking them (Kitzler 1941; Kramer 1937). In another experiment, male *L. vivipara* courted conspecific males painted as females, but females painted as males, or painted black, were also courted (Bauwens et al. 1987), suggesting that males can probably identify females by characteristics other than their colour pattern. Moreover, sex recognition seems to rely on chemoreception, but not on coloration, for other species of reptiles such as gekkons, which are known to use chemoreception of skin-derived chemicals in sex recognition (Greenberg 1943; Mason & Gutzke 1990). In fact, chemical cues are known to play an important role in the intraspecific communication of lizards (Mason 1992; Halpern 1992; Cooper 1994), and several studies have shown pheromonal detection in different species (e.g. Cooper & Vitt 1984; Alberts 1989; Gómez et al. 1993). Thus, we hypothesized that in many lizards pheromonal identification of sex might take precedence over the chromatic cue.

The Iberian wall lizard, *Podarcis hispanica*, is a small (50–70 mm adult snout-to-vent length, SVL) diurnal lacertid lizard of the Iberian Peninsula, common in rocky habitats or artificial walls (Martín-Vallejo et al. 1995). The sexes differ in dorsal pattern and coloration (see below). Sexual dichromatism is more apparent in the belly that is orange in adult males during the mating season, whereas it is white in females and juvenile males (Pérez-Mellado & Galindo 1986). On the other hand, *P. hispanica* have well-developed chemosensory abilities, and can discriminate between prey and non-prey odour impregnated cotton swabs (Cooper 1990) and, at least, between conspecifics and heterospecifics by chemical cues alone (Gómez et al. 1993).

In this paper, we aimed to examine whether colour pattern or odours, or both, are used by male *P. hispanica* in female recognition. We experimentally manipulated the coloration and odour of female *P. hispanica*, thereby creating groups with all the combinations between coloration and odour of males and
females. Using data from staged encounters, we compared the responses of resident unmanipulated males to the different groups of manipulated females and their response to unmanipulated males and females. We hypothesized that responding males should behave in the experiments as they normally did with unmanipulated individuals. Thus, they should respond to manipulated individuals that they recognize as males as if they were intruders in their home ranges, and display aggressive behaviours towards them. Whereas individuals recognized as females should not elicit aggressive behaviour but should be courted. The outcome of aggressive vs. courtship displays should indicate whether visual or odoriferous cues, or both, are used in sex discrimination and which cues serve to stimulate courtship of females by male *P. hispanica*.

**Materials and Methods**

**Species and Study Site**

We captured adult *P. hispanica* (12 males and 24 females) on rocky outcrops in an oak forest near Cercedilla (40°44′ N, 4°02′ W; Madrid province, Spain). We captured lizards by noosing in different places over a large area to ensure that individuals had not been in previous contact, which may have affected the outcome of the interactions (Olsson 1994a). Lizards were housed individually at ‘El Ventorrillo’ Field Station (5 km from the capture site) in outdoor plastic cages (60 × 50 × 50 cm) containing sand substrate and rocks for cover. We provided mealworms dusted with a multivitamin powder as food and water ad libitum. The experiments were carried out during Apr. and May 1998, which coincided with the mating season of lizards in their original natural population (López & Martín, unpubl. data). All lizards were healthy during the trials and, at the end of the experiment, were released at their capture sites.

**Experimental Procedure**

We experimentally manipulated colour and odour of female *P. hispanica*. We painted individuals to resemble either male or female natural coloration and then we impregnated them with either male or female odours (taken from other individuals). Manipulated females (n = 16) were size-matched by SVL and randomly assigned to each different treatment (four groups of four females each). Other control individuals were used as unmanipulated (four males and four females) and some females were left with normal unmanipulated colour but were impregnated with other female odour (four females). Each manipulated or control individual participated in two tests within the same treatment with different responding males (n = 8).

Before the treatment, lizards were placed in a refrigerator for 10 min to immobilize them and facilitate the manipulations. We first attempted to eliminate odoriferous skin secretions with a treatment that has been effective in removing sexual pheromones of snakes (Noble 1937; Ross & Crews 1978) and lizards
Lizards were first washed with 96° alcohol, with special attention devoted to removing scents from the more odorous areas such as the cloacal and femoral regions. We then painted individuals and thereafter coated them with non-odoriferous vaseline to eliminate odoriferous scents. Lizards were painted by using OcaldoR water colours (Calder colours Ltd, Ashby-de-la-Zouch, UK), mixing them to achieve good visual matches with the natural colours and patterns of *P. hispanica* in our study area (see Arnold & Burton 1978; Pérez-Mellado & Galindo 1986). The background (brownish-olive) was first painted covering the dorsum of the lizard’s body. The belly was then painted with orange (for male-like treatment) or white colours (for female-like treatment). Head, hands and the tail remained unpainted. After drying at room temperature, we painted with the tip of a pen small pale white spots forming a discontinuous and irregular longitudinal row along each side and a few small blue spots along the edge of the belly (male-like treatment), or two longitudinal white stripes along each side (female-like treatment). Lizards were replaced in the refrigerator until the paint had dried before using them in experiments.

Male and female odours were transferred to the experimental individuals immediately preceding each trial by rubbing with cotton swabs impregnated in distilled water, the head, neck, trunk and tail skin and the cloacal area and femoral pores of a male or a female lizard, against the corresponding skin areas of the experimental individual. We made an effort to ensure odour transfer in those areas that are more frequently and intensely investigated by tongue-flicking during social encounters. This technique has been successfully employed to transfer odours between individuals in other lizard species (Cooper & Vitt 1987).

We attempted to match natural male or female coloration and odour. Lizards might, however, respond to cues which are not in the spectrum visible to the human eye such as ultraviolet radiation (Fleishman et al. 1993), which might not be accurately imitated or concealed by the paint used. We are confident that this was not the case because we conducted observations of the responses of males towards unmanipulated males and females. The results showed that responding males did not change their normal expected behavioural responses as a result of this manipulation. Thus, the number of aggressive responses to manipulated females painted as females with female odour, to females with normal unmanipulated colour but impregnated with other female odour, and to unmanipulated females were not significantly different (One-way repeated measures ANOVA: $F_{2,14} = 1.00, p = 0.39$) (Fig. 1). Thus, the experimental treatments seemed to be effective in resembling the natural colour and odour. Also, other species of lizards seem to respond normally to artificially painted individuals that resemble natural coloration (Cooper & Vitt 1988; Thompson & Moore 1991; Olsson 1994b; Martín & Forsman 1999).

**Staged Interactions**

We staged encounters between pairs of lizards in the home cage of the responding male and, thus, the responding male acted as the owner and the
manipulated female acted as an intruder. With this design we tried to mimic a natural field situation where a resident male *Podarcis hispanica* and an experimental intruding manipulated female, an unmanipulated male (UM; hatched bars) or female (UF; hatched bars), or a female with normal unmanipulated colour but impregnated with female odour (UF/oF). Manipulated females were painted as males (pM) or as females (pF), and then impregnated with odours from males (oM; black bars) or from females (oF; open bars). Results from the first 5 min of the trials and from the total of responses during the 15 min of the trials are shown.

![Fig. 1: Mean (+SE) number of aggressive responses in staged encounters between a responding resident male *Podarcis hispanica* and an experimental intruding manipulated female, an unmanipulated male (UM; hatched bars) or female (UF; hatched bars), or a female with normal unmanipulated colour but impregnated with female odour (UF/oF). Manipulated females were painted as males (pM) or as females (pF), and then impregnated with odours from males (oM; black bars) or from females (oF; open bars). Results from the first 5 min of the trials and from the total of responses during the 15 min of the trials are shown](image)

To begin a trial, we gently took one manipulated lizard from its cage, placed it gently in the middle of the responding male’s cage and, from a blind, we recorded their behaviour. In agonistic contests, we considered a ‘neutral response’ when the two individuals were together but no response or non-aggressive interaction was observed, or an ‘aggressive response’ if the responding male approached another individual with aggressive display and forced the other
individual to retreat or run away either without contact, by touching him on the flanks or, occasionally, by giving quick bites, especially on the snout or head. This fighting behaviour was readily discernible from ‘courtship behaviour’ (Verbeek 1972; López & Martín 2001). Approaching males employ threatening postures, strut toward an opponent on raised, stiff forelegs with their neck arched and the snout pointing slightly down, whereas, during courtship displays, the male approaches the female slowly and begins to lick the tail or the surrounding substrate. He then grips and shakes the female’s tail with a gentle bite, which does not result in any discernible wounds. If the female is receptive she will allow mounting. Thus, the body posture of the male and the intensity of his approaching and bites unequivocally characterize the difference between courtship and aggressive behaviours.

We noted the number and characteristics of the interactions (‘aggressive’ vs. ‘neutral’) between individuals during each trial, and the numbers of courtships observed. Also, females often showed foot shaking, a behaviour that seemed to deter the aggressive response of a male and that could be dependent on the treatment. Thus, we noted the frequency of such foot-shaking events. Each event consisted of several continuous and quick movements of the legs during sequences of variable duration in response to a male approach. A trial was interrupted after 15 min. Also, we decided to stop observations if persistent attacks or desperate attempts to escape were recorded. This was, however, not necessary 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. None of the individuals suffered physical injuries or showed physical stress during or after the trials, and all of them had maintained or increased their original body mass at the end of the trials. The paint on experimental lizards was removed immediately with water after they had completed each trial. No damage or necrosis of tissue due to the paint were ever recorded. Although agonistic interactions between lizards were not very aggressive, we use a minimal number of animals over the minimum time necessary to test our hypothesis.

To compare the number of aggressive interactions, number of neutral interactions, and the number of courtships observed in the 15 min of the trial across treatments, we used two-way repeated measure analyses of variance (ANOVA), examining the effects of colour and odour treatments (within-subject factors) on the responses of the same responding male. We included the interaction between colour and odour treatments to determine whether responses to a determined colour treatment changed under different odour treatments (Sokal & Rohlf 1995). Data were log-transformed to ensure normality. Tests of homogeneity of variances (Hartley’s Fmax test) showed that, in all cases, variances were not significantly heterogeneous. In a second step, we analysed only the responses in the first five minutes of the trials as an indication of a long-distance response by the owner, because males responded firstly to the intruding individual from long distance before any chemical testing could occur. Significance level was 0.05 and all tests were two tailed.
Results

The type of experimental treatment of manipulated females had a significant effect on the aggressive responses of responding males; manipulated females impregnated with male odour received a significantly greater number of aggressive responses than those impregnated with female odour, independently of their coloration (Two-way repeated measures ANOVA: colour effect, $F_{1,7} = 1.08$, $p = 0.33$, odour effect, $F_{1,7} = 15.32$, $p = 0.006$, interaction, $F_{1,7} = 2.40$, $p = 0.16$) (Fig. 1). Nevertheless, coloration seemed to be important in long-distance sex recognition, as suggested when we analysed only the responses in the first five minutes of the trials. Thus, in addition to the significant effect of odour treatment, manipulated females painted as males received, in the first five minutes, a significantly higher number of aggressive responses than those painted as females (Two-way repeated measures ANOVA: colour effect, $F_{1,7} = 7.20$, $p = 0.03$, odour effect, $F_{1,7} = 9.31$, $p = 0.018$, interaction, $F_{1,7} = 0.47$, $p = 0.51$).

Similarly, the number of neutral responses also depended on the experimental treatment. Females impregnated with odour of females elicited a higher number of neutral responses than those impregnated with odour of male, independently of coloration (Two-way repeated measures ANOVA: colour effect, $F_{1,7} = 2.85$, $p = 0.13$, odour effect, $F_{1,7} = 19.13$, $p = 0.003$, interaction, $F_{1,7} = 2.74$, $p = 0.14$).

The occurrence of foot-shaking behaviour in females was also dependent on the experimental treatment, probably as a consequence of the different responses of resident males. Thus, females with male odour showed a significantly higher rate of foot-shaking behaviour independently of coloration (Two-way repeated measures ANOVA: colour effect, $F_{1,7} = 0.21$, $p = 0.66$, odour effect, $F_{1,7} = 6.12$, $p = 0.04$, interaction, $F_{1,7} = 4.34$, $p = 0.08$) (Fig. 2).

![Fig. 2: Mean (+SE) number of foot-shaking behaviour events in staged encounters between a responding resident male *Podarcis hispanica* and an experimental intruding manipulated female painted as a male (pM) or as a female (pF), and then impregnated with odours from a male (oM; black bars) or from a female (oF; open bars)](image_url)
Some females of all manipulated groups were courted by males. Both colour and odour were important in eliciting male courtship. However, the manipulations that render females more similar to actual females elicited more courtships and, thus, females painted as females and with female odour were preferentially courted (Two-way repeated measures ANOVA: colour effect, F1,7 = 12.60, p = 0.01, odour effect, F1,7 = 8.33, p = 0.02, interaction, F1,7 = 7.00, p = 0.03) (Fig. 3).

Discussion

The results of our experiment indicate that, at least at close range, odoriferous cues are more important than colour patterns in sex recognition by male *Podarcis hispanica*. Thus, the reaction of responding males to manipulated females was mainly dependent on the scent, whereas colour seemed to be less important. Moreover, when a conflict between the meaning of the visual and scent signals arose, males seemed to rely on the scent to identify the sex of the intruding conspecific. Therefore, pheromonal identification of sex seems to take precedence over the chromatic cue in the lizard *P. hispanica*. The presence and relative concentration of pheromone components in lizards vary not only between sexes but also among individuals, which may convey information on the individual identity and serve a variety of functions (Alberts 1992). Therefore, in lizards, discriminations based on pheromone components may be more reliable and also may provide more detailed information on the conspecific than might be obtained from colour patterns alone. Nevertheless, the analysis of the first responses of
male *P. hispanica* to manipulated females suggests that coloration may be more important in long-distance communication, whereas pheromone identification may be the most important and confirmatory cue when two individuals are close together.

Other unrelated stimuli could have also influenced our results. For instance, individuals might be also identified by other morphological traits such as sexual dimorphism in body size and head shape, or certain behavioural cues associated to their sex, and these presumably may explain some mixed responses of responding males. Thus, some females from all the groups were courted irrespective of their manipulation. When a male came nearby, females often exhibited submissive behaviour such as tail twitches and foot shaking that seemed to deter the aggressive response of a male. Although these behaviours were also exhibited by young males towards adults, they were infrequent in encounters between two males with similar body size (López & Martín, unpubl. data).

Responding males were less aggressive towards individuals impregnated with scent of females, suggesting that female-like scent indeed reduces the aggressive response of territorial males. Thus, females may inhibit aggression and remain in male territories by signalling their sex through chemosensory cues, which are reliable as they are probably dependent on female-specific hormones. In this context, the fact that resident males responded aggressively towards intruders bearing odours of males, independently of their actual sex or coloration, could be interpreted in terms of acquired ability to recognise competitor males by chemosensory cues. By using a female-like dull coloration, a male with a low resource-holding potential might evade aggression from a dominant male, adopting a satellite-sneaking mating strategy (Martin & Forsman 1999). However, lizards could easily recognize cheating males by chemosensory cues. Thus, even females painted as females but with male odour received a higher aggressive level from the responding male, probably because they were considered as male cheaters (i.e. actual males with female-like coloration). Nevertheless, in the garter snake *Thamnophis sirtalis*, a small proportion of males, the so-called ‘she-males’, seem to be able to produce the female sex attractant pheromone, eliciting courtship from normal males (Mason & Crews 1985). This attractiveness seems to impart a selective advantage to them, because in competitive mating trials these she-males mate more than twice as often as normal males.

Male *P. hispanica* attempted to court individuals impregnated with female scent, independently of their coloration. This suggests that female pheromones are enough, and probably also necessary, to elicit male courtship in this lizard. The same pattern might be found in many other species of reptiles. For example, in an experiment with the snake *T. sirtalis parietalis*, certain pheromone signals released by females were necessary for eliciting male courtship, which began with a chemosensory investigation of the female’s body (Garstka & Crews 1981). Furthermore, male snakes receiving an application in the skin of serum from females were also courted. Similar results were obtained in another experiment in which tubes of female skin were placed on male *T. sirtalis sirtalis* (Gillingham & Dickinson 1980). Also, in the skink *Eumeces laticeps* an urodeal pheromone from
females induced male courtship of post-reproductive females when transferred to the skin surfaces of the latter (Cooper et al. 1986). Thus, chemosensory cues would reliably indicate not only the sex of the bearing but also the reproductive status.

However, our experiment indicates that coloration was also important eliciting courtship. Females with scent of females but painted as males were less courted, than females painted as females and with female odour. Thus, both coloration and chemical cues were important to elicit courtship. In fact, other studies of the related species *Podarcis bocagei* showed that some females developed a male-like dorsal coloration when they were gravid (i.e. unreceptive) and that this coloration may deter the courtship of males (Galán 2000). This development of bright coloration during pregnancy in female lizards has been described in many other lizards as a way to avoid male harassment (Cooper & Greenberg 1992). We have not observed changes of coloration in female *P. hispanica* in our population, but it is likely that male coloration, or the absence of female coloration, might also inhibit courtship in this species.

We conclude that chemosensory cues seem to be more important than visual ones in sex recognition by *P. hispanica*, at least at close range. We hypothesize that because most lizards seem to be able to detect pheromones from conspecifics (Mason 1992), chemical recognition of sex taken precedence over chromatic cues might be more widespread than expected among lizards, but also among other animals with chemoreceptive capacities, as a way to effectively identify the sex of a possible intruder. We also conclude that both colour and odour are important in eliciting courtship of females and that this may be the origin in other species of changes in female coloration when they are pregnant. Our findings point out the need for further studies of sex recognition and intraspecific communication taking into account the different roles and the relative importance of colour and chemical cues.

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**Literature Cited**


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