

Discrimination of conspecific faecal chemicals and spatial decisions in juvenile Iberian rock lizards (*Lacerta monticola*)

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Abstract Faeces play a role in intraspecific chemical communication in many vertebrates, including lizards. Here, we hypothesised that juvenile Iberian rock lizards (*Lacerta* (=Iberolacerta) *monticola*) use substrate-borne conspecific faecal chemicals to assess the social environment and base their spatial decisions. To test this hypothesis, we prepared chemical stimuli by dissolving faeces in an organic solvent (dichloromethane, DCM) and conducted chemosensory trials where we quantified latency to the first tongue flick (TF) and TF rates when young captive-borne juveniles were placed in an arena compartment whose substrate was labelled by (1) a control (DCM), (2) their own faecal chemicals, (3) faecal chemicals of another juvenile, (4) faecal chemicals of an adult female or (5) faecal chemicals of an adult male. Following TF observations, we removed a partition that separated the labelled compartment from a chemically unlabelled one, and recorded for 5 min when juveniles first crossed to the unlabelled compartment and total time spent in the labelled compartment. Each juvenile was tested with all stimuli in a randomised order. In addition, juveniles belonged to different families and were unrelated to and unfamiliar with faeces donors. Taken together, TF and spatial

responses toward stimuli indicate that juveniles discriminated between faecal chemicals of conspecific juveniles, adult females and males, and that they avoided remaining in substrates labelled by adult male faecal chemicals. We suggest that juveniles assess the social environment based on conspecific faecal chemicals thus avoiding aggression and cannibalistic risks undertaken from encounters with adult males.

Keywords Chemical communication · Conspecific discrimination · Faecal chemicals · Juvenile lizards · Tongue flick

Introduction

Animals often rely on information gathered from conspecifics for their decision-making processes (Danchin et al. 2004) and, when choosing where to settle, they may be attracted to conspecifics because prior occupancy of a habitat provides an indication of its suitability (Stamps 1987). Conspecific attraction during settlement should be pronounced in individuals unfamiliar with the habitat, such as new arrivers or juveniles, and that was confirmed experimentally on several taxa (Stamps 1987; Muller et al. 1997; Muller 1998). For instance, settlement by juvenile *Anolis aeneus* lizards is encouraged by the presence of juvenile conspecifics, as prospective settlers are attracted to juvenile territorial residents, settle nearby them and prefer moving into a previously occupied territory (Stamps 1987, 1988, 1991). However, when juveniles suffer aggression from larger-sized territorial adults, or inclusively cannibalistic predation from adults, they may be expected to avoid them (Stamps 1983). For instance, juvenile *A. aeneus* (Stamps 1983) and chameleons (*Chamaeleo chamaeleon*;

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Keren-Rotem et al. 2006) use different habitats than adults, thus avoiding cannibalism. Conspecific cues used to assess the social environment include visual and behavioural interaction with residents (Stamps 1987) and the detection of auditory (Muller 1998) and chemical (Burke 1986) signals produced by them.

In lizards, intraspecific communication by chemical cues, or pheromones, is widespread (Mason 1992; Cooper 1994; Schwenk 1995). Pheromones are often complex mixtures of proteins and lipids that are secreted from the skin (Mason and Gutzke 1990), epidermal (Alberts 1993) and cloacal (Cooper and Gartska 1987) glands, and that are present in faeces (Bull et al. 1999a). The chemical composition of pheromones is highly variable between individuals, partly genetically based (Alberts et al. 1993), and allows for the discrimination of self vs non-self (Aragón et al. 2001; Moreira et al. 2006), familiar vs unfamiliar individuals (Aragón et al. 2001), group vs non-group members (Bull et al. 1999b, 2001; O'Connor and Shine 2005) and kin vs non-kin (Main and Bull 1996; Léna and De Fraipont 1998). Pheromones also convey information about individual attributes, such as age (Martín and López 2006a) and sex (López and Martín 2005). In addition, lizard discrimination of conspecific chemicals is often functional in juveniles (Hanley et al. 1999; Font and Desfilis 2002) and, in the common lizard (*Lacerta vivipara*), was shown to play a role in dispersal; philopatric and disperser juveniles differed in their responses toward conspecific chemical cues (Léna et al. 2000; Aragón et al. 2006a, b; Cote and Clobert 2007).

Faeces play a role in intraspecific communication in many vertebrates (Müller-Schwarze 2006) and are used for signalling visually and chemically the resident status of individuals or territory ownership in frogs (Waldman and Bishop 2004), salamanders (Jaeger et al. 1986; Simmons et al. 1994), mammals (Johnson 1973; MacDonald 1980), as well as lizards (Duvall et al. 1987; Carpenter and Duvall 1995; Bull et al. 2001). In Iberian rock lizards (*Lacerta* (= *Iberolacerta*) *monticola*), faeces are deposited on visually conspicuous sites, and the faecal chemicals of adult males allowed for self-recognition, discrimination of familiar vs unfamiliar males, and signalling of male body size, thus suggesting that faeces play a role in territorial scent marking (López et al. 1998; Aragón et al. 2000). However, in these lizards, as in other species, there is little knowledge regarding the influence of conspecific faeces on juvenile spatial decisions.

Here, we hypothesised that juvenile Iberian rock lizards use substrate-borne conspecific faecal chemicals to assess the social environment and base their spatial decisions. In contrast to femoral secretions, which are produced only by males during the mating season to scent mark territories (López et al. 2006; Martín et al. 2007), whereas juveniles are born 3–4 months later (Moreira 2002), faeces are

produced by all conspecifics in all seasons, thus possibly allowing newborn juveniles to comprehensively assess the social environment. To test this hypothesis, we prepared chemical stimuli by dissolving faeces in an organic solvent (dichloromethane, DCM) that maintained faeces pheromonal properties in other lizards (Bull et al. 1999a), thus preventing test juveniles from discriminating faeces visually (Duvall et al. 1987; López et al. 1998). Then, we conducted trials where we quantified tongue flick (TF) and spatial responses when 16 captive-borne juveniles of 16 to 32 days old were presented with five stimuli in a randomised order: (1) a control (DCM), (2) their own faecal chemicals, (3) faecal chemicals of another juvenile, (4) faecal chemicals of an adult female and (5) faecal chemicals of an adult male. Juveniles belonged to different families and were unrelated to and unfamiliar with the faeces donors. We predicted that juveniles should discriminate between faecal chemicals from different conspecifics since micro-habitat sharing with juveniles, adult females or males should entail differential costs and benefits. Specifically, we predicted that juveniles should discriminate and avoid substrates labelled by adult male faecal chemicals because Iberian rock lizard males aggressively defend territories (Aragón et al. 2004, 2006c), chase, bite (pers. observations), and even cannibalise juveniles (Martinez-Solano 2001).

Materials and methods

Study population

Iberian rock lizards (*Lacerta* (= *Iberolacerta*) sp.; phylogenetic revision in Mayer and Arribas 2003) are small lacertids that inhabit high mountains and northwest coastal regions of the Iberian Peninsula. The subspecies *L. (I.) m. monticola* (Mayer and Arribas 2003) occurs at Serra da Estrela, Portugal, and is distributed between 1,400 m and the mountain top ('Torre') at 1,993 m of altitude (Moreira et al. 1999). Activity starts soon after snow melting in March–April and lasts to October–November. Mating takes place in April–June, and females lay a single clutch of 2–12 eggs yearly in June–July (Moreira 2002). Females do not attend the eggs and juveniles hatch in August–September (Moreira 2002). Lizards mature at 1–2 years of age at lower altitudes of the population range, and at 2–3 years of age at the mountain top. Longevity exceeds 10 years. Adult female and male snout-vent length (SVL) ranges 65–95 and 70–85 mm, respectively (Moreira et al. 1999).

Study animals

Adult lizards (female SVL 74–81 mm; male SVL 74–79 mm; $n=32$ per sex) were captured by noosing at the

surroundings of 'Torre' in April 2005, shortly after emerging from winter hibernation. They were maintained at 7°C from the capture date to the start of experiments, in May. Males (in groups of four during the initial 10 days and isolated after becoming intra-sexually aggressive) and females (continuously in groups of four) were housed indoor in 50×25×25 cm glass cages, provided with cardboard substrates, cardboard rolls for shelter, and wire mesh lids. Cages were heated (60 W spotlights provided a 28–35°C gradient that encompasses the species' preferred temperature) and overhead lighted (36 W full-spectrum daylight tubes) on a 10:14 light/dark cycle, and were screened from each other using cardboard. Mealworm (*Tenebrio* sp. larvae) dusted with multivitamin supplements and water were continuously available.

After males shed the skin and females developed ovarian egg follicles detectable by abdominal palpation (onset of sexual activity), we presented females with males in the male's home cage. Following copulation (or after 15 min when copulation did not occur) females were returned to their cages. This procedure was repeated in consecutive days until females underwent two copulations with the same male. In addition, each female copulated with a different male. When females became distended with eggs, cages were provided with plastic boxes with incubation medium (1:10 ml water/vermiculite size '2') for oviposition. Females were inspected twice daily and, upon laying, eggs were incubated at 28°C, inside 27×15×4 cm plastic boxes with incubation medium and divided into 18 equal-sized chambers. We incubated one egg per clutch in a different box and in a randomised chamber position. Towards the end of the incubation period, eggs were inspected daily. Juveniles hatched between 17 July and 4 August and could not move between chambers, thus being ascribed to their families. They were measured (SVL to the nearest 1 mm using a ruler), weighed (to the nearest 0.01 g using a digital balance) and marked individually by toe clipping. Thereafter, they were housed in groups of 20 per cage (50×25×25 cm), established according to hatching date, since juveniles do not show escalated fights (pers. observations). Juvenile cages were heated, lighted and provided with cardboard substrates and shelters as for adults. Vitamin-supplemented small mealworm and water were continuously available. After the study, adults and juveniles were released at capture locations.

Chemosensory trials

To prepare chemical stimuli, we collected and preserved by freezing two faeces per juvenile donor and one faeces per adult in 13–14 August. Faeces were collected directly into Eppendorf tubes while gently pressing the lizards' abdomen

to induce defaecation, thus allowing us to obtain fresh faeces that were uncontaminated by other body secretions. On the day of trials, faeces were thawed at room temperature and transferred to separate glass vials with 2 ml DCM using clean forceps. Vials were closed with Teflon-lined caps, shaken vigorously and allowed to rest for 1 h at room temperature. To standardise the amount of faeces between stimuli, we used two faeces per juvenile and a similar quantity (judged visually) of an adult faeces. Control stimuli were prepared in the same manner without addition of faeces.

Chemosensory trials were conducted outdoor, on sunny days, between 17 and 21 August. We tested four juveniles per day and altogether tested 16 juveniles. Each juvenile belonged to a different family and was unrelated to and unfamiliar (i.e. not exposed visually or chemically prior to the trials) with faeces donors. We used a repeated measures design where each juvenile was tested with all stimuli in the same day, in a randomised order, and 30 min elapsed between consecutive trials. Stimuli were: (1) a control (DCM, solvent used to dissolve faecal chemicals), (2) their own faecal chemicals, (3) faecal chemicals of another juvenile, (4) faecal chemicals of an adult female and (5) faecal chemicals of an adult male. We did not assign the sex of test and donor juveniles because sexes cannot be easily distinguished at early ages, and instead chose juveniles randomly.

Trials were conducted between 1030 and 1330 h. One hour before starting the trials, test juveniles were placed in glass flasks in an incubator at 28°C so as to standardise body temperature. As arenas, we used 34×22×10 cm plastic boxes divided in two equal-sized compartments (17×22×10 cm) by means of a drawn line and a removable opaque partition across the centre. Each compartment was provided with a fresh blot paper (12×15 cm) fixed to the centre. We placed arenas on a grass lawn and mounted 8-mm video cameras on an overhead view. Then, we collected 1.5 ml of the faecal chemical solution with a clean syringe, avoiding the collection of solids, applied it homogeneously to the blot paper on one of the arena compartments (labelled compartment; right or left side was assigned randomly) and allowed 5 min for the blot paper to dry (DCM is highly volatile and evaporated completely before starting a trial). The blot paper on the alternate compartment (unlabelled compartment) was not manipulated. Trials were started by placing a juvenile on the centre of the labelled compartment by gently turning the flask. Because juvenile TFs cannot be reliably quantified on videotapes, one experimenter sat motionless beside the arena, and dictated TF observations to a second experimenter, who recorded and timed events with a chronometer. Juvenile behaviour did not appear to be affected by the experi-

menter's presence. We recorded time elapsed between placement of the juvenile and the first TF (i.e. latency to the first TF) and number of TFs elicited during 1 min, counting from the first TF. Then, filming was started and the arena partition was removed, thus allowing juveniles to move freely between the two compartments. Trials were terminated following 5 min, and juveniles were returned to their glass flasks and put in the incubator. Upon completion of a day's trials, juveniles were measured and weighed (as above) and returned to their home cages.

From the videotapes, we recorded the time after removal of the arena partition that juveniles crossed between the two compartments (i.e. when the snout crossed the drawn line). We defined the following variables: (1) time when juveniles first crossed from the labelled to the unlabelled compartment (i.e. first transition); (2) number of times that juveniles crossed between compartments; and (3) time spent in the labelled compartment (i.e. time in stimulus).

Data analyses

Data were tested for normality of distribution within stimulus (Shapiro–Wilk test W , $n=16$ in all cases) and for homogeneity of variance between stimuli (Levene's test F , $df=4,75$ in all cases), and were transformed when normality and homoscedasticity were not verified. For comparing latency to the first TF (non-transformed), time of first transition ($\log x+1$ transformed) and time in stimulus (non-transformed) between stimuli, we used repeated measures ANOVAs and Newman–Keuls a posteriori tests because data were normally distributed ($W>0.89$, $P>0.06$ in all cases) and homoscedastic ($F>1.91$, $P>0.12$ in all cases). For comparing number of TFs elicited during 1 min between stimuli, we used a Friedman ANOVA because data were not normally distributed in two cases ($W<0.79$, $P<0.03$ for faecal chemicals of another juvenile and of adult males; $W>0.91$, $P>0.13$ for the remainder stimuli) and could not be normalised through data transformation. In this case, a posteriori paired comparisons followed Siegel and Castellan (1988). To determine whether TF and spatial responses were influenced by juvenile morphology (SVL and body mass) and age, we calculated the differences in latency, number of TFs, time of first transition and time in stimulus when juveniles were tested with each stimulus and when they were tested with their own faecal chemicals (i.e. TF and spatial responses with each stimulus relative to responses with own faecal chemicals; hereafter termed relative latency, relative number of TFs, relative time of first transition, relative time in stimulus). Then, we analysed the correlations (Spearman r_s) of those differences on juvenile morphology and age on the date of trials. Data are presented as mean \pm 1SE (range).

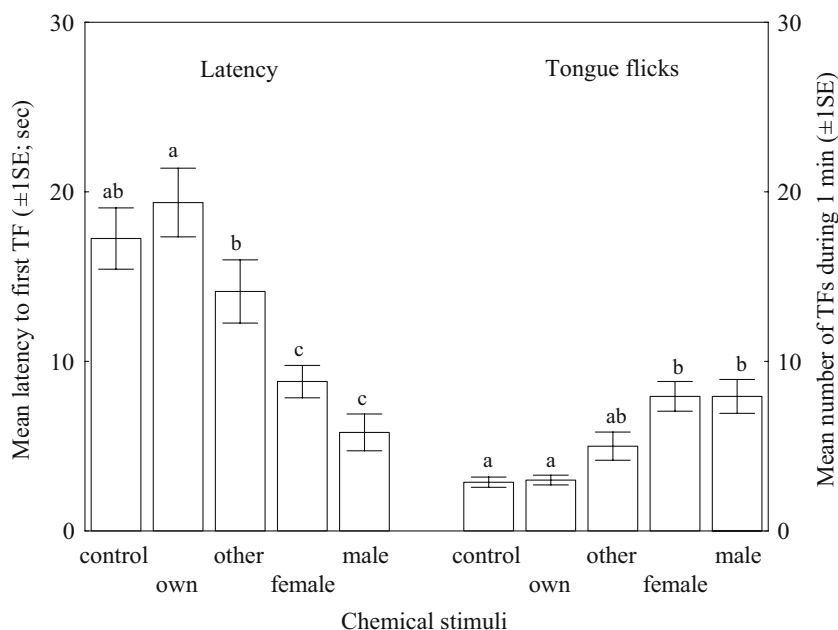
Results

Latency to the first TF ($F=18.53$, $df=4,60$, $P<0.001$) and number of TFs elicited during 1 min ($\chi^2=47.18$, $df=4,60$, $P<0.001$) differed significantly between stimuli (Fig. 1). A posteriori tests showed that latency was significantly higher, and the number of TFs was significantly lower for the group of stimuli comprising the control and own faecal chemicals than for the group comprising faecal chemicals of adult females and males. Moreover, latency and the number of TFs toward faecal chemicals of another juvenile showed intermediate values between those observed for those two groups; latency differed significantly from that observed for own faecal chemicals, faecal chemicals of adult females and males, but the number of TFs did not differ significantly from that observed for any of the remainder stimuli (see a posteriori statistical results in Fig. 1).

Juveniles were highly active during trials and crossed between arena compartments a mean of 6 ± 1 times (0–25 times) during 5 min. They remained in the labelled compartment for the entire 5 min in only four (5%) of the 80 trials. These cases involved four different juveniles when they were tested with their own faecal chemicals (two cases) and with the faecal chemicals of another juvenile (two cases). The time that juveniles first crossed to the unlabelled compartment (i.e. first transition; $F=3.79$, $df=4,60$, $P=0.008$) and the time that juveniles spent in the labelled compartment (i.e. time in stimulus; $F=3.04$, $df=4,60$, $P=0.024$) differed significantly between stimuli (Fig. 2). A posteriori tests showed that the time of first transition was significantly lower for faecal chemicals of adult males than for own faecal chemicals, faecal chemicals of another juvenile and faecal chemicals of adult females, whereas time in stimulus was significantly lower for faecal chemicals of adult males than for own faecal chemicals, and lower ($P=0.06$ was short of statistical significance) for faecal chemicals of adult males than for female faecal chemicals (see a posteriori statistical results in Fig. 2).

On the day of their trials, juveniles were 23 ± 1 days old (16–32 days), 34 ± 1 mm SVL (30–41 mm) and 0.77 ± 0.04 g (0.48–1.13 g) in body mass. Relative latency, relative number of TFs, relative time of first transition and relative time in stimulus did not correlate significantly with SVL and body mass for any stimuli ($P>0.05$ in all cases). Age did not affect TF responses, but appeared to affect spatial responses. In fact, relative latency and relative number of TFs did not correlate significantly with age for any of the stimuli ($P>0.32$ in all cases). However, relative time of first transition correlated positively with age for adult male faecal chemicals ($r_s=0.54$, $P=0.03$) but not for the remainder stimuli ($P>0.08$ for control, other and female stimuli). Also, relative time in stimulus correlated positively

Fig. 1 Latency to the first TF and number of TFs elicited during 1 min differed significantly between substrates labelled by the ‘control’ (DCM, solvent used to dissolve faecal chemicals), own faecal chemicals (‘own’), faecal chemicals of another juvenile (‘other’) and faecal chemicals of adult females (‘female’) and males (‘male’). A posteriori significant differences between stimuli in latency ($P < 0.02$ in all cases) and number of TFs ($P < 0.001$ in all cases) are indicated by *different letters*. P values for a posteriori non-significant differences between stimuli were > 0.09 in all cases



with age for the control ($r_s = 0.61$, $P = 0.01$) and for faecal chemicals of another juvenile ($r_s = 0.52$, $P = 0.04$), but not for the remainder stimuli ($P > 0.42$ for female and male stimuli). Thus, juveniles of relatively older age showed weaker reduction in time of first transition or in time in stimulus toward faecal chemicals from adult males and from another juvenile when responses toward their own faecal chemicals were taken as reference.

Discussion

Despite the recognition that faeces serve for territorial scent marking in lizards (Duvall et al. 1987; Carpenter and Duvall 1995; Bull et al. 2001), including Iberian rock lizards (López et al. 1998; Aragón et al. 2000), there is little knowledge regarding the influence of conspecific faecal chemicals on juvenile spatial decisions. Studies on common

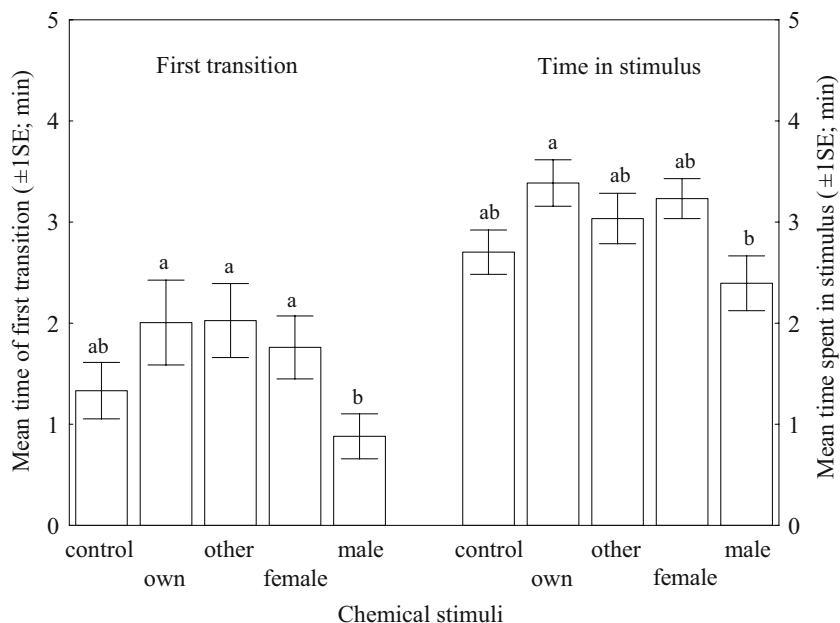


Fig. 2 Time of first transition from the labelled to the unlabelled arena compartments and time spent in the labelled compartment differed significantly between substrates labelled by the ‘control’ (DCM, solvent used to dissolve faecal chemicals), own faecal chemicals (‘own’), faecal chemicals of another juvenile (‘other’) and faecal chemicals of adult females (‘female’) and males (‘male’). A

posteriori significant differences between stimuli in time of first transition ($P < 0.03$ in all cases) and in time in stimulus ($P = 0.03$) are indicated by *different letters*. P values for a posteriori non-significant differences between stimuli were = 0.06 for the comparison of time in stimulus between ‘female’ and ‘male’ and > 0.13 for the remainder comparisons

lizards showed that conspecific chemicals influence juvenile dispersal (Léna et al. 2000; Aragón et al. 2006a, b; Cote and Clobert 2007) but those chemicals were of mixed unidentified body sources. In the present study, we collected faeces directly from the donors' cloaca so as to prevent contamination with other body secretions, and conducted chemosensory trials using faecal chemical solutions, thus preventing visual faeces discrimination. Results indicate that 16 to 32 days old Iberian rock lizard juveniles discriminated between faecal chemicals of conspecific juveniles, adult females and males, and that they avoided remaining in substrates labelled by adult male faecal chemicals. These results support the hypothesis that juveniles use substrate-borne conspecific faecal chemicals to assess the social environment and make spatial decisions, thus avoiding aggression (pers. observations) and predation risks (Martinez-Solano 2001) undertaken from encounters with adult males.

Chemosensation in many reptiles works in a hierarchical fashion, with chemicals being first received through the nares, processed by the nasal organs, and triggering TF-mediated vomerolfaction (Halpern 1992; Cooper 1994; Schwenk 1995). As such, different latencies to the first TF or the number of TFs elicited toward stimuli indicate distinct chemosensory activities and, thus, stimuli discrimination (Cooper 1994). Accordingly, observed differences in latency and number of TFs between the group of stimuli comprising the control and own faecal chemicals and the group comprising faecal chemicals of adult females and males indicate that juveniles discriminated between these stimuli groups. In addition, latency (but not the number of TFs) differed significantly between faecal chemicals of another juvenile and the remainder faecal chemicals, indicating discrimination between the former and latter chemicals. Nonetheless, the lack of differences in TF responses between stimuli does not imply non-discrimination, since animals may be shown to respond differently at other behavioural levels. In this respect, results further indicate that juveniles discriminated between faecal chemicals of adult females and males. Whereas latency and number of TFs did not differ significantly between female and male faecal chemicals, time of first transition was significantly lower for male than female faecal chemicals, and time in stimulus was lower for male than female faecal chemicals. Accordingly, taken together, TF and spatial responses toward stimuli indicate that juveniles discriminated between faecal chemicals of conspecific juveniles, adult females and males. We also observed that spatial responses were influenced by juvenile age. Spatial responses toward chemosignals were shown to change with age in another lizard (Labra et al. 2003), and our results raise the possibility that some ontogenetic shifts occur early in life as juveniles were only 16 to 32 days old.

Juveniles showed stronger avoidance toward adult male faecal chemicals than toward the remainder faecal chemicals, since they moved away more quickly and spent less time in the labelled arena compartment when it contained the former chemical. These results suggest that juveniles in the natural population may use chemical cues to avoid encounters with adult males. At the study population, females often lay communally and distantly from their mating season grounds in sites characterised by the availability of flat rocks over a loose soil, and these circumstances determine that a large number of juveniles disperse from localised natal sites into surrounding habitats (Moreira 2002). The ability to discriminate conspecific faecal chemicals from an early age might allow dispersing juveniles to prospect for and settle in areas where aggression (pers. observations) and predation risks (Martinez-Solano 2001) undertaken from encounters with adult males are minimised. In accordance with this hypothesis, juveniles of other lizards were shown to innately discriminate chemical cues from their predators (Van Damme et al. 1995) and to suffer increased chronic stress when exposed to chemosignals from socially dominant males (Alberts et al. 1994).

Because lizards were all fed on similar diets (diet can influence chemosignals; Martín and López 2006b), juvenile discrimination of conspecific faecal chemicals may be hypothesised to be based on age and sex differences in faeces chemical composition. In fact, studies on other lizards found that the lipophilic fraction of epidermal gland secretions differed according to age (Martín and López 2006a) and sex (López and Martín 2005), and male faeces may contain high steroid concentrations even outside the mating season (Atkins et al. 2002). Alternative explanations for our results may be postulated, but do not appear to be more parsimonious. Since juveniles were reared in groups, and were not exposed to adults prior to the trials, juvenile habituation to juvenile faecal chemicals may have determined weaker TF (lower latency and higher TFs) and spatial (higher time of first transition and time in stimulus) responses toward juvenile faecal chemicals than toward adult faecal chemicals. Nonetheless, juveniles were unhabituated to both adult female and male faecal chemicals, and still showed differential responses toward these stimuli. In addition, we reared females in groups, whereas males were isolated for the most part of the study. Social influences on lizard chemosignals are little studied (but see Aragón et al. 2006a, b), but we might expect that female faecal chemicals would convey information regarding higher degree of social interactions (or higher crowding conditions) than faeces from isolated males, which would possibly result in stronger avoidance by juveniles, which was not the case.

In conclusion, our study shows that juvenile Iberian rock lizards discriminated between faecal chemicals of conspe-

cific juveniles, adult females and males, and that they avoided remaining in substrates labelled by adult male faecal chemicals. These results suggest that juveniles may use conspecific faecal chemicals to assess the social environment and minimise aggression and predation risks undertaken from encounters with adult males. Juvenile discriminatory abilities may be based on age and sex differences in faeces chemical composition, but this hypothesis needs to be investigated.

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