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ORIGINAL ARTICLE

Proteins from femoral gland secretions of male rock lizards *Iberolacerta cyreni* allow self—but not individual—recognition of unfamiliar males

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Abstract

Individual recognition (IR) plays an important role in modulating social interactions of several animal species. IR may work at two fundamental levels: at class-level (CIR), if it allows group membership identification (e.g. familiar/unfamiliar), or at individual-level (true IR; TIR), if it allows uniquely recognizing conspecifics. Direct and indirect evidences suggest that many lizards are able to recognize conspecifics, notably using the secretions from femoral glands, specialized epidermal structures located in the cloacal region. Such secretions are made of a mix of lipids and proteins, the latter having been poorly studied but hypothesised to convey identity-related information. Using male Iberian Rock lizards, we set up bioassays to test the role of the protein fraction in IR, and specifically whether lizards: (1) can detect proteins from femoral glands, (2) can recognize their own proteins from those of an unfamiliar male (CIR) and (3) can distinguish between two unfamiliar protein signals (TIR). We found that femoral proteins can be actually detected, and the protein signal was enough to allow self-recognition, but not to distinguish between two unfamiliar males. These outcomes support the hypothesis that proteins from lizard femoral glands are used in intraspecific communication, at least at CIR level. The lack of TIR detection has three possible explanations: (1) finer IR abilities are actually absent in this species; (2) TIR requires a more complex and complete chemical signal or (3) a more prolonged and complete set of previous interactions among individuals is needed to lead to familiarity and TIR.

Significant statement

The ability to individually distinguish conspecifics is at the basis of many social behaviours. While the sensorial channel through which individual recognition is achieved may vary, the structure of the signal is usually complex and it could be expected different components to play different roles, so which part of the signal is actually responsible for individual recognition? Lizards use the secretion of specialized epidermal glands for intraspecific chemical communication, including individual recognition. Being a mixture of lipids and proteins, we wondered if it was the protein fraction of the secretion (i.e. the heritable part) which conveys identity. Using male Iberian Rock lizards, we showed that proteins allow for self but not for true individual recognition, suggesting that a partial signature may be not enough.

Keywords Chemical communication · Femoral glands · Individual recognition · Lizards · Proteins · Tongue-flicking

Introduction

Individual recognition (IR) is the ability to identify a conspecific on the basis of some distinctive characteristics (Dale et al. 2001). IR is expected to evolve in those social contexts where

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repeated encounters among the same individuals are likely to occur (Tibbetts and Dale 2007), and when the advantage of being identified overcomes the disadvantage (Johnstone 1997; Dale et al. 2001; Tibbetts and Dale 2007). Examples of such contexts may include kin and offspring recognition (e.g. Berger et al. 1997; Pitcher et al. 2015), familiar/unfamiliar discrimination (e.g. d'Ettorre and Heinze 2005; Ancillotto and Russo 2014) and dominance hierarchy establishment (e.g. Martín et al. 2007b; Keller-Costa et al. 2015) or territory definition (e.g. Gosling and Roberts 2001; Brennan and Kendrick 2006). Being the above situations widespread in the animal kingdom, IR can be observed in many different taxa, like insects (Sheehan and Tibbetts 2011), crayfish (Van der Velden et al. 2008), fish (Myrberg et al. 2005), amphibians (Bee and Gerhardt 2002), reptiles (Carazo et al. 2008), birds (Dhondt and Lambrechts 1992) or mammals (Kazial et al. 2008).

According to the way information about the identity cues is processed by the receiver, IR may be distinguished into 'true' IR (TIR) and class-level IR (CIR) (Beecher 1989; Johnston and Jernigan 1994; Tibbetts and Dale 2007). Even though the above distinction has been debated (Steiger and Müller 2008; Tibbetts et al. 2008), TIR assumes that the receiver is able to associate the signaller individual characteristics to a set of specific and univocal information about the signaller itself, which in turn evokes a unique response by the recipient (Sherman et al. 1997; Tibbetts and Dale 2007; Tibbetts et al. 2008). In contrast, CIR allows assigning the signaller to a category, a group of conspecifics, triggering the same response for all its members (e.g. male/female; familiar/unfamiliar) and ignoring the individual level (Beecher 1989; Tibbetts and Dale 2007; Tibbetts et al. 2008). TIR has been considered a higher-order ability compared with CIR (Johnston and Jernigan 1994), as the cognitive process involved is expected to be more complex (Sherman et al. 1997; Johnston and Bullock 2001; Mateo 2004), and to require specific adaptations and selective pressures (Johnstone 1997; Sheehan and Tibbetts 2010). Consequently, assessing the IR level (CIR or TIR) is of primary importance to understand the social function of signals, the forces promoting signal evolution and the role signals play in fostering individual phenotypic variability (Carazo et al. 2008; Sheehan and Tibbetts 2009; Tibbetts et al. 2017).

Despite reptiles have been traditionally neglected by research specifically focused on assessing the level of IR abilities (Wilkinson and Huber 2012), correlative and experimental evidences have suggested that TIR may actually occur and could explain some widespread social traits in this taxon, such as the ability to discriminate among self, familiar and unfamiliar conspecifics (Alberts 1992; Labra and Niemeyer 1999; Font and Desfilis 2002; Ibáñez et al. 2012), or the occurrence of the "dear enemy effect" related to territoriality (Whiting 1999; López and Martín 2002; Van Dyk and Evans 2007; Whiting and While 2017). Indeed, the two studies (LaDage and Ferkin 2006; Carazo et al. 2008) explicitly designed to explore the actual IR level in reptiles, notably lizards, found support to the hypothesis of TIR abilities. LaDage and Ferkin (2006), using an habituation-dishabituation approach, showed that male leopard geckos (*Eublepharis macularius*) are able to become familiar with two different females (CIR ability), and discriminate between them (TIR ability). Following the same experimental paradigm (habituation/dishabituation), Carazo et al. (2008) demonstrated that male Iberian wall lizards (*Podarcis hispanicus*) can individually distinguish rival males based on their scent alone (TIR ability) and can also remember the spatial location of the rival marks (Carazo et al. 2008). These findings suggest that in lizards: (i) TIR could be a widespread ability, associated to different social contexts (e.g. mate choice, rival recognition) and (ii) the chemical modality should play an important role in conveying IR (both TIR and CIR). Indeed, while all sensory channels (e.g. visual, Tibbetts 2002; chemical, Johnston and Bullock 2001; acoustic, Sousa-Lima et al. 2002; electric, Crawford 1991) are potentially useful in IR, the chemical one may be particularly suitable in all those contexts where IR has to be feasible in the absence of the signaller, like in territorial dynamics (Thom and Hurst 2004).

Lizards widely use the chemical modality in intraspecific communication (Martín and López 2011; Baeckens 2019) due to a combination of fine sensory structures (i.e. vomero-nasal organ coupled with tongue flicking behaviour) (Schwenk 1995), and specialized epidermal glands, located in the precloacal region (pre-cloacal glands) or along the inner part of the thighs (femoral glands) (Cole 1966; García-Roa et al. 2017a). Their waxy secretions are left on the substrate and may inform conspecifics about many different characteristics of the signaller, like age (Khannoon et al. 2011), dominance (Martín et al. 2007b), parasite load (Martín et al. 2007a), fighting ability (Labra 2006), colour morph (Mangiacotti et al. 2019a) and immune response (López et al. 2006). In addition, gland secretions have been shown to be involved in IR: many lizard species are able to distinguish their own scent from that of other individuals (e.g. Alberts 1992; Aguilar et al. 2009; Baird et al. 2015; Mangiacotti et al. 2019b), or can discriminate between familiar and unfamiliar conspecifics on a chemical basis (e.g. Alberts and Werner 1993; Aragón et al. 2001c; Font and Desfilis 2002; López et al. 2002), although TIR based on chemical cues has been demonstrated in only one species (Carazo et al. 2008). In all the above cases, anyway, almost no attempt has been done to discover which chemicals are specifically involved in IR (but see Alberts and Werner 1993; Mangiacotti et al. 2019b). Secretions are indeed made of a mixture of proteins and lipids (Cole 1966; Alberts 1990; Baeckens et al. 2015; Mangiacotti et al. 2017) in variable proportions according to the species (Alberts 1990; Escobar et al. 2001; Mangiacotti et al. 2019c) and season (Alberts et al.

1992; Mangiacotti et al. 2019c). Both fractions play a communication role (Alberts and Werner 1993; Martín and López 2015; Mangiacotti et al. 2019b), but lipid composition may vary temporally depending on changes in individual condition (Martín and López 2015). However, proteins, given their synthetic origin and their direct permanent link to genes, seem the best candidate to convey the individually stable information that allows chemical IR (Tibbetts et al. 2017; Mangiacotti et al. 2017, 2019a). Experimental support to such hypothesis comes from two phylogenetically distant lizards: the green iguana (*Iguana iguana*) and the common wall lizards (*Podarcis muralis*). In two separate trials, green iguanas showed that they are able to discriminate self-, familiar- and unfamiliar secretions, and that they increase tongue flicking rate (a proxy for interest; Cooper 1994) towards proteins compared with lipids (Alberts and Werner 1993). Common wall lizards, on the other hand, changed their movement patterns when facing a proteinaceous stimulus extracted from their own secretions compared with the one from an unfamiliar conspecific, demonstrating a discrimination ability between self and non-self cues (Mangiacotti et al. 2019b).

The aim of this study is therefore to specifically assess which level of IR can be actually mediated by the protein fraction of the femoral gland secretions. As model species, we used the Iberian rock lizard (*Iberolacerta cyreni*), a small lacertid lizard inhabiting rocky mountain habitats of Central Spain (Crochet et al. 2004; Sillero et al. 2014), and whose chemical communication has been long studied (e.g. López et al. 2002; López and Martín 2005; Aragón et al. 2006; Martín and López 2013a; García-Roa et al. 2017b). Notably, males of this species are able of self-recognition and to distinguish femoral secretions of familiar and unfamiliar males (Aragón et al. 2001a, c), and to use this information in decision-making processes (Aragón et al. 2003, 2007; Martín and López 2011). We performed three different experiments each answering a specific question: (1) Are males able to discriminate proteins from femoral glands from other chemicals (including other not-specific proteins)? (2) Does the proteinaceous signal allow self-other recognition (CIR ability)? and (3) Can male Iberian rock lizards individually distinguish other, unfamiliar males basing on femoral proteins alone (TIR ability)?

Material and methods

Sampling and housing lizards

At the end of May 2018, we captured by noosing male Iberian rock lizards ($N = 16$) at different areas around Puerto de Navacerrada (Guadarrama mountains, Central Spain) between 1800 m and 2000 m a.s.l. Noosing is a common and harmless practice to capture lizards (Vargas et al. 2000; Willson 2016), and we did not record injuries or damages in lizards due to such technique. Lizards were taken inside individual cloth bags to 'El Ventorrillo' MNCN-CSIC field station, 5 km from the capture site, where they were housed in outdoor individual plastic containers (60 × 40 × 44 cm, length × width × high). The terraria contained a brick for shelter and a small container with water ad libitum. We daily fed lizards crickets (*Achaeta domestica*) and mealworms (*Tenebrio molitor*), which were dusted with a vitamin complex and calcium powder every 2 days.

We measured snout-to-vent length (SVL) of males and classified them as 'small' (SVL range: 71–75 mm) or 'large' (SVL range: 77–81 mm), which corresponded to different age categories (López et al. 2003). Since different age/size classes may influence the response to chemical stimuli (Aragón et al. 2000; López et al. 2003), we used these two-dimensional classes to ensure that in all the experiments focal males matched the donors' size. Also, to ensure that lizards responded to unfamiliar males, we used in the trials scent stimuli from males that had been captured at more than 100 m of the capture site of the responding male.

All lizards were healthy during the experiments and were released with good conditions 1 month after capture, at the end of these and other trials, at their exact places of capture.

Preparation of the proteinaceous stimuli

To prepare the proteinaceous stimuli for all the experiments we followed Mangiacotti et al. (2019b). Immediately after capturing lizards, well before starting the experiments 2 weeks after, we obtained femoral gland secretions of all individual males by gently pressing around the femoral pores and collecting secretions directly in glass vials that were maintained in a freezer at − 20 °C. Donor lizards also were subsequently used in the experiments as responding lizards. However, because this is an innocuous procedure made only once and many days before the trials, we are confident that it should not affect the lizards' responses. In the laboratory, we extracted the protein fraction by a two-step protocol. First, we defatted the secretions by adding 200 μL of *n*-hexane directly into the vial, vortexing and incubating the sample at room temperature for 30 min. Then, we centrifuged the samples and removed the supernatant containing lipids. We repeated this step three times and, in the end, we air-dried the samples to completely remove any hexane trace. We dissolved the remnant pellets into 400 μL 10 mM (pH 7.4) phosphate-buffered saline (PBS) and incubated them at 37 °C for 4 hours. Then, we transferred the supernatant containing the dissolved proteins into new vials and we assessed protein concentration using DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). The calibration curve was obtained with standard bovine gamma globulin (Bio-Rad). Vials with protein solution were stored at − 20 °C until their subsequent use in the experiments.

This extraction procedure was made only once and provided enough proteins for all the subsequent trials as the amount needed in each test with cotton swab was actually minimal.

Additionally, for the first experiment (see below), we prepared two protein solutions by mixing respectively ten randomly selected samples from large males, and ten from small ones. Each mix was obtained by balancing the contribution from each donor (50 μg of proteins/sample) and adding PBS until 5 mL final volume (0.1 M). In the end, we also prepared a 0.1 M control protein solution by dissolving 500 μg of standard bovine gamma globulin (Bio-Rad, Hercules, CA, USA) into 5 mL PBS final volume.

Chemosensory tests

To assess chemosensory detection and discrimination of proteins from femoral gland secretions of conspecifics, we used tongue-flick (TF) behaviour in response to different chemical stimuli as a bioassay. This method assumes that differential rates of tongue extensions to the different chemical stimuli indicate detection and discrimination of these different stimuli (Cooper and Burghardt 1990; Cooper 1994). We compared TF rates of lizards in response to different scents categories of conspecifics in different experiments.

All the trials were made during June 2018, coinciding with the mating season of lizards, in sunny days, with appropriate temperatures, between 1100 and 1300 h (GMT) when lizards were fully active.

In the first experiment (discrimination test), we tested whether male lizards ($N = 16$) could detect and discriminate the protein fraction from femoral gland secretion. We compared the responses of adult male lizards with different chemical stimuli: (a) the 10 mM PBS used in protein extraction as a clean control; (b) the 0.1 M gamma globulin solution, as a control of a non-femoral protein (Cooper et al. 2002) or (c) the 0.1 M protein mix solution (see above) from femoral secretions of a pool of ten unfamiliar males matching the size of the focal ones. By using pooled protein instead of protein from a single donor, we intended to control for a potential individual effect. We used a repeated measures design where all individual lizards responded to all the treatments. We tested each lizard once per day with one stimulus and tested the rest of the scent stimuli in subsequent days in a counterbalanced order. We conducted experiments in each of the home terraria of lizards, from which we had previously removed the water container and the refuge temporarily 15 min before the trials. This setup allowed observing the lizard responses while they behave normally (i.e. without showing any signs of stress such as rapid escape locomotion or freeze defensive behaviour) as they were acclimatized to their respective terraria. Because chemoreceptive abilities are temperature dependent in lizards (Van Damme et al. 1990), we left lizards to bask and thermoregulate for at least 1 h before trials, so that they could attain optimal body temperatures. We prepared the stimuli by dipping the cotton tip (1 cm) of a wooden applicator (10 cm) in one of the vials containing one of the different scent stimuli. A new stimulus was used in each trial, and the order of presentation of the stimuli was counterbalanced. To begin a trial, the same experimenter in each experiment, who was blind to the treatments, slowly approached a terrarium and slowly moved the cotton swab to a position 2 cm anterior to the lizard's snout. In all tests, lizards responded to the scent stimuli and directed TFs to the swab in all conditions. We recorded latency to the first TF (i.e. the period between presentation of the cotton swab and the first TF directed at the swab) and numbers of TFs directed to the swab during 60 s beginning with the first TF. The differences in number of TFs and latency to first TF among treatments were separately assessed using general linear models (GLMs) where the different scent stimuli were set as the repeated measure factor. Data were log-transformed to ensure normality and homogeneity of variances (tested with Levene's test). We used Tukey's honestly significant difference (HSD) tests for post hoc pairwise comparisons among treatments (Sokal and Rohlf 1995).

The second experiment (CIR test) tested male lizards ($N = 16$) for self-recognition based on proteins from the femoral gland secretion alone. We used a functional modification of a classical habituation-dishabituation procedure (e.g. Johnston and Bullock 2001; Mateo 2006; Carazo et al. 2008). Each lizard was first tested three times repeatedly with the same cotton swab impregnated in the same stimuli. In each presentation, we exposed the swab to the lizard and counted the numbers of directed TFs in 60 s starting from the first TF. Then, we removed the swab, waited for 1 min and started a new test to record TFs using the same swab. Immediately following these three habituation trials, we waited for 1 min and conducted one dishabituation trial in which we tested the lizard with a new cotton swab impregnated in the treatment stimulus and recorded TF rates. If lizards were able of detecting and discriminating the treatment stimulus on the basis of chemical cues, then TF rates of subject lizards should change during the dishabituation trial in comparison with the TF rates to the stimulus used several times in the previous habituation trial. For the habituation trials, each male was first tested three repeated times with either (a) a clean cotton swab impregnated in PBS ('control' treatment) or (b) with the proteins dissolved in PBS extracted from his own femoral gland secretion ('self-other' treatment) or (c) with the proteins from gland secretions of an unfamiliar male of the same size category as the focal lizard ('other-self' treatment). Then, we made the dishabituation trial in which we tested again each lizard with a new cotton swab impregnated in either (a) PBS (for the 'control' treatment) or (b) proteins of an unfamiliar male ('self-other' test) or (c) his own proteins ('other-self' treatment). We used a repeated measures design as in the previous experiment where all individual lizards were used in all the treatments, one per day, in a counterbalanced order. We hypothesised that if TF rates during the dishabituation trial increased in the 'self-other' treatment, did not change or decreased in the 'other-self' treatment (due to the previous knowledge of the self-stimulus), and did not change or decreased in the 'control' treatment (because there was no actual change in the dishabituation trial), then this would indicate self-recognition i.e. CIR ability.

The third experiment (TIR test) wanted to assess if male lizards ($N = 16$) were capable of individual discrimination on the basis of proteins alone. We again used a habituation–dishabituation procedure (as in the second experiment), but here each lizard was first tested three times with the same swab with either (a) control PBS ('control' treatment) or (b) proteins from an unfamiliar male of the same size category than the responding lizard ('other1-other2' treatment). Then, we conducted the dishabituation trial with a new swab with either (a) PBS ('control' treatment) or (b) proteins from a different unfamiliar male ('other1-other2' treatment). We also used here a repeated measures design testing all individual lizards in the two treatments, one per day, in a counterbalanced order. We predicted that, if lizards were capable of TIR on the basis of proteins alone, then subject lizards' chemosensory exploration rates in the 'other1-other2' treatment should increase in the dishabituation trial compared with the last habituation one, in order to gain more information about the novel stimulus. Indeed, we learned from previous studies on this species (López et al. 1998; Aragón et al. 2000; López and Martín 2011) and the closely related *I. monticola* (Moreira et al. 2006) that TF rate usually increases in response to a novel stimulus from conspecifics, supporting the idea that novelty should elicit a raise in TF rate.

To examine differences in log-transformed TF rates of lizards among treatments in both the second and the third experiments, we used repeated measures General Lineal Models (GLMs) with 'trial' (four levels: the three habituation trials and the dishabituation trial) and 'treatment' (control and the different protein treatments) as within factors, including the interaction in the models. We used post hoc Tukey's tests for comparisons within each treatment of TF rates in response to (1) swabs with control PBS or proteins in the first vs. third habituation trials to assess habituation to repeated samples of the same chemical stimuli and (2) the swab in the third habituation trial vs. the swab bearing proteins or control PBS in the dishabituation trial to test for detection of the new chemical stimuli.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Discrimination test

Latency times to the first TF were significantly different among scent stimuli ($F_{2,30} = 39.78$, $P < 0.0001$; Fig. 1a). All males had significantly longer latencies to swabs impregnated with the blank control (PBS) than both to globulin dissolved in PBS (Tukey's test, $P = 0.00017$) and to the mix of proteins from femoral secretions of unfamiliar males dissolved in PBS ($P = 0.00012$). Latencies to globulin were significanlty longer than to proteins from femoral glands ($P = 0.0022$).

There were also significant differences among scent stimuli in TF rates ($F_{2,30} = 33.94$, $P < 0.0001$; Fig. 1b). All males elicited significantly lower TF rates to PBS alone than to globulin (Tukey's test, $P < 0.005$), and to proteins of unknown males ($P = 0.00012$). TF rates to globulin were significantly

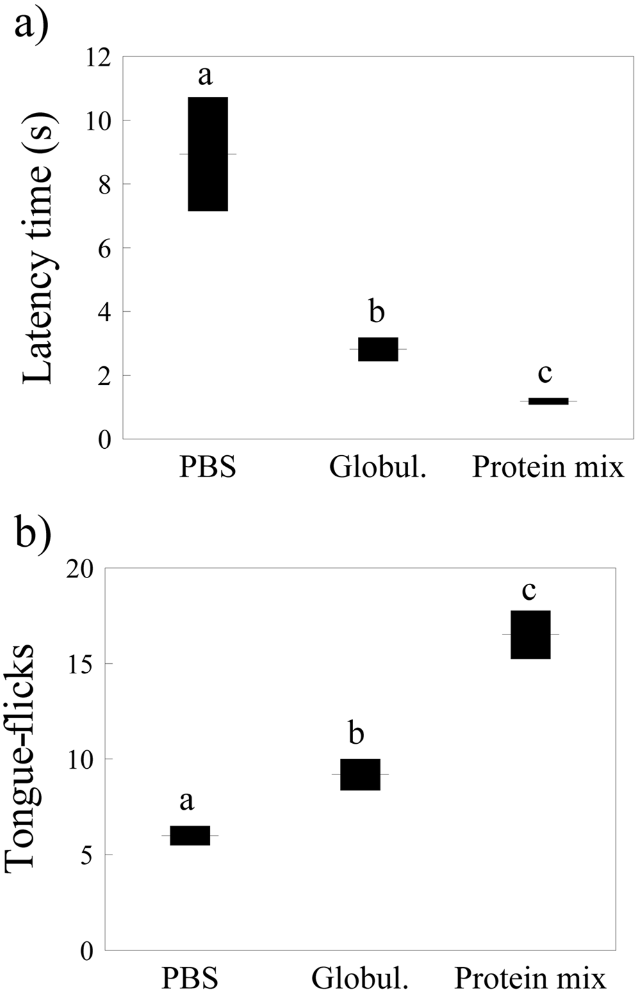

Fig. 1 **a** Latency time (mean ± SE; s) to the first tongue-flick and **b** number (mean ± SE) of directed tongue-flicks emitted by male *I. cyreni* lizards in response to different scent stimuli presented on cotton swabs. We used: PBS alone (neutral control), standard bovine gamma globulin solved in PBS (protein control) and the mix of proteins extracted with PBS from femoral secretions of a pool of unfamiliar males

lower than to the mix of proteins from femoral glands ($P = 0.00024$).

CIR test

The TF rates of males differed significantly among repeated trials within a test ($F_{3,45} = 10.83$, $P < 0.0001$) and among treatments ($F_{2,30} = 8.68$, $P = 0.001$), and the interaction between trial and treatment was significant ($F_{6,90} = 5.04$, $P < 0.0002$) (Fig. 2). Post hoc tests showed that males had no significant differences in TF rates comparing the first vs. the third habituation trials in the PBS 'control' (Tukey's test, $P = 0.16$), the 'self-other' ($P = 0.27$) or the 'other-self' treatments ($P = 0.15$). However, while in the 'control' treatment, there were no significant differences in TF rates between the dishabituation trial and the previous third habituation trial ($P = 0.97$); in the 'self-other' treatment, responses of males to proteins of other individual male in the dishabituation trial were significantly higher than to their own proteins in the previous third habituation trial ($P = 0.0004$). In contrast, in the 'other-self' treatment, responses of males to their own proteins in the dishabituation trial did not significantly differ from responses to proteins of other individual male in the previous third habituation trial ($P = 0.99$). Nevertheless, responses of males to their own proteins in the dishabituation trial were significantly lower than to the proteins of other individual male in the previous first ($P = 0.028$) and second ($P < 0.05$) habituation trials (Fig. 2).

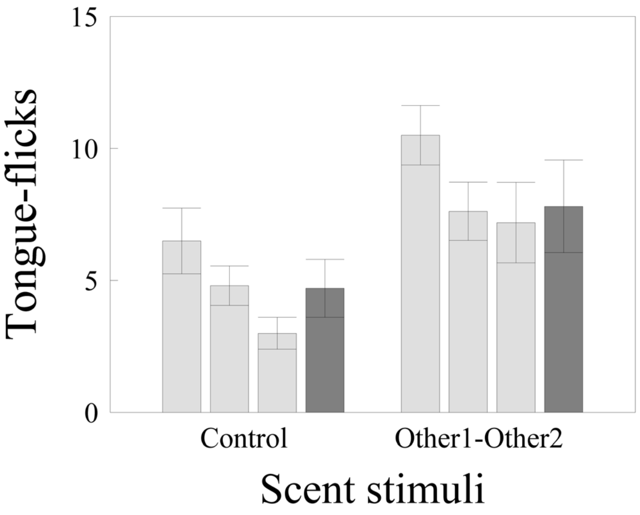

Fig. 2 **a** Self recognition in male *I. cyreni* lizards. Number (mean ± SE) of directed tongue-flicks emitted by lizards in 60 s in response to scent stimuli presented on cotton swabs. For each treatment, we made three habituation trials (light grey) with swabs bearing PBS (Control) or the proteins extracted from femoral secretions of the responding lizard (Self-Other) or from an unfamiliar male of the same size category (Other-Self) solved in PBS, and a dishabituation trial (dark grey) with swabs bearing PBS or proteins from an unfamiliar male (Self-Other) or from the responding male (Other-Self)

TIR test

The TF rates of males differed significantly among repeated trials within a test ($F_{3,45} = 6.07$, $P = 0.0015$) and between treatments ($F_{1,15} = 9.68$, $P = 0.007$), and the interaction between trial and treatment was not significant ($F_{3,45} = 0.24$, $P = 0.87$) (Fig. 3). Post hoc tests showed that males had no significant differences in TF rates comparing the first vs. the third habituation trials in the PBS 'control' (Tukey's test, $P = 0.16$) or the 'other-other' treatments ($P = 0.09$). Also, there were no significant differences in TF rates between the dishabituation trial and the previous third habituation trial both in the 'control' treatment ($P = 0.83$) and in the 'other-other' treatment ($P = 0.99$) (Fig. 3).

Discussion

Our experiments showed that male Iberian rock lizards are able to discriminate proteins from femoral gland secretions and to distinguish their own proteins from those of conspecific males, fixing CIR ability. In contrast, lizards cannot distinguish different unfamiliar males basing only on their protein signal, suggesting the lack of an automated, protein-based TIR.

Lizards have well developed chemoreception aptitude (Martín and López 2011; Baeckens 2019) and a very sensitivity vomeronasal organ, notably towards heavy molecules (Schwenk 1995; Filoramo and Schwenk 2009), so their ability to discriminate proteins i.e. large and not volatile compounds, is somewhat expected having regard to the previous findings using bovine gamma globulin in the lacertid *Podarcis lilfordi* (Cooper et al. 2002). The discrimination test confirmed this

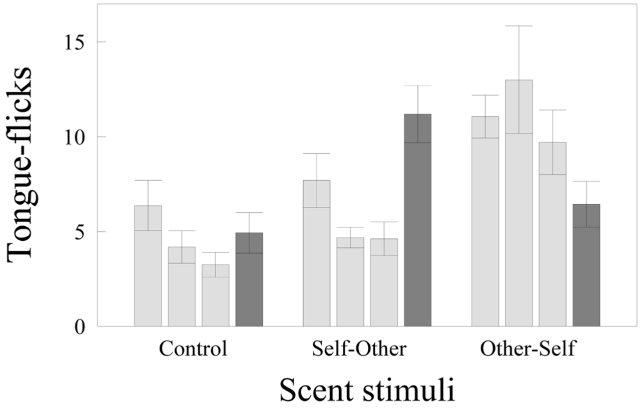

Fig. 3 Individual recognition in male *I. cyreni* lizards. Number (mean ± SE) of directed tongue-flicks emitted by lizards in 60 s in response to scent stimuli presented on cotton swabs. For each treatment, we made three habituation trials (light grey) with swabs bearing PBS (Control) or the proteins extracted from femoral secretions of an unfamiliar male of the same size category than the responding male (Other1-Other2) solved in PBS, and a dishabituation trial (dark grey) with swabs bearing PBS or proteins from a different unfamiliar male (Other1-Other2)

result also in *I. cyreni*, and additionally, it showed that proteins from femoral glands can elicit more interest (increased tongue-flicking and reduced latency), not only than a neutral control (PBS, the solvent) but also compared with bovine gamma globulin, a protein not found in the lizards' gland secretions. Detectability is a pre-requisite for the hypothesis that proteins can be actually used in intraspecific communication, as already suggested in other lizard species (Alberts and Werner 1993; Alberts et al. 1993; Mangiacotti et al. 2017) and tested in the Common Wall lizard, *P. muralis* (Mangiacotti et al. 2019b).

The actual use of proteins in intraspecific communication is further confirmed by our second experiment, which showed male Iberian rock lizards to be able to classify the proteinaceous stimuli at least into two categories: self or not-self. Indeed, while in the 'self-other' treatment TF rates increased in the dishabituation trial, in the reverse treatment 'other-self' they did not, but they rather decreased compared with the first two habituation trials. This differential response demonstrates self-recognition and, consequently, CIR. These results corroborate what has been already observed in the Common Wall lizard, concerning the identity information that proteins from femoral glands may convey (Mangiacotti et al. 2019b). Furthermore, they are consistent with the findings from previous studies having used TF rate to investigate self-recognition based on chemical cues: TF rate usually increases when lizards are tested against the complete femoral gland secretions (proteins and lipids) of another male, both in *I. cyreni* (Aragón et al. 2001a) and in the closely related species *I. monticola* (Moreira et al. 2006), and even in other phylogenetically distant lizards (e.g. Labra et al. 2001; Aguilar et al. 2009; Baird et al. 2015). Male chemical self-recognition may be considered the class-level basic form of recognition in territorial species (Thom and Hurst 2004), which may foster a spatial modulation of aggressive behaviour by allowing, for instance, the switch between the 'resident' and 'intruder' mode in asymmetric male-male encounters (Olsson 1992; López and Martín 2001; Aragón et al. 2006; Sacchi et al. 2009; Titone et al. 2018). Conversely, it is not enough for a finer adjustment of male-male aggressiveness, like those involving neighbourhood dynamics (Carazo et al. 2008) and previous contest experience (López and Martín 2001, 2011).

The protein fraction of the femoral gland secretions did not seem to enable TIR, since, in our third experiment, the scents from two unfamiliar males used for the habituation-dishabituation trials elicited the same level of 'interest' or 'chemical exploration' (i.e. tongue-flicking) and were not discriminated. Therefore, we could conclude that, though being actually able to process chemical signals from conspecifics, male Iberian rock lizards do not use such information to calibrate their own response according to the specific features of each individual. Despite we cannot rule out this possibility, this conclusion seems us not completely convincing. Previous observations on *I. cyreni* and the closely related species *I. monticola* showed that these rock lizards share several traits with the related *P. hispanicus*, where chemically based TIR was actually demonstrated (Carazo et al. 2008): notably, the importance and use of chemical cues in intraspecific communication (López et al. 2002; Martín and López 2006, 2013b; Moreira et al. 2006), the occurrence of a 'dear-enemy' effect (Aragón et al. 2000, 2001b, c, 2003, 2007; López and Martín 2011) and the ability of chemical cues to inform about the potential threat posed by a rival male (López et al. 2006; Martín et al. 2007b; López and Martín 2011). In such context, chemical TIR has been suggested to be the underlying mechanism modulating territorial defence in lizards (Carazo et al. 2008). Indeed, two alternative explanations can be made assuming that chemical TIR actually occurs in *I. cyreni*. First, the proteinaceous fraction alone is not enough to elicit TIR, and combination with the lipophilic fraction might be crucial to the recognition process. Only in this way, the chemical signature could reach the needed complexity to allow an inter-individual variability enough to minimize identification mismatches (Johnstone 1997; Tibbetts and Dale 2007). Indeed, in the only case where chemical TIR has been proved in a lizard (Carazo et al. 2008), a complete collection of chemical cues (femoral gland secretions, skin exudates, faeces) including lipids and proteins was used as stimulus. In this scenario, proteins may allow self but not individual recognition, for which a complete chemical signal may be needed. The alternative view concerns the need of a previous familiarity with other individuals before they could be individually recognized (Johnston and Jernigan 1994). However, the conditions of our experiments, notably the use of unfamiliar donors, do not allow us to test this hypothesis. Developing recognition at the individual level (i.e. TIR) requires the individual-specific information acquired during previous interactions to be associated with the memories of those interactions (Johnston and Bullock 2001). If this training phase does not occur, recognition cannot work until the individual level but stops at a lower one (e.g. self/non-self) (Johnston and Jernigan 1994). Accordingly, the lack of discrimination between the last two trials in the 'Other1-Other2' experiment (Fig. 3) may indicate that lizards deemed both stimuli to be equally unfamiliar males i.e. they bore the same level of novelty. A similar observation, based on a cognitive approach, was done in golden hamster (*Mesocricetus auratus*), where chemical individual recognition of females, in a habituation/dishabituation test, occurred only if males had previously interacted with them both (Johnston and Jernigan 1994; Johnston and Bullock 2001). The two studies having reliably proven TIR occurrence in lizards (LaDage and Ferkin 2006; Carazo et al. 2008) took advantage of the habituation phase to attain familiarization. Compared with our experimental protocol, in both cases, habituation was longer, provided access to more comprehensive stimuli (the whole animal or the whole repertoires of chemical cues) and allowed a free exploration of the stimuli, so it might be argued that any experimental setup involving an habituation phase with few stimulations over short time as we did in this study might not enable familiarization with the scent donor, preventing the detection of an individual discrimination. On the contrary, the same approach is fully suitable to work with self-recognition (CIR experiment) because an individual is necessarily familiar with itself.

In conclusion, we confirmed that proteins from lizard femoral gland secretions may play an important role in intraspecific communication (and therefore should not be neglected in future studies of lizard chemical communication), and that proteins can actually convey identity-related information, allowing an individual recognition at least at the class-level. The conclusive assessment of their importance in higher-order IR, like TIR, will require further investigations with a different experimental design, able to explicitly include the cognitive nature of recognition processes (Johnston and Jernigan 1994; Johnston and Bullock 2001).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The captures and experiments enforced all the present Spanish laws and were performed under licence from the Ethical and Environmental Organisms of Madrid Community Government (Ref. 10/165952.9/18) where they were carried out.

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