



# First experimental evidence that proteins from femoral glands convey identity-related information in a lizard

Marco Mangiacotti<sup>1,2</sup>  · Sofia Gaggiani<sup>1</sup> · Alan Jioele Coladonato<sup>1</sup> · Stefano Scali<sup>2</sup> · Marco Alberto Luca Zuffi<sup>3</sup> · Roberto Sacchi<sup>1</sup>

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## Abstract

Transferring identity-related information (IRI) to conspecifics may give advantage in effectively tuning intraspecific behaviour. Some lizard species use the secretions of specialized epidermal glands (femoral or cloacal) to convey IRI. Those secretions are made of lipids and proteins, the former been suggested to inform about signaller quality, the latter suspected to communicate IRI to conspecifics. Here, we tested the hypothesis that proteins broadcast IRI by analysing the movement patterns of 28 male common wall lizards (*Podarcis muralis*) under strictly controlled experimental conditions. Lizards were videotaped in plastic terraria where the substrate scent was manipulated by filling it with a solution bearing (i) the proteins extracted from the secretions of the tested lizard (SELF); (ii) the proteins from a never-met donor from other nearby populations (NON-SELF); and (iii) the solvent alone. Lizards showed higher behavioural response to the NON-SELF treatment with respect to both CTRL and SELF ones. Further, protein concentration did not affect behavioural response, suggesting an all-or-nothing effect. Both results agree with the hypothesis that proteins may be used in chemical communication and convey IRI, demonstrating for the first time that they can be used as intraspecific signal.

**Keywords** Unfamiliar recognition · Chemical communication · Lizards · Femoral glands · Proteins · Lipids · Identity signals · Quality signals · Residence in space and time analysis · Movement pattern

## Introduction

The ability to transfer identity-related information (IRI) to conspecifics gives undoubted advantage in effectively tuning intraspecific behaviour and fostering decision-making processes (Johnstone 1997a; Dale et al. 2001; Thom and Hurst 2004; Bradbury and Vehrencamp 2011). Inbreeding avoidance (Berger et al. 1997), offspring recognition (Stoffel et al. 2015), sexual display modulation (Baeckens et al. 2016), aggressiveness adjustment (Ancillotto and Russo 2014), and

territory definition (Gosling and Roberts 2001) are just few examples of biologically relevant contexts where such information flow plays a pivotal role.

Most lizard species are able to detect conspecific IRI, as well as to adjust a differential behavioural response (Alberts 1992; Ladage et al. 2006; Van Dyk and Evans 2007; Lopez et al. 2009; Baird et al. 2015). Although all available communication channels can virtually be recruited for IRI (Johnstone 1996; Dale et al. 2001; Thom and Hurst 2004), the chemical one is the most widespread among lizards, probably following the general importance and development of the chemosensory pathway in squamates (Cooper 1994; Schwenk 1995; Mason and Parker 2010; Robinson et al. 2015; García-Roa et al. 2017; but see: Van Dyk and Evans 2007). Consequently, lizards are expected to use chemical scents to convey IRI.

About one fourth of lizard species (96.8% of Lacertoidea; García-Roa et al. 2017) have a series of follicular epidermal glands in the pre-cloacal or femoral region (Cole 1966; García-Roa et al. 2017), which are suggested to be designed for intraspecific communication (Alberts 1993; Martín and López 2011; Mayerl et al. 2015; Baeckens et al. 2017b).

✉ Marco Mangiacotti  
marco.mangiacotti@gmail.com

<sup>1</sup> Dipartimento di Scienze della Terra e dell'Ambiente, Università degli Studi di Pavia, I-27100 Pavia, Italy

<sup>2</sup> Museo di Storia Naturale di Milano, Corso Venezia 55, I-20121 Milan, Italy

<sup>3</sup> Museo di Storia Naturale dell'Università di Pisa, Via Roma 79, I-56011 Calci, PI, Italy

These glands are often sexually dimorphic, being larger in males (Cole 1966; Martín and López 2011), and respond to androgen levels (Padoa 1933; Alberts et al. 1992; Mangiacotti et al. 2017a; Baeckens et al. 2017a). They secrete a mixture of protein and lipids (Cole 1966; Alberts 1990; Martín and López 2011; Baeckens et al. 2015; Mangiacotti et al. 2017b) left on the substrate and used as chemical cues (Alberts 1990). Lipids are the best studied fraction (Martín and López 2011; Mayerl et al. 2015; Baeckens et al. 2017b) and have been related to quality and condition of the signaller (Cooper and Pèrez-Mellado 2002; Martín and López 2007, 2015; Martín et al. 2008; Khannoon et al. 2011; Kopena et al. 2014). Much less is known about proteins (Mayerl et al. 2015; Mangiacotti et al. 2017b), which has been suggested to be used in intraspecific communication, potentially in conveying IRI (Alberts 1990; Alberts and Werner 1993). Proteins, indeed, keep two important properties required by a signal to transfer IRI (Dale et al. 2001): high genetic determination and high variability (Mangiacotti et al. 2017b). Then, lipids and proteins may be used together in a complementary way, to simultaneously transmit quality- and identity-related information (Johnstone 1997b; Tibbetts et al. 2017; Mangiacotti et al. 2017b). The two sides need to be closely tied for the communication system to properly work, as, being chemical cues potentially detectable even in the absence of the signaller, the quality signal is useless if not accompanied to IRI (Endler 1993; Bradbury and Vehrencamp 2011).

The previous hypothesis, combined to the lizard ability in IRI detection (Ladage et al. 2006; Van Dyk and Evans 2007; Baird et al. 2015), leads to the prediction that the protein fraction alone of a conspecific scent should be enough to elicit a behavioural response in a target lizard. In the present study, such prediction was tested using the common wall lizard (*Podarcis muralis*) as a model species. It is a medium-sized lacertid lizard relatively widespread in Central and Southern Europe (Sillero et al. 2014), which has already been the focus of studies on chemical communication (Martín et al. 2008; Heathcote et al. 2014; Pellitteri-Rosa et al. 2014; Sacchi et al. 2015; Baeckens et al. 2017a; MacGregor et al. 2017), and for which preliminary information about the protein fraction are available (Mangiacotti et al. 2017a, b). In detail, we used the proteinaceous fraction of femoral gland secretions as stimulus to verify if males are able to discriminate between their own proteins (SELF) and those from an unfamiliar (NON-SELF) male and the potential effect of protein concentration on the response.

## Material and methods

### Lizard collection and housing

Sixty adult male common wall lizards (snout-vent length, SVL range 54–70 mm) were captured during

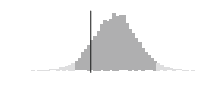
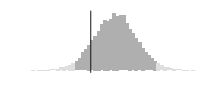

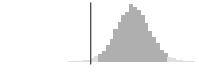
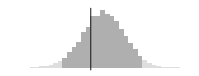
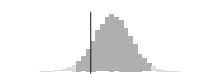

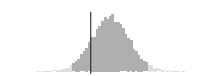
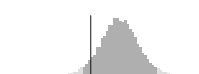

spring 2017 (20th March–20th May): one half was noosed in the botanic garden of Pavia (Northern Italy) and constituted the experimental focal sample. The other half was caught in different sites around Pavia, at least 5 km apart from the previous ones, and formed the donor sample. Lizards were transferred at the university lab (in Pavia) where their SVL was measured at nearest millimetre (using a ruler) and their femoral gland secretions collected into glass vials, with the help of a steel spatula. Vials were stored in a freezer (−20 °C) until subsequent analysis (Mangiacotti et al. 2017b). Donors and focal males never came into contact during the transportation or lab operations. The donor lizards were released at their capture sites immediately after lab procedures. The focal lizards were individually housed in 20 × 30 × 20 cm transparent plastic boxes, with a sheet of blotting paper as substratum, a flat brick as shelter/basking site, and a small bowl of water. Mealworms were provided as food everyday (one/day). The housing room was maintained between 15 and 30 °C (the natural temperature range for the season), and natural daylight was guaranteed. One week was set as the minimum acclimation period before starting the trials, and all lizards were released at their capture sites at the end of the experiments, after maximum two weeks from their capture. No animal was intentionally or accidentally injured or killed, and all lizards looked healthy at release.

### Extraction, quantification, and preparation of the proteinaceous stimuli

All the collected samples (from focal and donor lizards) underwent a two-step protein extraction protocol, slightly modified from Mangiacotti et al. (2017b), due to different final use. The lipophilic fraction was first solubilized by adding 200 µL of n-hexane to each secretion sample. After vortexing and incubating for 2 h at room temperature, the samples were centrifuged (14,000 rpm for 5 min), the hexane removed, and the residual pellet air-dried. To ensure in depth defatting, the procedure was repeated three times. The obtained protein pellet was then dissolved in 1500 µL of 10 mM (pH 7.4) phosphate-buffered saline (PBS). After extraction, protein concentration was assessed by the bicinchoninic acid assay (BCA) (Smith et al. 1985) using bovine serum albumin as the standard protein for the production of the calibration curve. Extraction worked well for all the samples and protein concentration was similar for focal and donor groups (mean ± standard deviation; focal 4.92 ± 2.99 µg/µl; donor 5.70 ± 2.21 µg/µl; see Table 1 and results for statistical support). Protein solutions as well as the PBS used in the extraction procedure were stored in freezer (at −20 °C) until their use in experiments.

**Table 1** Parameter estimates for model 0, I, and II. The half sample mode ( $\beta$ ) and the 95% highest density intervals (HDI<sub>95</sub>) are given for each parameter; the graphical representation of the posterior distribution of the

estimates is also reported (dark grey areas, HDI<sub>95</sub>) and compared with the null value (black vertical line). SVL (model II) is the lizard snout-to-vent length, proxy for its size

Model	Parameter	$\beta$	HDI <sub>95</sub>		
			lower	upper	
0	Proteinaceous concentration difference (NON-SELF minus SELF)	0.735	-0.530	2.113	
	Intercept	0.499	0.437	0.570	
I	Treatment <sub>NON-SELF</sub>	0.110	0.023	0.196	
	Treatment <sub>SELF</sub>	0.028	-0.061	0.112	
	Temperature	0.025	-0.014	0.064	
II	Intercept	0.612	0.541	0.686	
	Concentration	0.046	-0.031	0.119	
	Temperature	0.052	-0.025	0.128	
	SVL	0.007	-0.065	0.089	

## Experimental setup

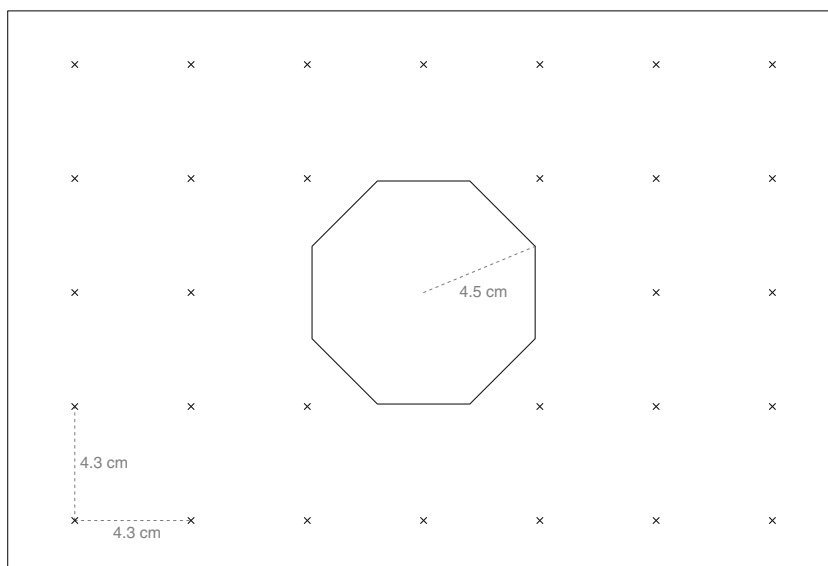
The experimental protocol resembles those typically used to investigate the response to chemical scent of predators (e.g. Thoen et al. 1986; Mencia et al. 2016; Prada et al. 2018) and already employed to address questions concerning lizard intraspecific communication (e.g. Alberts 1992; Labra and Niemeyer 1999; Aragón et al. 2003; Aguilar et al. 2009; Baeckens et al. 2016). The protocol was adapted to allow for the use of manipulated scents.

A clean and empty plastic box identical to that used for acclimation was prepared for each trial. To avoid visual disturbance during the experiments, the four sides of the box were externally covered by white paper. A sheet of blotting paper (same type and size of the one used for the acclimation)

was used as substrate. A grid was superimposed to the sheet (Fig. 1) to mark the 30 regularly spaced points where to release 50  $\mu$ L of the stimulus solution (a total of 1500  $\mu$ L); this design allowed the same distribution of the stimulus solution from trial to trial. The central scent-free area (octagon in Fig. 1) was used to start the experiment.

Before each trial, the focal lizard was heated for 5 min using a 75-W halogen infrared lamp (Reptiles-Planet.com) positioned 40 cm above the acclimation box. After switching off the lamp, the body temperature was measured with a handheld infrared thermometer (Lafayette TRP-39, Lafayette Instrument Co., Lafayette, IN, USA; sensitivity 0.1 °C; precision  $\pm$  2%). Then, the lizard was transferred to the experimental box and maintained for 5 more minutes inside an opaque plastic tube laid in the middle of the octagon, in order to reset the escaping

**Fig. 1** Scheme of the grid used to scatter the chemical solution bearing the stimulus on the blotting paper used in the experiments: multiplication symbols mark the points where 50  $\mu$ L of stimulus solution were dropped; the central octagon represents the scent-free zone to start the trial



behaviour, which typically follows manipulation. After the acclimation period, the tube was removed and the movements of the lizard recorded using a webcam (Microsoft LifeCam HD 3000) mounted on an easel, 60 cm above the box, and connected to a laptop by a 3-m cable. Recording was managed by Free2X software v1.0.0.1 (freely available at: <http://www.free2x.com/webcam-recorder/>), setting quality to  $800 \times 600$  pixels and 15 frames/s. Recording duration was set to 20 min (18,000 frames), starting 5 s after the tube removal (Mencia et al. 2016). Room temperature was set to 28  $^{\circ}$ C to reduce thermal loss during the experiments. Experiments took place between 10:00 and 14:00. Each focal lizard made three sequential trials, on three subsequent days, with a different stimulus: PBS (used as control, CTRL); protein solution of its own secretion (SELF); and protein solution from a never-met donor (NON-SELF). The order of presentation was balanced within treatment (Font and Desfilis 2002). After each trial, the lizards were returned to their original acclimation boxes. If the lizard did not move after 10 min from the start, the experiment was repeated the subsequent day.

### Lizard movements

We used idTracker (Pérez-Escudero et al. 2014) to extract the 2D trajectories (18,000 set of sequential xy coordinates) from the video files of each trial. The software searching parameters (intensity threshold; minimum size) were tuned in order to avoid bias in the trajectory extraction, and the final results were visually inspected using idPlayer (Pérez-Escudero et al. 2014). Then, each point in the trajectory was classified according to the “residence in space and time” (RST) method (Torres et al. 2017), which classifies each spatial point on the basis of the relation between the time spent and the distance travelled around it (see Torres et al. 2017 for further details). According

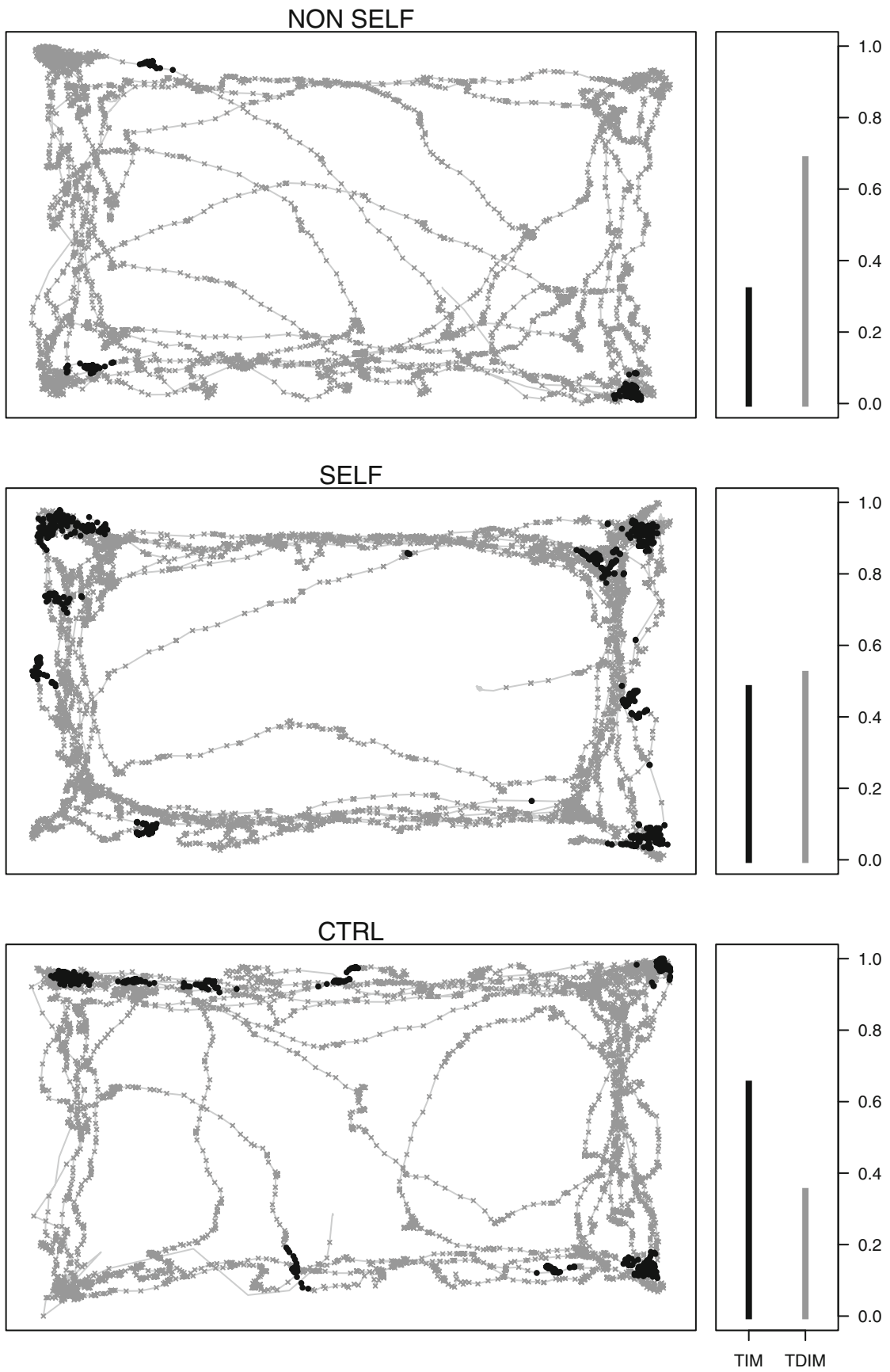
to RST analysis, there are three possible and biologically meaningful movement states: (i) transit movement (TM), when time and distance are low; (ii) time-intensive movement (TIM), when high time corresponds to low distance (e.g. freeze behaviour in our case); and (iii) time and distance intensive movement (TDIM), when time and distance are high (e.g. exploration, escaping attempt). The above classification requires a search radius  $R$  to be set a priori.  $R$  is a function of the mean transit speed ( $\bar{v}$ ) and time intervals ( $\Delta t$ ) between subsequent points (Torres et al. 2017):  $R = (\bar{v} \times \Delta t) / 2$ . According to the speed performance of *Podarcis muralis* measured in the field (Braña 2003), we used  $\bar{v} = 43.99$  cm/s (average maximum exploration speed during explorative movements) and  $\Delta t = 0.067$  s (the inverse of the frame/s),  $R$  resulted 1.47 cm. The proportion of each category within a trajectory describes the movement pattern associated to each focal lizard (Torres et al. 2017).

### Statistical analyses

Three models (0, I, and II) were used to address as many specific questions. Model 0 was fitted to exclude the potential effect of protein concentrations in the stimulus among treatments: the vector of paired differences between concentrations of NON-SELF and SELF trials was estimated and then compared to the null value (Kruschke 2010).

A linear mixed model (model I) was built to investigate if lizard behaviour was differentially affected by the stimuli.

**Fig. 2** Exemplificative RST analyses of NON-SELF (top), SELF (centre), and CTRL (bottom) trajectories obtained for the focal lizard ORT107. For each panel: on the left is reported the recorded trajectory (grey line) with the corresponding RST point classification (grey multiplication symbol indicates TDIM; black dots indicate TIM); on the right, it is shown the relative proportion of TIM and TDIM points. TM points were omitted since they are always less than 0.2% of the total



TDIM proportion was set as the response variable (TM was near zero, and consequently, TIM proportion was anti-correlated to TDIM); stimulus (three-levels factor) was the main predictor; lizard temperature (standardized) was the covariate to control for; lizard identity (id) entered the model as a random factor on the intercept to account for replicates (Kéry 2010), and for all other individual traits which remain constant over the trials (e.g. size, personality).

In the end, a second linear model (model II) was fitted on the NON-SELF subsample, to test if and how different concentrations of proteinaceous stimuli were able to alter lizard behaviour. In this case, TDIM proportion was still the response, protein concentration was the main predictor, temperature was maintained as the control variable, and SVL was used to account for potential effect of focal lizard size on the movement pattern.

All the models were fitted using JAGS 4.3.0 (<http://mcmc-jags.sourceforge.net/>), using flat normal priors for coefficients ( $\mu = 0$  and  $\sigma = 0.001$ ) and uninformative gamma priors for errors and random intercept ( $a = 0.001$  and  $b = 0.001$ ). Three independent chains were run, with 100,000 iterations each; first 10,000 values were discarded, and thinning was set to 15, to break within-chain autocorrelation (Kéry 2010). Convergence was checked and results from the posterior distribution are reported as the half sample mode (Bickel and Frühwirth 2006) plus the 50% and/or 95% highest density intervals (HDI<sub>50</sub>; HDI<sub>95</sub>) (Kruschke 2010). Data preparation, model settings, call to JAGS, and posterior elaborations were done in R 3.5.0 (R Core Team 2018) using the package R2jags (Su and Yajima 2015), modeest (Poncet 2012), and HDInterval (Meredith and Kruschke 2018).

## Results

Out of the 30 focal lizards tested, two were excluded because they did not move for more than 10 min even repeating the

trial. Consequently, the analysis is based on 28 lizards, for a total of 84 videos (one for each treatment for each focal lizard). On average, 54.90% of trajectory points were classified as TDIM, 0.02% as TM, and 45.08% as TIM (see Fig. 2 for an exemplification of RST analysis).

The paired difference in the protein concentration between NON-SELF and SELF treatment was slightly larger than zero (Table 1: model 0), the null value being well encompassed within HDI<sub>95</sub>.

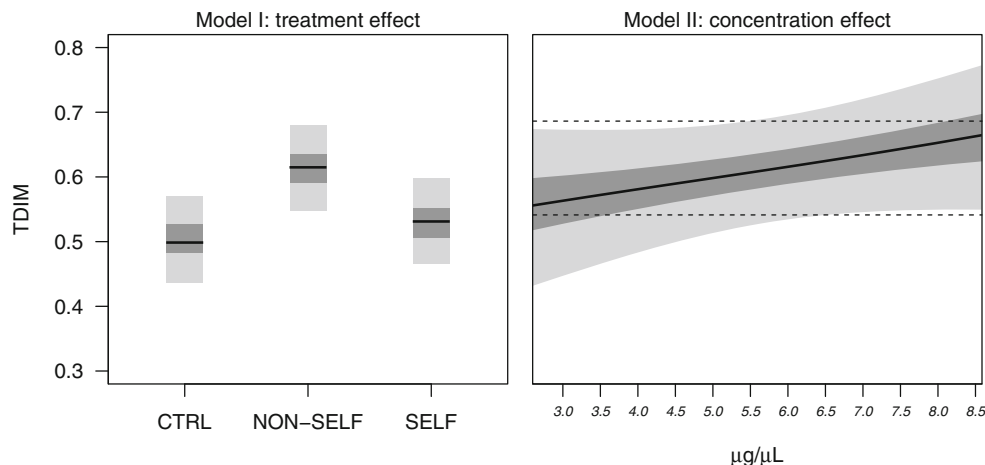
According to model I, TDIM was positively affected by NON-SELF (Table 1: model I), but not by SELF treatment, which did not differ from CTRL (Table 1: model I). NON-SELF treatment predicted larger value for TDIM than CTRL (Fig. 3;  $P_{\text{NON-SELF} > \text{CTRL}} = 0.992$ ) and SELF (Fig. 3;  $P_{\text{NON-SELF} > \text{SELF}} = 0.968$ ). Body temperature at trial start had no effect (Table 1: model I), as well as the proteinaceous concentration and the focal lizard size in NON-SELF treatment (Table 1: model II; Fig. 3).

## Discussion

We showed that male common wall lizards responded differently to the proteins secreted by an unfamiliar conspecific compared to a neutral stimulus and to their own proteins. Notably, when proteins from femoral gland secretions of a never-met male were used to mark the substratum, TDIM increased by 1.23 times the CTRL and 1.16 the SELF value (Fig. 3). The observed outcome was not affected by experimental contingency (i.e. body temperature, proteinaceous concentration, or lizard size), as their respective effects are not credible (Table 1).

In the present study, TDIM corresponds to escaping attempts (climbing and scratching the box walls and corners, jumping) or exploratory activity (slow movements along the perimeter often accompanied by tongue-flicking). A TDIM intensification in the NON-SELF treatment can reflect a

**Fig. 3** Posterior predictions of the effect of treatment (left) and protein concentration (right) on the response variable (TDIM). Black solid lines indicate mode of the posterior distribution; dark grey areas indicate HDI<sub>50</sub>; light grey areas indicate HDI<sub>95</sub>; dashed lines indicate HDI<sub>95</sub> of the model II intercept (i.e. the most probable values of the response in the absence of a concentration effect)



situation where an intruder enters the territory marked by the scent of an unfamiliar male: perceiving the odour of the unknown rival without being able to see it may trigger more explorative, and “nervous” movement patterns. Most studies having used a comparable experimental setup (Labra and Niemeyer 1999; Aragón et al. 2003; Van Dyk and Evans 2007; Aguilar et al. 2009) consistently found non-self (or unfamiliar) cues to elicit an increase of the intruder’s movements, with few exceptions: Aragón et al. (2001), who found no significant difference, but the same trend; and Font and Desfilis (2002), who found a significant opposite trend (familiar > unfamiliar), but working with juveniles (see discussion therein for interpretation). Further, in agonistic contests staged to test the occurrence of a residence effect in lizards, intruders typically increase avoidance behaviours (e.g. running, climbing, scratching the cage walls; López and Martín 2001; Aragón et al. 2006; Sacchi et al. 2009; Titone et al. 2018). All the above responses require some abilities for rival recognition (Glinski and Krekorian 1985; Whiting 1999; López and Martín 2001; Thom and Hurst 2004; Tibbetts and Dale 2007; Carazo et al. 2008) and, therefore, imply a IRI transferring. Applied to the present study case, this is equivalent to say that proteins from femoral glands are able to convey IRI, as they were the only available cue to identify the conspecific as a stranger.

A circumstantial evidence supporting the previous conclusion may come from combining model I and II outcomes. Within the NON-SELF treatment (i.e. the treatment level giving the maximum response to chemicals), the proteinaceous concentration in the solution did not affect the focal lizard response (Table 1: model II). Hence, the increase in TDIM did not depend upon the amount of proteins (model II), but only by their occurrence at a perceivable level (model I). Such all-or-nothing response is expected for an IRI signal, since it has not to be related to signaller quality or condition (Dale et al. 2001; Tibbetts et al. 2017). Indeed, a response proportional to the concentration of specific compounds has been already observed in lizards (e.g. López and Martín 2005; Martín and López 2006, 2007; Martín et al. 2007), but only when lipophilic substances or the complete (proteins and lipids) secretions were used. Coherently with the properties of a quality signal (Dale et al. 2001; Tibbetts et al. 2017), the abundance of such elements was found to correlate to qualitative traits (size, fighting ability, immune-response level, parasites load; Martín and López 2015). This progressive effect in the response has disappeared when the lipophilic fraction was removed, still preserving the ability to inform about the secretion provenience (NON-SELF vs SELF) to the proteinaceous remain. The lack of correlation may then suggest proteins to inform about discrete traits (like identity or strategy, sensu Tibbetts et al. 2017). We do admit that alternative explanations might be considered, such as an artefact due to the reduced sample size (28 lizards with one replicate), or

more complex effects: e.g. protein concentration is proportional to donors’ size, which may affect the behaviour of focal lizards in a non-linear way, depending on the opponent size (Sacchi et al. 2009; Titone et al. 2018), or lack of lipids may have reduced the detectability or the efficacy of the signal (Alberts and Werner 1993), thus masking the relation.

In conclusion, the present study provides for the first time (as far as we are aware) experimental support to the hypothesis that proteins from lizard femoral glands can be used as intra-specific signal, and can convey information about conspecifics familiarity. Even if the experimental design was not fit to investigate the actual level of individual recognition (Thom and Hurst 2004) nor the underlying mechanism (targeted studies are needed to shed light on these topics), results are promising and widen the perspective on the study of chemical communication in lizards, constrained for decades to the lipids fraction (Mayerl et al. 2015).

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