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Protein components of femoral gland secretions in a polymorphic lizard (*Podarcis muralis*)

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To my wife and sons

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Abstract

In animal intraspecific communication, the information conveyed by a signal is determined by the social function of the signal itself, and allows predicting its pattern of variability: an identity signal will exhibit a high inter-individual variation, an ontogenetic stability, a strong genetic determination, and no relation with quality traits. A strategy signal will share with the previous the genetic determination, and the independence from the condition or quality of the individual, but it will be discrete and multimodal. On the opposite, quality cues will be continuous traits, less variable than identity signals, and strongly related to condition and quality of the signaller.

The chemical channel is the most ancient one, and the only one able to continue signalling also in the absence of the signaller. Such property becomes very important in decision-making processes, when territorialism or multiple strategies occurred in a population.

Lizards are good models to study chemical communication. Most species have a set of epidermal glands (femoral glands) which produce waxy secretions used as cues in social context. They are a mixture of lipids and proteins, the former used to communicate individual quality and condition, the latter, far less studied, maybe involved in signalling. The proteins nature, and some preliminary observations has suggested that they may convey identity-related information.

The aim of my research is to assess if proteins from femoral glands are actually used as signals and if they convey identity- or strategy-information. As study species I chose the Common Wall lizards (*Podarcis muralis*), a small lacertid presenting a ventral colour polymorphism.

I first investigated the intra- and inter-populations patterns of variability of the protein assemblage, using one-dimensional electrophoresis, to demonstrate that signal is variable enough to support the identity-signal hypothesis. I then moved to the comparison of the protein patterns of the three main colour morphs. I used two-dimensional electrophoresis to obtained a finer resolution, and spectrometric analysis to identify proteins. I expected to find a morph-specific protein composition, according to the strategy-signal prediction. To obtain an experimental evidence for the

communication role of proteins, I set up a behavioural test in neutral arenas in order to demonstrate that lizards can decrypt the information encoded into proteins alone. Male behaviour was observed in presence of the protein scent of its own, of that by an unfamiliar male, and a control: a treatment effect would have been interpreted as the prove that proteins were detectable and informative. Finally, I investigated if and how the lipids and proteins co-varied along the activity season, to verify the prediction that only lipids, as quality-signals, would have shown a variation in their composition.

Results from the first three steps of the research agreed with the hypothesis that proteins are identity- and strategy-signals: (1) the among-individuals variation was large, and accompanied by a genetic correlation with clade and population of origin (identity-signal); (2) colour morphs had their own protein pattern, with specific spots in the two-dimensional electrophoresis maps (strategy-signal); (3) lizards responded differently to the proteins from an unfamiliar males (detectable and informative signals). The final step introduced some unpredicted responses: while some parts of the protein signal were, as expected, seasonally stable, some others were not, and varied according to the lipids content. This outcome requires a more complex hypothesis about the protein roles, which will remain speculative until a clear protein identification will be attained. Unfortunately, the identification attempts I performed during the different research steps failed, due to the lack of specific databases against which to match spectrometry data. So, further work should focus on this specific point.

CHAPTER 1

General introduction

Animal signalling: identity, quality, and behavioural strategy

Ecological and social interactions among animal species have promoted the evolution of a great variety of communication systems and signalling (Johnstone, 1997a): calls of frogs and whales, bright colours of butterfly and birds, pheromones of ants and mice, electric cues of fishes, ritualized postures of lizards, all represent just a subsample of such variety. As a general definition, we can consider signals all those «*acts or structures produced by signallers, which evolved for the purpose of conveying information to recipients, such that the information elicits a response in recipients, and the response results in fitness consequences that, on average, are positive for both the signaller and the recipient*» (Laidre & Johnstone, 2013). In Iberian wall lizards (*Podarcis guadarramae*), males signal the goodness of their immune system to potential mates by enriching their scent with a precursor of vitamin D_3, a costly compounds obtained from diet (López & Martín, 2005). By preferring males with provitamin D_3-enriched secretions, females choose partners that will make their offspring more viable. Being more frequently chosen, these males will increase their progeny. This example of mate choice perfectly matches the definition: the signal elicits a response in the recipient, which confers a fitness advantage to both the signaller (male) and the receiver (female).

A key point in the evolution of animal signalling is its social function, which determines the information content and the structure of the trait used as signal (Beecher, 1989; Alberts, 1992; Johnstone, 1997a; Ossip-Klein *et al.*, 2013). A cue that informs about the signaller's quality is expected to show a different pattern of variation, and to originate through a distinct selective mechanism, compared to a signal of group membership (Tibbetts *et al.*, 2017). In the former case, the trait variation should be continuous, with unimodal distribution, and correlated to other quality-related traits (Dale, Lank & Reeve, 2001); further, it should be under strong directional selection (Kingsolver *et al.*, 2001). On the contrary, a group-membership signal is expected to be multimodal, with as many modes as the number of groups, to show a little within-group variability, and to be uncorrelated to quality traits, and to undergo negative frequency-dependent selection (Sinervo & Svensson, 2002).

Among the possible information a signaller may be interested in conveying to a recipient, three are particularly relevant, as they are often used in intraspecific communication to drive decision-making processes (Tibbetts *et al.*, 2017): identity, quality, and strategy. This information may overlap in a single cue, but their different nature let them be recognizable in the signal structure and design (Dale *et al.*, 2001; Tibbetts *et al.*, 2017).

Identity signals allow conspecific to recognize and/or discriminate among individuals. They are expected to evolve when the signaller pays the cost of being confused (Johnstone, 1997b; Tibbetts & Dale, 2007), which is quite common in those social context where individuals may interact repeatedly, i.e., to establish spatial or subordination relationships, neighbourhood dynamics, or cooperative interactions like pair-bonding (Tibbetts & Dale, 2007). Many examples of such signals exist both in vertebrates and invertebrates, and considering the different communication modalities. In the paper wasp (*Polistes fuscatus*) individual recognition is mediated by the facial and abdominal patterns, a combination of yellow and black marks (Tibbetts, 2002), and it is used by nest-mates to maintain an established hierarchy and reduce reciprocal aggressiveness. Similarly, cofounding queens of the ant *Pachycondyla villosa* reduce their aggressive interactions through individual recognition based on the cuticular chemical profiles (D'Ettorre & Heinze, 2005). Among vertebrates, the little brown bat (*Myotis lucifugus*) is able to recognize conspecifics individually by their calls (Kazial, Kenny & Burnett, 2008), while mice (*Mus domesticus*) have evolved a set of urinary proteins (MUP; Major Urinary Proteins) specifically aimed at signalling mice identity (Hurst *et al.*, 2001; Mucignat-Caretta & Caretta, 2014). The common denominator of all the above cases is the design of the signal, which is characterized by: a high among-individual variability; an ontogenetic stability; a genetic determination; a low cost of production and maintenance, and, consequently, no relation to condition and quality traits (Dale *et al.*, 2001).

Quality signals are used to inform conspecifics about the signaller's worth, i.e., its genetic constitution, physical condition, immunocompetence, abilities (Searcy & Nowicki, 2005). They are typically associated to sexual selection, and higher quality signals correlate to higher probability for

being chosen as mate or for winning intrasexual contests (Andersson & Simmons, 2006). For example, male house finches (*Haemorhous mexicanus*) bearing bright red colouration are preferred by females, and show a better ability in feeding their mate and, later, offspring (Hill, 1991). The rattle in male barn swallows (*Hirundo rustica*) songs reflects the condition and testosterone level of the signaller, and is used in intrasexual competition (Galeotti *et al.*, 1997). The amount of cholesterol in the scent of male Iberian rock lizards (*Iberolacerta monticola*) is used to assess rivals' fighting abilities (Martín & López, 2007). Independently from the recruited channel, quality signals share three main properties: i) they are less variable than identity signals (Sheehan & Tibbetts, 2010); ii) they have high cost of production; iii) they are strongly related to condition and context (Dale *et al.*, 2001; Tibbetts *et al.*, 2017).

A third kind of information conveyed by intraspecific signals is strategy, i.e., the signaller's behavioural type chosen among a discrete, and equally viable, set of alternative behavioural categories (Tibbetts *et al.*, 2017). For example, male side-blotched lizards (*Uta stansburiana*) belong to three different colour morphs (Sinervo & Lively, 1996), each corresponding to a territorial strategy: orange-throated males are ultra-dominant, defend large territories, and mate with all females who enter their home range; blue-throated males are mate-guarder, defend smaller territories and watch over a single female at a time; yellow males are "sneakers", defend no territory, resemble females, enter territories of orange males, and mate opportunistically. Females modulate their mate choice with flexible rules depending on morph frequencies, and are informed about the strategy of males by the throat colour (Alonzo & Sinervo, 2001). Besides the cases of colour polymorphism (Wellenreuther, Svensson & Hansson, 2014), also marks of sexual recognition can be considered "strategy signals", as they inform conspecifics about the signaller's reproductive tactic, which is unrelated to its identity or quality (Tibbetts *et al.*, 2017). Males of the African electric fish *Pollimyrus isidori* can distinguish females from males basing on their electric cues alone, and start courtship or aggressive behaviour accordingly (Crawford, 1991); the same occurs in the damselfly (*Ischnura elegans*), where males use chemical cues to discriminate

conspecifics' sex and modulate their behavioural response (Frati *et al.*, 2015). Whatever the context, the common features characterizing a strategy signal may be synthetize as follows: i) showing a multimodal frequency distribution, each mode matching a strategy, with low variation within mode (Tibbetts *et al.*, 2017); ii) being highly heritable, as predicted by the definition of strategy itself (Calsbeek, Hasselquist & Clobert, 2010; McKinnon & Pierotti, 2010); iv) having a low correlation with signaller' condition, since individual quality is assessed within each strategy, and not among them (McKinnon & Pierotti, 2010; Cuthill *et al.*, 2017).

Even if the three kinds of information (identity, quality, strategy) are independent among each other, they can be encoded in the same communication channel (Johnstone, 1996; Dale *et al.*, 2001). It has been shown, for example, that in the passerine bird *Queleas queleas* (red-billed queleas) the appearance of head and breast are able to code for them all (Tibbetts *et al.*, 2017): the combination of mask brightness and breast hue (from yellow to red) is used for individual recognition of males; the beak redness is used to inform about male quality; the discrete colouration of the beak (red or yellow) is used to discriminate between aggressive and territorial males (red) and non-territorial females (yellow). Hence, also in this case, the different information contents of the signal and their social function are better predicted by analysing the pattern of variation, the cost for production and maintenance, and the condition-dependence of the signal itself (Dale *et al.*, 2001; Ossip-Klein *et al.*, 2013; Tibbetts *et al.*, 2017).

Communication modalities and chemical cues

A variety of channels or modalities may be used in animal communication. A modality is the sensory channel through which information passes from the signaller to the recipient (Marler, 1967), and three main groups can be identified according to the physical nature of the medium (Shorey, 1976): chemical (olfaction and taste), mechanical (tactile or sonic), and radiational (visual *s.l.* or electric). These major communication channels occur in widely diverse groups of animals, from protozoans through the complex higher vertebrates. The adoption of a specific modality within

a given group or species depends upon its evolutionary history, and the signal is shaped by the combination of information content, context and physical environment (Endler, 1992; Ossip-Klein *et al.*, 2013).

Among the different modalities, chemical communication represents the most ancient and widespread cue animals use to gain and transfer information to conspecifics (Shorey, 1976). Mate choice, recognition of rivals, neighbours, or kin, evaluation of genetic quality, reproductive status, condition, dominance, fighting abilities, etc., are all examples of situation where intraspecific information transfer involves chemical signals (Endler, 1993; Martín & López, 2015; Tibbetts *et al.*, 2017). Differently from the others (visual, acoustic, tactile, electric), the chemical communication channel is based on molecules movement, hence bearing some unique properties: it is not instantaneous, nor necessarily synchronous with the signaller's presence (it may persist over time); and it may be highly specific (Wyatt, 2003). As a consequence, the chemical modality is particularly suitable in those social contexts where communicating individual identity or the behavioural strategy plays an important role (Johnstone, 1997b; Dale *et al.*, 2001; Tibbetts *et al.*, 2017).

Following the general scheme of communication, also intraspecific chemical signalling requires a signaller (who sends the message), a receiver (who gets the message), and a medium (the semiochemical, i.e., the substance used as signal) (Shorey, 1976; Wyatt, 2003; Bradbury & Vehrencamp, 2011). A variety of *ad hoc* secretory organs has independently evolved in different taxa to produce semiochemicals, usually by specialization of already existing structures: e.g., facial glands of pinnipeds (Hardy, Roff & Smith, 1991), sternal glands of male Koalas (Salamon & Davies, 1998), uropygial gland of ducks (Caro & Balthazart, 2010), Dufour's gland of Imenoptera (Jackson & Morgan, 1993), pheromone glands of gypsy moths (Hollander, Yin & Schwalbe, 1982), head-wart epithelium of the snail *Euhadra peliomphala* (Takeda & Tsuruoka, 1979). On the receiver side, receptors are usually pre-adapted structures (mainly olfactory-like), originally devoted to detect environmental chemicals, which still maintain their primary function (Shorey, 1976;

Wyatt, 2003). Similarly, semiochemicals have usually evolved from pre-existing compounds, belonging to different chemical categories (hydrocarbons, lipids, proteins), which have been selected for communicating based on their ability to elicit the receiver's sensory system (Wyatt, 2003; Bradbury & Vehrencamp, 2011), accounting for the environmental features (Wyatt, 2014).

Given the possible social and ecological contexts, the species life-history traits, the arrangement of secretory structures, semiochemicals, and receptors, a huge variety of intraspecific chemical communication pathways may evolve, still maintaining the same general features unique to the chemical modality.

Chemical signalling in lizards

Lizards represent an ideal models to study chemical communication. Even though also the other channels are used (Fox, McCoy & Baird, 2003), the chemical one is strengthened by the development of two specific structures devoted at sending and receiving chemical signals, respectively (Schwenk, 1995; García-Roa *et al.*, 2017).

Like all squamates, lizards have an additional sophisticated system for chemoreception, constituted by the combination of vomeronasal organ (VNO) and tongue flicking behaviour: the tongue extrusion is used to sample chemicals occurring on the substrate or in the air, which are then delivered to the paired VNO openings above the roof of the mouth (Schwenk, 1995). "Vomerolfaction" is functionally, but not anatomically, linked to the main olfactory system (Keverne, 1999), which may serve to drive tongue-flicking behaviour when more volatile chemicals has been detected by the olfactory system (nares and nasal organs) (Schwenk, 1995; Halpern & Martínez-Marcos, 2003). Molecules delivered in the VNO openings reach the lumen where they are detected by the sensory epithelium, which, after transduction, relays information via the accessory olfactory nerves to the accessory olfactory bulbs of the telencephalon (Rehorek, Firth & Hutchinson, 2000). In some cases, it has been demonstrated that vomerolfaction takes precedence over olfaction and gustation (Cooper & Alberts, 1991).

In parallel, most lizard species has developed specialized exocrine epidermal glands in the cloacal region (pre-cloacal glands) or along the inner part of the thighs (femoral glands) (Cole, 1966; Mayerl, Baeckens & Van Damme, 2015). They originate from the invagination of the *stratum germinativum*, and maintain their contact with the external epidermis through a duct (Imparato *et al.*, 2007). Glands vary their position, morphology, and number among the different lizard groups (Baeckens *et al.*, 2015; Mayerl *et al.*, 2015; García-Roa *et al.*, 2017), though conserving their holocrine nature: secretory cells, after undergoing a four-stages differentiation, produce a solid plug, which protrudes externally, and is gradually consumed during lizard motion (Cole, 1966; Khannoon, Dollahon & Bauer, 2013; Mayerl *et al.*, 2015). Plugs have a waxy appearance, being made of a mixture of proteins and lipids (Alberts, 1990; Alberts, Pratt & Phillips, 1992a; Escobar, Labra & Niemeyer, 2001), in variable proportion: 80% proteins and 20% lipids in the desert iguana (*Dipsosaurus ornatus*) (Alberts, 1990); from 65% to 87% proteins in the green iguana (*Iguana iguana*) (Alberts *et al.*, 1992b); 32.5% protein in *Liolaemus belli* (Escobar *et al.*, 2001). Glands are more active in males, being often vestigial in females, and are under androgen control: after providing testosterone, females' glands start secreting, while they almost stop the activity in castrated males (Padoa, 1933; Fergusson, Bradshaw & Cannon, 1985). Furthermore, secretion amount positively correlates with testosterone level (Alberts *et al.*, 1992a; Baeckens *et al.*, 2017), and typically varies according to the time of the year, with a peak during the breeding season, and an abrupt drop afterwards (Padoa, 1933; Fergusson *et al.*, 1985; Alberts *et al.*, 1992a).

The characteristics of the femoral (or pre-cloacal) glands have suggested they play an important role in intraspecific chemical communication, notably in sexual communication (Padoa, 1933; Fergusson *et al.*, 1985; Mayerl *et al.*, 2015). Indeed, it has been demonstrated that secretions are actually used as chemical cues in inter- and intrasexual interactions: females are able to choose males on the basis of the chemical scent from femoral glands alone (López & Martín, 2005; Gabirot, Lopez & Martín, 2013); males, for their part, are able to use the same secretions to assess

rival fighting ability or identity (Alberts & Werner, 1993; López & Martín, 2001; López, Martín & Cuadrado, 2002a; Martín & López, 2007; Carazo, Font & Desfilis, 2008).

Proteins, lipids and signal design

In the last decades, lot of studies has tried to decode the whole information conveyed by lizard femoral secretions (Martín & López, 2011, 2015; Mayerl *et al.*, 2015). Surprisingly, almost all of them has focused on the lipophilic fraction, and has ignored proteins (Font *et al.*, 2012; Mayerl *et al.*, 2015), with the few exception of the promising works by Allison C. Alberts and colleagues (Alberts, 1990, 1991; Alberts & Werner, 1993; Alberts, Phillips & Werner, 1993) on the green and desert iguanas (*Iguana iguana* and *Dipsosaurus ornatus*). Consequently, an unbalanced knowledge exists about the two components of femoral gland secretions (Mayerl *et al.*, 2015).

The lipophilic fraction lists different chemical compounds (e.g., steroids, fatty acids, alcohols, esters, tocopherol, squalene; (Louw *et al.*, 2007; Weldon, Flachsbarth & Schulz, 2008; Khannoon, El-Gendy & Hardege, 2011; Martín *et al.*, 2011, 2013a; Martín, Ortega & López, 2013b; Martín *et al.*, 2015a; Khannoon, 2012; Jara *et al.*, 2018), which are typical precursors, products, or by-products of fats metabolism (Weldon *et al.*, 2008; Martín & López, 2015). Since these compounds accomplish or regulate many important physiological functions (e.g., immunological, antioxidant, endocrinal, sexual, accretive) (Martín & López, 2015) their occurrence in femoral secretions is costly, and thus can be used as a reliable and honest proxy of individual quality (Zahavi, 1975; Martín & López, 2015; Tibbetts *et al.*, 2017). The ability of specific lipids from femoral secretions to transfer quality-related information has been experimentally proved in several species. For example, in the lacertid *Iberolacerta monticola*, the amount of ergosterol (provitamin D_2) in the secretion is related to male quality (immunity and asymmetry), and females consistently prefer territories marked by male scent enriched with ergosterol (Martín & López, 2006), supporting the hypothesis that it conveys information about male quality. On the male side, cholesterol was found to correlate with dominance and fighting ability (Martín & López, 2007) and experimental trials

found that the artificial increase of cholesterol content induces avoidance behaviour in conspecific males of *I. monticola* and *Acanthodactylus boskianus* (Khannoon *et al.*, 2011). Further, the variability of lipid profiles according to season (Alberts *et al.*, 1992b), environmental features (Gabirot, López & Martín, 2012; Heathcote *et al.*, 2014; Martín, Ortega & López, 2015b), androgen levels (Baeckens *et al.*, 2017), health status (López, Amo & Martín, 2006; Martín, Amo & López, 2008), and male condition (López, Muñoz & Martín, 2002b; Carazo, Font & Desfilis, 2007), agrees with what is expected for a quality signal (Dale *et al.*, 2001; Tibbetts *et al.*, 2017).

On the contrary, no data is available about the composition of the protein fraction, and its keratinous nature has been just speculated (Padoa, 1933; Cole, 1966). Similarly, the few information about their potential function comes from three studies having analysed the proteins pattern of variation through one-dimensional electrophoresis (Alberts, 1990, 1991; Alberts *et al.*, 1993), and from the side outcomes of a behavioural experiment that tested the ability of iguanas to discriminate the familiarity of a conspecific by means of chemical cues (Alberts & Werner, 1993). The analysis of patterns showed that they are stable across time (Alberts, 1990), and are characterized by a structured variability, i.e., patterns vary among species (Alberts, 1990, 1991), between sexes (Alberts *et al.*, 1993), between relatives and non-relatives (Alberts *et al.*, 1993), and among individuals (Alberts, 1990, 1991; Alberts *et al.*, 1993). In green iguanas (*Iguana iguana*), the protein fraction seems also able to elicit tongue-flicking more than lipids do (Alberts & Werner, 1993), supporting the hypothesis that they can be actually detected by VNO, as already demonstrated for proteins of preys (Cooper, 1991). Altogether, these observations support the potentiality for the femoral gland proteins to be actually used as signals and, notably, to convey identity or strategy information (Tibbetts *et al.*, 2017), as already happens in other taxa (Lazar *et al.*, 2004; Touhara, 2008; Wyatt, 2014). The observed pattern of variation, indeed, fits the predictions for both types of signals: great variability and/or multimodal distribution, high genetic determination, and cheap production (Tibbetts *et al.*, 2017).

Seen in this light, the two components of femoral secretions may build up a complete signal, which simultaneously convey information about quality (lipids), identity, and strategy (proteins). This may be pivotal in territorial species like lizards (Fox *et al.*, 2003), because in such contexts quality-related information (important in decision-making processes like mate choice or conflicts modulation) needs to be associated to individual identity, since chemical signals are detected also without seeing or being in contact with the signaller. Further, when more than one strategy is played in a population, also this kind of information is expected to be conveyed, in order to allow conspecifics to tune their behaviour accordingly (e.g., in polymorphic species: assortative mating, or morph-specific aggressiveness) (Abalos *et al.*, 2016; Sacchi *et al.*, 2018a).

Common wall lizard: the model species

To investigate lizards chemical communication, I focused on the Common Wall lizard (*Podarcis muralis*), as model species.

P. muralis is a small-to-medium-sized lacertid lizard (adult male total length 16-23 cm; females are smaller) (Corti & Lo Cascio, 2002), widespread in central and southern Europe, spanning from northern Spain to Turkey (Sillero *et al.*, 2014). It is phenotypically quite variable within its geographic range, especially in colouration, being dorsally brownish or greenish, with stripes or reticulated black motives (Corti & Lo Cascio, 2002). Many clades has been identified, highlighting a large within-species genotypic diversity (Giovannotti, Nisi-Cerioni & Caputo, 2010; Schulte *et al.*, 2012; Salvi *et al.*, 2013). It is a generalist, feeding mainly on ground invertebrates (Corti & Lo Cascio, 2002; Scali *et al.*, 2016), and able to occupy a variety of habitats, even urban, characterized by the occurrence of vertical surfaces (walls, rocks, trees) (Corti & Lo Cascio, 2002; Lazić *et al.*, 2013; Sacchi *et al.*, 2018b). It is sexually dimorphic, being males longer, heavier, with larger head and shorter trunk compared to females (Sacchi *et al.*, 2015b). During the mating season (April - June) males become territorial and aggressive, and male-male combats can be easily observed (Corti & Lo Cascio, 2002; Sacchi *et al.*, 2009).

The Common Wall lizards shows a ventral colour polymorphism in both sexes (Sacchi *et al.*, 2013), with three distinct morphs characterized by red, white, and yellow belly and throat, respectively (Sacchi *et al.*, 2013). Also intermediate colours can be observed, resulting from the additive effect of two main morphs at a time (Sacchi *et al.*, 2013). The colour morphs represent alternative strategies played along trade-offs of life-history traits (Calsbeek *et al.*, 2010), involving reproduction (Galeotti *et al.*, 2013), physiology (Sacchi *et al.*, 2007, 2017; Galeotti *et al.*, 2010), behaviour (Pérez i de Lanuza, Font & Carazo, 2013; Pérez i de Lanuza, Font & Carretero, 2016; Scali *et al.*, 2013; Sacchi *et al.*, 2018a), and ecology (Scali *et al.*, 2016; Perez i de Lanuza & Carretero, 2018; Pérez i de Lanuza, Sillero & Carretero, 2018). Colour polymorphism has been also found to weakly affect the composition of the lipophilic fraction of the femoral gland secretions (Pellitteri-Rosa *et al.*, 2014).

Concerning the intraspecific chemical communication and the role of femoral gland secretions, the species has been the target of many studies which, on the one side, have already characterized the composition of the lipophilic fraction (Martín *et al.*, 2008; Heathcote *et al.*, 2014; Pellitteri-Rosa *et al.*, 2014; Baeckens *et al.*, 2017; MacGregor *et al.*, 2017), and, on the other side, have allowed highlighting the importance of the chemical modality in this species (Sacchi *et al.*, 2015a; While *et al.*, 2015; Heathcote *et al.*, 2016). Therefore, the suitability of the Common Wall lizard as model species for investigating the potential role of proteins in chemical communication seems justified, since this lizard shows: (1) phenotypic variability at different level (individual, population, clade); (2) different strategies played within population (colour morphs); (3) social context which promotes identity, quality and strategy signalling; (4) preference for the chemical channel.

Thesis outline

The general aim of my thesis is to find support to the hypothesis that proteins from femoral gland secretions of the Common Wall lizard play a communication role. Notably, given their nature, I

expect proteins may convey at least two kinds of information: individual identity, and colour morph (i.e., morph strategy).

To test my hypothesis, in **Chapters 2** and **3** I analyse the pattern of variations in the protein content in order to assess if it agrees with what is expected from a signal that conveys identity (**Chapter 2**) or strategy (**Chapter 3**). I use 1-dimensional and 2-dimensional gel electrophoreses, which allow characterizing the variability of the pattern, and high performance liquid chromatography and mass spectrometry for proteins and peptides identification.

Specifically, in **Chapter 2**, I investigate the sources of variability of the protein patterns by comparing samples from individuals coming from same/different populations or clades, and searching for a correlation between protein composition and level of genetic similarities, a complexity in the signal, and an independence from quality traits.

In **Chapter 3** I compare the protein pattern of the three main colour morphs, trying to establish the occurrence of a morph-specific protein expression, and identify such differential proteins.

In **Chapter 4** I set up a behavioural test to check if proteins alone allow male lizards to discriminate between their own scent and that of an unfamiliar males. A behavioural approach is a fundamental step to obtain experimental evidence for the protein communication role, and corroborate the results from the correlative analyses on protein patterns.

Finally, in **Chapter 5**, I study the co-variation of the two components of the signal (lipids and proteins) over the activity season, while controlling for the effect of testosterone, which promotes glandular activity. Under my hypothesis of conveying identity- and strategy-related information, proteins should not respond to testosterone and should not be correlated to lipids.

At the end, in **Chapter 6**, I sum up the main findings of my thesis and put them into a broader context including potential avenues for future research.

The Chapters from 2 to 5 are thought as stand-alone manuscripts, ready for publication. Therefore, some repetitions cannot be avoided, and the overall structure may follow a too self-

supporting rationale. In all these chapters I am the primary author and principle contributor, and all co-authors are informed and gave their permission to include the manuscript in the thesis. **Chapter 2** and **4** are published journal articles: chapter 2 is included according to the journal policy (CC BY-NC 4.0); chapter 4 is the accepted version of the published manuscript (The final publication is available at link.springer.com). These are:

Mangiacotti, M., Fumagalli, M., Scali, S., Zuffi, M.A.L., Cagnone, M., Salvini, R. & Sacchi, R. (2017). Inter- and intra-population variability of the protein content of femoral gland secretions from a lacertid lizard. *Current Zoology* **63**, 657–665.

Mangiacotti, M., Gaggiani, S., Coladonato, A.J., Scali, S., Zuffi, M.A.L. & Sacchi, R. (2019): First experimental evidence that proteins from femoral glands convey identity-related information in a lizard. *Acta Ethologica*. doi: 10.1007/s10211-018-00307-1.

Chapters 3 is a submitted article:

Mangiacotti, M., Fumagalli, M., Scali, S., Zuffi, M.A.L., Cagnone, M., Salvini, R. & Sacchi, R. (*under revision*). Morph-specific proteins in the femoral gland secretions of a colour polymorphic lizard. *Scientific Reports*.

Finally, **Chapter 5** is a ready-to-submit manuscript:

Mangiacotti, M., Pezzi, S., Balestrazzi, L., Fumagalli, M., Coladonato, A.J., d'Ettorre, P., Bonnet, X., Zuffi, M.A.L., Scali, S. & Sacchi, R. (*in prep.*). Seasonality of intraspecific chemical communication in lizards: a protein story.

CHAPTER 2

Inter- and intra-population variability of the protein content of femoral gland secretions from a lacertid lizard

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Article

Inter- and intra-population variability of the protein content of femoral gland secretions from a lacertid lizard

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Abstract

Femoral glands of male lizards produce waxy secretions that are involved in inter- and intraspecific chemical communication. The main components of these secretions are proteins and lipids, the latter having been extensively studied and already associated to male quality. On the opposite, the composition and role of proteins are nearly unknown, the only available information coming from few studies on iguanids. These studies got the conclusion that proteins might have a communicative function, notably they could signal individual identity. A generalization of these findings requires the extension of protein analysis to other lizard families, and the primary detection of some patterns of individual variability. Using the common wall lizard *Podarcis muralis* as a model species, the protein fraction of the femoral pore secretions was investigated to provide the first characterization of this component in a lacertid lizard and to explore its source of variability, as a first step to support the hypothesized communicative role. Samples of proteins from femoral secretions were collected from 6 Italian populations and subjected to 1-dimensional electrophoresis. The binary vector of the band presence/absence was used to define the individual profiles. Protein fraction is found to have a structured pattern, with both an individual and a population component. Although the former supports the potential communicative role of proteins, the latter offers a double interpretation, phylogenetic or environmental, even though the phylogenetic effect seems more likely given the climatic resemblance of the considered sites. Further studies are necessary to shed light on both these issues.

Key words: chemical communication, femoral glands, lizards, *Podarcis muralis*, proteins, SDS-PAGE.

Chemical communication is among the most primitive and widespread way to obtain and transfer information in the animal kingdom (Bradbury and Vehrencamp 2011). Lizards do not make an exception and the chemical pathway has been favored by the acquisition of a highly specialized chemosensory system (i.e., the vomeronasal system) (Cooper 1994; Schwenk 1995) and by the development of specialized epidermal glands (Mayerl et al. 2015). Notably, some lizard species have 2 series of glands along the ventral side of the thighs or proximal to cloaca which open outside through modified scales (femoral pores) and produce waxy secretions passively or actively left on the substrate (Gabe and Saint Girons 1965; Cole 1966). Femoral pores are sexually dimorphic,

being appreciably reduced and often vestigial in females (Padoa 1933; Cole 1966), and their development and activity follow plasma testosterone concentration (Padoa 1933; Forbes 1941; Fergusson et al. 1985; van Wyk 1990; Alberts et al. 1992a; Baeckens et al. 2016), with a productivity peak in the breeding season (Padoa 1933; Cole 1966; Alberts et al. 1992b; Martín and López 2015). Consequently, their biological role has been immediately associated to reproduction, although a variety of speculations about their exact function (e.g., fastening male to female during copulation, quieting females, marking territories, facilitating sexes pairing; Cole 1966) have been raised. Since Cole's review (1966), several studies have investigated the semiochemical properties of these secretions and it is now accepted that they play an important role in the lizard communication system (Martín and López 2015; Mayerl et al. 2015). Nevertheless, the decryption of the chemical code is still ongoing and a comprehensive framework about this topic is even lacking (Martín and López 2015).

Femoral gland secretions are made of an unbalanced mixture of proteins and lipids (Cole 1966; Alberts 1990; Escobar et al. 2001; Weldon et al. 2008), the former being sometimes the most abundant component (e.g., 80% in *Dipsosaurus ornatus*; Alberts 1990). The lipophilic fraction comprises different chemical compounds (e.g., steroids, fatty acids, alcohols, esters, tocopherol, squalene; Louw et al. 2007; Khannoon et al. 2011b; Martín et al. 2011, 2013a, 2013b, 2015; Khannoon 2012), which are typical precursors, products, or byproducts of fat metabolism (Weldon et al. 2008; Martín and López 2015). Since these compounds accomplish or regulate many important physiological functions (e.g., immunological, antioxidant, endocrinal, sexual, accretive; Martín and López 2015), their occurrence in femoral secretions imposes a cost to the emitter, and thus can be used as a reliable and honest proxy of individual quality (Zahavi and Zahavi 1999; Martín and López 2015). The ability of specific lipids from femoral secretions to transfer quality-related information has been experimentally proved in behavioral tests with manipulated scents. For example, in the lacertid *Iberolacerta monticola*, Martín and López (2006) found that the amount of ergosterol (provitamin D2) in the secretion is related to male quality (immunity and asymmetry), and females consistently prefer territories marked by male scent enriched with ergosterol, supporting the hypothesis that ergosterol mediates information about male quality. On the male side, cholesterol was found to correlate with dominance and fighting ability (Martín and López 2007) and experimental trials found that the artificial increase of cholesterol content induces avoidance behavior in conspecific males of *I. monticola* (Martín and López 2007) and *Acanthodactylus boskianus* (Khannoon et al. 2011a). Further, the high variability of lipid profiles, depending on season (Alberts et al. 1992b), environmental features (Gabirot et al. 2011; Heathcote et al. 2014; Martín et al. 2015), androgen levels (Baeckens et al. 2016), health (López et al. 2006; Martín et al. 2008), and male condition (López et al. 2002; Carazo et al. 2007), agrees with the hypothesis that lipophilic fraction mainly signals quality and condition (Martín and López 2015; Mayerl et al. 2015; but see Pellitteri-Rosa et al. 2014).

Proteins are known to be used as chemical signal in other vertebrates (elephants, Lazar et al. 2004; rodents, Wyatt 2014; newts and frogs, Touhara 2008). In reptiles, they represent a significant fraction of the femoral gland secretions, spanning from 32.5% by mass in *Liolaemus* sp. (Escobar et al. 2001), to 87% in *Iguana iguana* (Alberts et al. 1992b). Surprisingly, both their composition and their function have been poorly investigated in lizards (Font et al. 2012; Mayerl et al. 2015). The only studies (as far as we are aware) that analyzed the protein component have been carried out by Alberts and colleagues (Alberts 1990, 1991, 1992; Alberts and Werner 1993; Alberts et al. 1993), who focused on iguanas (*Dipsosaurus dorsalis* and *I. iguana*) and showed that: i) lizards were able to detect the protein fraction of the femoral secretions (Alberts et al. 1993) and can discriminate familiar and unfamiliar conspecifics on this basis (Alberts 1992; Alberts and Werner 1993); ii) the mono-dimensional electrophoretic patterns obtained by different individuals showed a structured variability, that is, patterns vary among species (Alberts 1990, 1991), between sexes (Alberts et al. 1993), between relatives and non-relatives (Alberts et al. 1993), and among individuals (Alberts 1990, 1991, 1993); iii) protein profiles seem to be stable across seasons (Alberts 1990). Altogether, these observations suggest the potentiality for the femoral gland proteins to be actually used as semiochemical and, notably, to transfer information about individual identity (e.g., species, population, sex, kinship, etc.; Alberts 1990; Alberts et al. 1993; Mayerl et al. 2015), as it already happens in other taxa (Wyatt 2014). Unfortunately, such a hypothesis was based on studies that have never been replicated in other lizard families and needs further support and greater generalization (Mayerl et al. 2015).

Over the last 30 years wall lizards (Lacertidae Gray, 1825) have been often used as animal models to address many different ecological, behavioral, and evolutionary issues (e.g., Van Damme and Verheyen 1990; Martín and López 1999; Carazo et al. 2007; Calsbeek et al. 2010b; Font et al. 2012; While et al. 2015). In this context, the studies on the femoral gland secretions have gained more and more popularity (Martín and López 2011, 2015; Font et al. 2012; Mayerl et al. 2015), but they have been always focused on the lipophilic fraction of the secretions, without considering the protein component (Mayerl et al. 2015). If proteins would actually be used to signal identity-related information, their exclusion from the analysis could have led to incomplete interpretations of the observed outcomes. So, to start filling the gap, the present study aims to: i) give a preliminary characterization of the femoral gland proteins in a lacertid lizard; ii) evaluate the occurrence of intra- and inter-populations variability in the protein patterns. The occurrence of some kind of variability represents a necessary prerequisite (even though not sufficient *per se*) to sustain the hypothesis of the communicative function of proteins, since without chemical variation one cannot diversify information (Beecher 1989; Tibbetts and Dale 2007). As model species we chose the common wall lizard *Podarcis muralis*, a small lacertid widespread in southern, central, and western Europe, which has been already used in many previous studies (e.g., Calsbeek et al. 2010a; Lazić et al. 2013; Scali et al. 2013; Sannolo et al. 2014; Sacchi et al. 2015a, 2015b; While et al. 2015; Baeckens et al. 2016), also on femoral gland secretions (Martín et al. 2008; Heathcote et al. 2014; Pellitteri-Rosa et al. 2014; Baeckens et al. 2016). We focused on Italian populations, which show a great genetic diversity (6 recognized clades: Southern Alps, Tuscany, Venetian, Romagna, Marche, and Southern Italy; Giovannotti et al. 2010; Schulte et al. 2012; Salvi et al. 2013), which partially matches with the observed phenotypic variability (e.g., greenness in the dorsal coloration; While et al. 2015), and thus allow comparing protein patterns of variation in a highly diversified genetic and phenotypic context.

Materials and Methods

Study site and sampling

Femoral gland secretions of mature males were collected from 6 distinct populations belonging to 3 out of the 6 Italian clades of

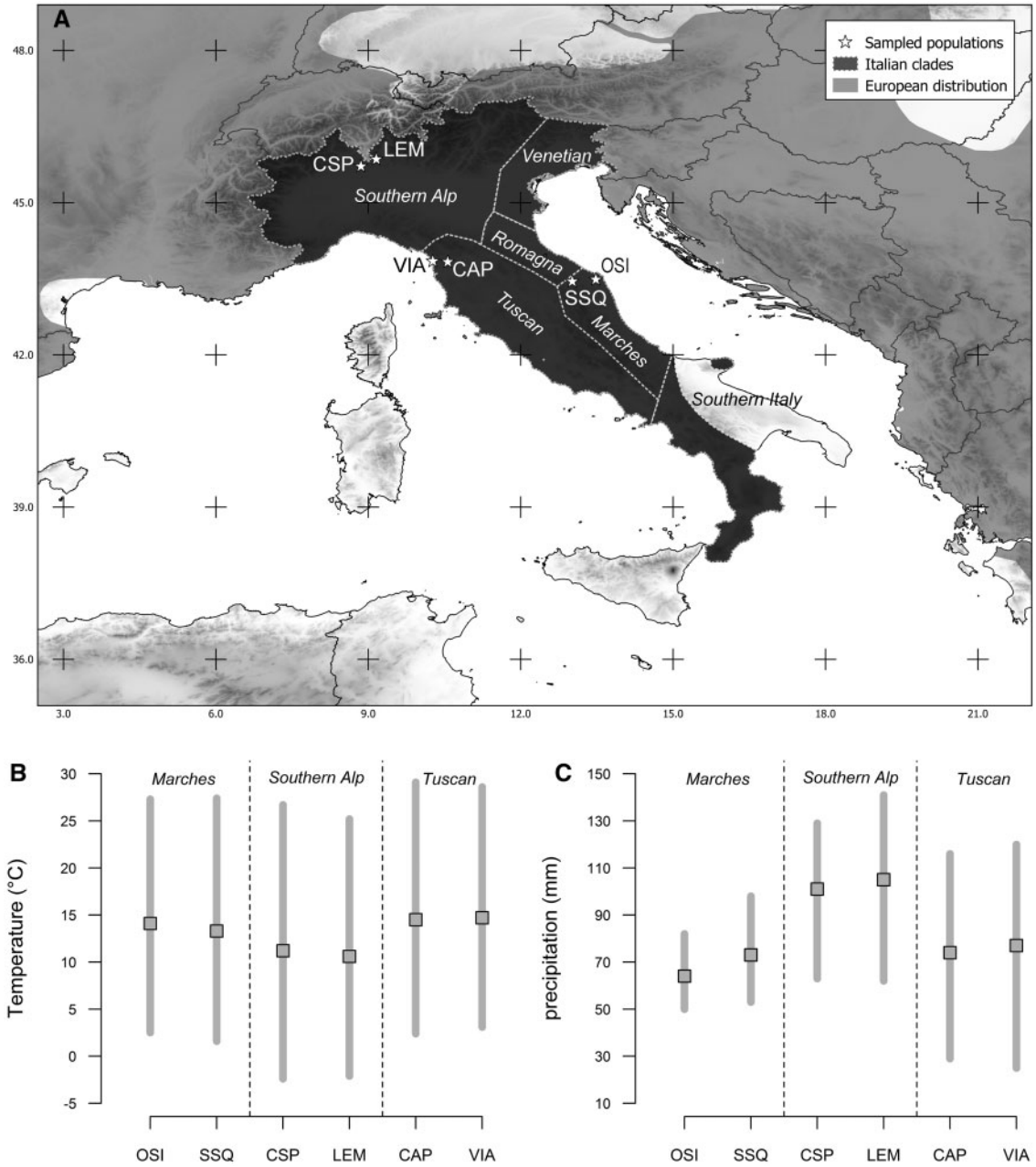

Figure 1. (**A**) Distribution map of Italian clades of the common wall lizard. The geographic delimitations of the clades follow Salvi et al. (2013). Stars represent the 6 considered populations, from North-West to South-East: Castelseprio (CSP), Lemna (LEM), Viareggio (VIA), Capannori (CAP), Serra San Quirico (SSQ), and Osimo (OSI). (**B**) Thermal characterization of the sampling sites: bars represent the annual temperature range (minimum of coldest month and maximum of warmest month); mean annual temperature is symbolised by squares. (**C**) Monthly precipitation variability: bars indicate the difference between the minimum and maximum precipitation of the driest and wettest month, while squares represent the mean monthly precipitation (annual precipitation/12). Climatic data were obtained from www.worldclim.org, ver. 1.4.

P. muralis (Figure 1A, Table 1): Southern Alps, Tuscan, and Marches clade, 2 populations each. The chosen clades represent 3 distinct lineages that express the 2 extreme phenotypes of the dorsal coloration (While et al. 2015): brownish (Southern Alp) versus greenish (Tuscan and Marches).

A general characterization of the climate of each site was obtained by the combination of 6 bioclimatic variables available at http://www.worldclim.org (last accessed: 15 July 2016) (Hijmans et al. 2005) as spatial raster at 30 arc second resolution: mean annual temperature (bio1), max temperature of warmest month (bio5), min temperature of coldest month (bio6), annual precipitation (bio12), precipitation of wettest month (bio13), and precipitation of driest month (bio14). These data were used to generate the plots of the temperature and precipitation for each site (Figure 1B,C).

Lizards were captured by noosing and measured for the snout-to-vent length (SVL) to the nearest 0.1 mm with a calliper. Samples of the femoral gland secretions from 5 to 10 lizards for each population were obtained by applying a gentle pressure around the thighs and collecting the protruding plugs directly into glass vials. Lizards were then released at the capture point and the vials transferred to

Table 1. Characteristics of the samples from the 6 considered populations

Site	Locality	Longitude	Latitude	Clade	n_{tot}	n_{eff}	SVL
OSI	Osimo	13.4785E	43.4884N	Marches	6	4	64.8 ± 2.5
SSQ	Serra San Quirico	13.0148E	43.4477N	Marches	5	4	67.0 ± 4.2
CSP	Castelseprio	8.8627E	45.7168N	Southern Alp	5	5	66.5 ± 1.2
LEM	Lemna	9.1586E	45.8584N	Southern Alp	8	6	70.5 ± 2.7
CAP	Capannori	10.5738E	43.8398N	Tuscan	5	5	63.5 ± 6.2
VIA	Viareggio	10.2715E	43.8506N	Tuscan	5	5	70.4 ± 5.9

Notes: n_{tot} = total number of individuals used in electrophoresis; n_{eff} = effective number of individuals that showed a clear protein pattern and were therefore used in the analysis; SVL = mean and standard deviation of the SVL (mm) based on n_{eff}. Longitude and latitude are in decimal degrees.

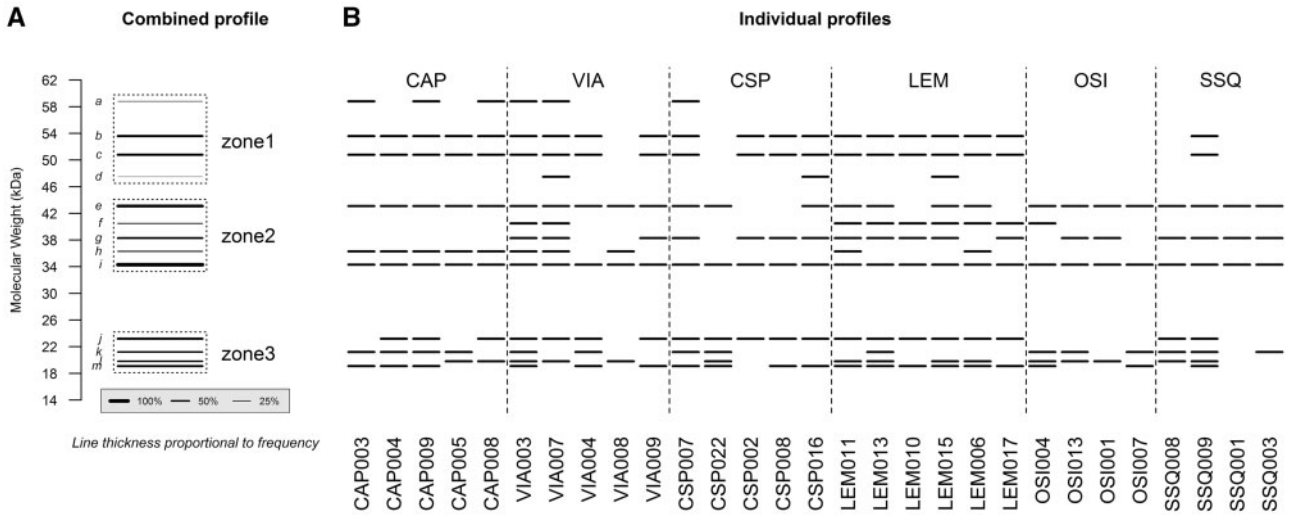

Figure 2. Schematized protein profiles after gel alignment and band detection. (**A**) Overall profile obtained by combining all the individual profiles: line thickness is proportional to the frequency of a band in the whole sample. (**B**) Individual profiles sorted by population of origin.

the laboratory and preserved at −20 °C until analyses (López and Martín 2005). Field work was conducted during spring 2014 and 2015.

Protein extraction and sodium dodecyl sulphate-PAGE analysis

Samples were defatted by incubation in n-hexane at room temperature for 24 h. After centrifugation, proteins (not dissolved in the organic solvent) were isolated as a pellet and air-dried. Protein pellets were dissolved in 50 mM Tris–HCl pH 6.8 containing 8 M Urea, 2% sodium dodecyl sulphate (SDS), 0.1% bromophenol blue, and 10% glycerol to obtain a final protein concentration of 1 μg/μL. To denaturate proteins, samples were incubated at 95 °C for 5 min. Electrophoretic runs were performed in a discontinuous mode (5% stacking gel and 12.5% running gel) by applying a constant voltage of 180 V for 1 h. Gels were stained with a 0.25% (w/v) Coomassie Blue R250 solution, containing 40% ethanol (v/v) and 10% (v/v) acetic acid. Once decolorated, gels were scanned and the obtained images (Appendix) individually passed in PyElph ver. 1.4 (Pavel and Vasile 2012) for band detection and alignment. From each gel image the following information were extracted: i) the binary matrix of band presence/absence; ii) the predicted band weights, estimated by a linear electrophoresis migration model applied to the lane of the standard molecular weights (Pavel and Vasile 2012).

The rows of the presence/absence matrix were compared with each other by the Sørensen similarity scores S (Sørensen 1948): for each lizards pair, the score corresponds to 2 times the number of shared bands divided by the total number of visible bands in the profile pair (Lynch 1990). This score can vary between 0 (no shared bands) and 1 (all bands are shared) and represents a conservative way to measure similarity (Lynch 1990). The pairwise similarity matrix was converted into a distance matrix by taking the squared root of $1–S^2$ (Legendre and Legendre 1998). Since the gel region below 17 kDa was partly contaminated by lipophilic residues which prevented clear band identification, we considered for the comparison only the region above this weight threshold (Appendix). Further, lanes with only 1 visible band were excluded from the analysis.

Statistical analysis

Within-population variability was evaluated by the direct comparison of the banding pattern and by the visual inspection of the score plot of the first principle coordinates axes generated by a principal coordinate analysis (PCoA) of the pairwise distance matrix (Legendre and Legendre 1998). The dispersion of the points is a measure of individual variability in protein profiles.

The among-populations variability was tested by a distance-based MANOVA (db-MANOVA; Anderson 2001) with the pairwise distance matrix as dependent, site as factor and SVL as covariate. SVL was used as a proxy to control for the amount of secretion and address possible quality-related effects on the protein occurrence. The homogeneity of dispersion required by the db-MANOVA was tested following Anderson (2006). The significance of the MANOVA was obtained via restricted permutations to take into account the potential error introduced by the non-simultaneousness of

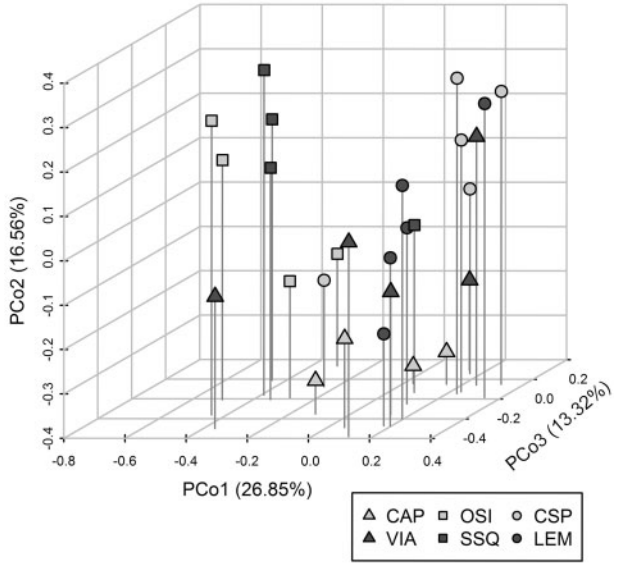

Figure 3. Principal coordinates analysis of the distance matrix computed on the individual protein patterns. The scores of the first 3 axes are used and the explained variance associated to each axis was reported as percentage. The shape of the symbols is clade-specific: triangles for Tuscan, squares for Marches, and circles for Southern Alp.

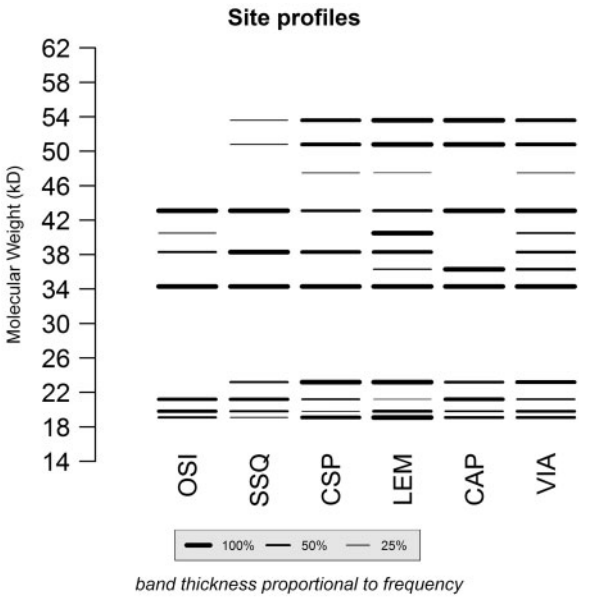

Figure 4. Weighted within-site protein profiles. The thickness of the bands is proportional to their frequency in each population.

the electrophoresis analysis: permutations were restricted to lanes within the same gel. All the analyses were performed in R 3.2.4 (R Development Core Team 2016), using vegan (Oksanen et al. 2015) and permute (Simpson 2015) packages.

Results

Out of the 34 samples loaded on gel (Table 1), 29 showed a clear banding, with more than 1 visible band, and therefore were considered in the analyses. Three samples were excluded due to bad coloration of the lanes (LEM028 from gel No. 1; LEM022 from gel No. 2A and SSQ011 from gel No. 2B; Appendix), while 2 more samples (OSI019 and OSI021 from gel No. 2B; Appendix) were not considered to avoid inflating false negative rate in band detection since they showed only 1 visible band.

The gel region corresponding to molecular weight larger than 17 kDa had a total of 13 identifiable band clusters, arranged in 3 distinct groups (Figure 2A): the first zone counted 4 bands with molecular weight ranging between 47.5 and 58.8 kDa: band *a* was clearly observable only in the first gel, and it was consequently excluded from the computation of the pairwise similarity score; bands *b* and *c* were widely shared among samples, while band *d* was rarer. The second zone comprised 5 bands between 34.3 and 43.1 kDa, with variable occurrence frequency: bands *e* and *i* were very common and the latter was the only 1 detected in all the lanes. The 4 bands in the third zone were quite near each other, ranging between 19.1 and 23.2 kDa, with almost equal occurrence frequency, with the exception of band *k*, which was less frequent.

An individual variability in the profiles was detectable directly in the original gels (Appendix), where both the occurrence and the intensity of the bands varied among individuals. The inspection of the schematized presence/absence pattern (Figure 2B) confirmed the same outcome, with only 1 pair of lanes that reproduced exactly the same scheme (LEM010 and LEM017; Figure 2B). Also the PCoA ordination (Figure 3) highlighted the occurrence of a within-population variability, most of which loaded by zones 2 and 3 (Figure 2A,B). Since the test of homogeneity of dispersion was not significant ($P > 0.39$; number of permutations = 9999), the within-population variability had to be assumed equal among the 6 study sites.

The db-MANOVA found a significant difference among populations (pseudo-$F = 2.97$; $P \leq 0.0001$; number of permutations: 9999), while SVL seemed having no effect on the protein pattern (pseudo-$F = 1.10$; $P > 0.29$). The factor "site" accounted for 39.15% of the total observed variation in the protein patterns. The most easily distinguishable populations were those from the Marches clade (Figure 4), where zone 1 was poorly represented (completely absent in OSI population). Viareggio (VIA) and Lemna (LEM) showed the highest level of banding complexity and the distinction between populations was based on banding frequency. Capannori (CAP) and Castelseprio (CSP) represented an intermediate case: CAP lacked bands *d*, *f*, and *g*; CSP missed bands *f* and *h*.

Discussion

Although a great number of studies focused on the role of femoral secretions in the Lacertidae family and demonstrated their importance in chemical signaling (Font et al. 2012; Martín and López 2015), they all focused solely on lipids as reference compounds (López and Martín 2005, 2006; Gabirot et al. 2008; Khannoon et al. 2011b, 2013, Martín et al. 2013a, 2015, 2016b; García-Roa et al. 2016). The present study analyzed for the first time the protein fraction of the femoral gland secretions in a model lacertid species, the common wall lizard, looking for indirect support to the hypothesis that also proteins may play a communicative role (Alberts 1990; Alberts et al. 1993).

The first outcome is that the protein fraction of femoral gland secretions appears well differentiated and structured, with a total of 13 clusters, clearly detectable and organized in 3 main zones. Actually, a fourth zone might be represented by the region below 17 kDa, although an improvement of the defatting procedure is urgently needed in order to obtain a reliable analysis also for low molecular weight proteins. This should become a priority in the view of the studies on iguanids (Alberts 1990; Alberts et al. 1993), where low molecular weight proteins (lower than 14 kDa) showed a high inter-individual variability, and were suspected to be important in individual recognition. Despite this limitation, the number of protein clusters observed in *P. muralis* falls within the variability range of the band count available for 16 iguanid species (Alberts 1991), where values range between 7 and 15 (median = 9.5; statistics from Table 1 in Alberts 1991). On the contrary, the distribution of the bands of *P. muralis* does not seem to match any previous pattern: in particular, bands between 24 and 32 kDa are lacking, while they are well represented in the iguanid species considered by Alberts (1991) and also in the gel images of *D. dorsalis* (Figure 2 in Alberts 1990) and *I. iguana* (Figure 1 in Alberts et al. 1993), where each species was replicated more than once and results can be considered more representative. This difference may reflect the phylogenetic distance between iguanids and lacertids, even though caution is needed because of the low number of the considered lacertid species. In general, the systematic occurrence of a well-structured banding model in phylogenetically distinct *taxa* suggests that the potential importance of the protein component of the femoral gland secretions has been probably understated (Font et al. 2012; Mayerl et al. 2015) and excludes that it is made only of keratin and/or melanin, as initially suggested by some authors (Cole 1966).

The second main finding is the occurrence of a within-population differentiation in the protein profiles: there is just 1 pair of samples showing the same banding scheme (Figure 2B). This result agrees with those obtained on desert iguanas (Alberts 1991, 1992) and green iguanas (Alberts et al. 1993), and supports the hypothesis that each lizard has its own protein profile, which may therefore be used to signal identity (Alberts 1990). Indeed, the ability of some lizard species (also lacertids) to recognize their conspecifics by means of chemical cues alone has been already proved (Alberts 1992, 1993; Aragón et al. 2001; Mason and Parker 2010; Font et al. 2012; Baird et al. 2015), suggesting that differences in chemical compounds at the individual level could actually occur and can be reliably used for individual recognition. Further, the stability of the protein composition within individuals found in iguanas (Alberts 1990) and the stronger relationship between proteins and genes makes them the ideal candidate to serve as an identity marker, as already found in mammals (*Mus musculus*; Hurst et al. 2001) and fishes (*Gasterosteus aculeatus*, Milinski et al. 2005).

The third and last result concerns the link between protein pattern variability and population of origin, which explained almost 39% of the profile variation. The effect of population may be interpreted as the product of the phylogeny (Alberts 1991; Alberts et al. 1993) as well as the adaptive response to site-specific environmental conditions. Indeed, the protein fraction may include either informative and non-informative compounds. These latter may play structural functions not related to identity (such as constituting lipophilic matrix, modulating lipids release, increasing visibility by UV emission), thus responding to the local environmental features as observed for lipids (e.g., temperature, humidity, windiness, substrate; Baeckens et al. 2015). To some extent, the environmental conditions experienced by different populations might consequently influence a portion of their protein profiles, and produce the observed among-populations patterns: a similar adaptive phenomenon at the intra-specific level has been already documented for femoral gland lipids (Khannoon et al. 2013; Heathcote et al. 2014; Martín et al. 2015). In the present study, the climatic conditions of the pair of sites belonging to the same clade are quite homogeneous (Figure 1B,C), while their protein patterns still maintain unique characteristics (Figure 4). This apparent discrepancy suggests that at least part of the among-populations variability may reflect their phylogenetic relationship (Alberts et al. 1993), as already suggested for the lipid differentiation of allopatric populations of *A. boskianus* (Khannoon et al. 2013). Nonetheless, it cannot be excluded that environmental effects can act also at a finer spatial scale or through ecological variables not considered nor correlated with the ones used to characterize the sites (e.g., windiness, substrate; Baeckens et al. 2015). Further, the relationship between chemical composition and environment may be hardly predictable (Martín et al. 2016a), even more in the absence of information about the identity and role of proteins.

In conclusion, proteins of the femoral gland secretions of the common wall lizard show a sufficient level of variability to make them hypothetically suitable to be used as chemical signals of individual identity. Surely, this potentiality still remains a hypothesis that needs an explicit demonstration, since the occurrence of individual variability alone does not necessarily imply that proteins are effectively used as chemical signals, nor that they actually transfer information about individual identity: the variability is a necessary but not sufficient condition (Beecher 1989). *Ad hoc* behavioral tests with manipulated scents combined with in-depth biochemical analysis which allows protein identification are therefore necessary in order to infer their actual role in femoral gland secretions. In addition, only by widening the geographic sampling and by combining proteomic and genetic data it will be possible to quantify and disentangle the environmental and phylogenetic effects on protein composition.

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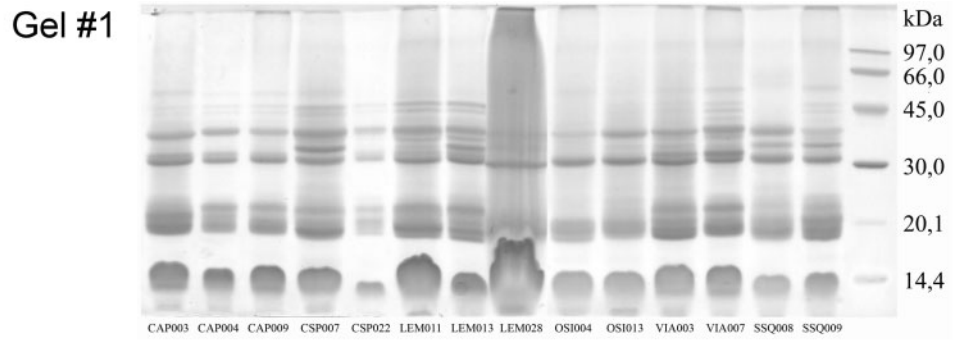
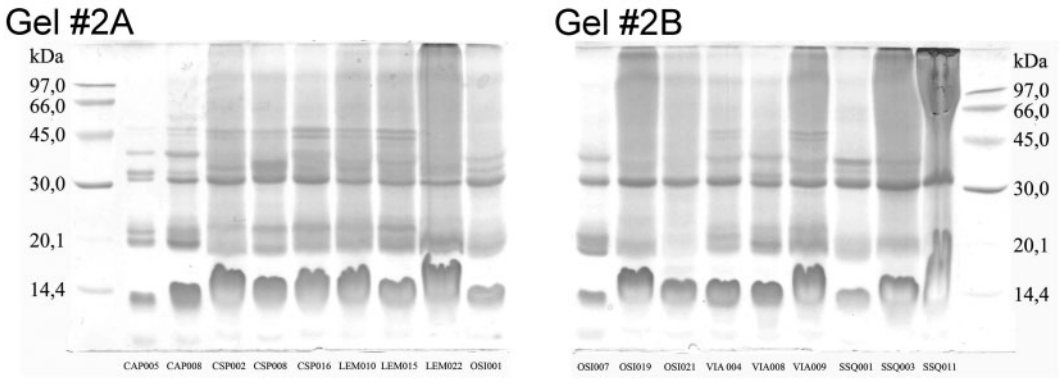
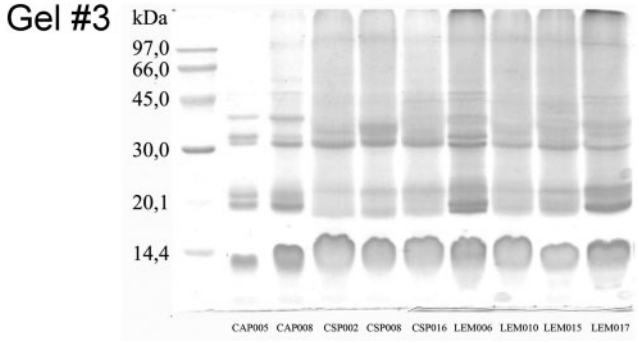
Appendix

Images of the 4 gels used in the analysis of protein pattern. Individual codes and standard molecular weights are also reported. The 3 letters of the individual code correspond to those used to indicate the study sites. Since most samples in Gel No. 3 were the same as in Gel No. 2A, only the lanes of unique ID were considered from this gel, that is, LEM006 and LEM017.







CHAPTER 3

Morph-specific protein patterns in the femoral gland secretions of a colour polymorphic lizard

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Morph-specific protein patterns in the femoral gland secretions of a colour polymorphic lizard

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ABSTRACT

Colour polymorphism occurs when two or more genetically-based colour morphs permanently coexist within an interbreeding population. Colouration is usually associated to other life-history traits (ecological, physiological, behavioural, reproductive …) of the bearer, thus being the phenotypic marker of such set of genetic features. This visual badge may be used to inform conspecifics and to drive those decision making processes which may contribute maintaining colour polymorphism under sexual selection context. The importance of such information suggests that other communication modalities should be recruited to ensure its transfer in case visual cues were insufficient. Here, for the first time, we investigated the potential role of proteins from femoral gland secretions in signalling colour morph in a polymorphic lizard. As proteins are thought to convey identity-related information, they represent the ideal cues to build up the chemical modality used to badge colour morphs. We found strong evidence for the occurrence of morph-specific protein profiles in the three main colour-morphs of the common wall lizard, which showed both qualitative and quantitative differences in protein expression. As lizards are able to detect proteins by tongue-flicking and vomeronasal organ, this result support the hypothesis that colour polymorphic lizards may use a multimodal signal to inform about colour-morph.

INTRODUCTION

Among the most intriguing phenomena able to recursively animate the debate and to stimulate theoretical work in evolutionary biology, colour polymorphism (CP) surely occupies a good standing[1,2]. Its usually preferred definition, which somehow encloses the reason itself for the interest, is that of Huxley[3], who slightly reformulated the original one by Ford[4]: CP occurs when two or more heritable colour morphs "*coexist in temporary or permanent balance within a single interbreeding population […] in such frequencies that the rarer cannot be due solely to mutation*" [3]. Colour is usually associated to other individual traits (physiological, morphological, ecological, reproductive, behavioural)[1,5,6], resulting the most apparent attribute among a set of correlated

ones[1,5–9]. Each morph can be viewed as an alternative combination of characters within a species, occupying a different peak in the adaptive landscape[1]. Understanding the mechanisms able to maintain (even "temporarily") a balanced morph composition against recombination and genetic drift, which should operate in the opposite direction, has been viewed as the key for a deeper comprehension of evolutionary processes[1,5,6,10–14]. Even if CP is generally regarded as any other polymorphism[1,3], it intrinsically and inevitably pertains also to the sphere of animal communication[15–17]. When CP is driven by sexual selection, colour represents the visible badge of the underlying set of correlated traits[6] and, as such, it is used to modulate the intra- and inter-specific interactions upon which CP maintenance is based[9,18]. Non-random pairing as well as morph-specific aggressiveness were often found to be the main behavioural mechanisms[6,9], which require colour to be the intraspecific signal mediating decision-making processes[18]. In such contexts, communicating the own morph to conspecifics is advantageous to both signaller and receiver, and the morph-identity function of colour is therefore promoted and maintained[19]. Communication plays such a pivotal role in the mechanism that one could expect that other (even all) channels must be recruited to ensure its reliability and efficacy[16,20,21]. Indeed, some evidence of non-visual communication modalities matching colour morphs have been already found in orchids[22–24], insects[25,26], fish[27–29], amphibians[30,31], and lizards[32–34]. In all the above cases, the role of non-visual channel is to make the visual one more effective, ensuring that the message will be delivered when colour alone is not enough or cannot be detected[35].

Lizards offer an ideal model to elucidate the interactions between visual and non-visual communication in association to CP. Firstly, CP is widespread and well-studied in this group[6], and has been extensively used for theoretical works[7,10–12,36–39]. Secondly, as sexual selection and social strategies seem to play a major role in maintaining CP in lizards[12,34,39–46], the need for an unbiased communication system is strengthened[16,18,21,47,48]. Finally, lizards have well-developed visual and chemical sensory systems, which constitute the hard-core of their social communication[11,49,58,50–57]. Notably, on the receiver side, chemoreception is powered by the vomeronasal organ associated to a

75 forked tongue and the tongue-flicking behaviour[58–61]. On the signaller side, most lizards species
76 have a series of specialized epidermal glands in the femoral and/or pre-cloacal region[62–64] producing
77 waxy secretions used to convey information about many signaller's traits, like species[65–67], sex[68–70],
78 identity[71–73], familiarity[50,74–76], status[77–80], and condition[81,82]. Therefore, the chemical path comes as
79 the ideal channel being combined to the visual modality explicitly recalled by CP.

80 Lizard femoral gland secretions are made of a mix of lipids and proteins[83,84] whose relative
81 proportion seems to vary with species considered [84–86] and along the activity season, following
82 androgen levels[86,87]. Unfortunately, only few data on a bunch of species are actually available[63,73].
83 The lipophilic fraction, which has been extensively studied, usually includes steroids, terpenes,
84 provitamins (D and E), long chain acids, alcohols, esters, ketones, aldehydes, all being precursors,
85 products or by-products of fat metabolism[83,88]. Given the cost they impose to the signaller, lipids
86 have been hypothesized to honestly convey quality- and condition-related information used by
87 conspecifics to make a decision in both intersexual (mate choice) or intrasexual interactions (male-
88 male combats)[53]. For example, females of the well-studied lacertid lizard *Iberolacerta monticola*
89 prefer territories marked by ergosterol-enriched scent of males with better immunity and
90 condition[89]. Males are still able to assess fighting ability of the potential opponent based on the
91 cholesterol level in the femoral secretions[78]. Similar evidences were also found in other lizard
92 species[65,90–92].

93 By contrast, the protein fraction is poorly known. The pioneering studies on the desert iguana
94 (*Dipsosaurus dorsalis*) and the green iguana (*Iguana iguana*) showed that proteins could be used as
95 signal, probably conveying identity-related information[69,76,84,87], and support to such function has
96 been recently confirmed for a lacertid species[93]. Combined to the expected strong relation between
97 proteins and genes, these findings suggest that proteins may play an important role in individual
98 recognition on a chemical basis[63,73,94], which is a key pre-requisite in driving lizard social
99 behaviour[80,95,96]. Since colour morph represents a genetic condition of the individual, not related to
100 its body condition[97], selection should promote the coevolution of: i) an encoding system of the

information about the signaller's morph, especially in the protein fraction of the femoral gland secretions, and ii) a decoding system of protein fraction associated to the vomeronasal organ[54] of conspecific males or females. This would be the only way by which information may help individuals to drive behavioural choices and therefore contribute to the CP maintenance[42,98].

To verify the hypothesis that proteins from femoral glands have the potential to convey information about colour morph, we analysed and compared the protein profiles from the three main morphs of the common wall lizard (*Podarcis muralis*)[42,99]. The ventral colouration (yellow, red/orange, and white) is genetically controlled[122], and has been already correlated to many other traits[41,43,100–105], even though a clear pattern has not still emerged. A potential environmental role in CP expression has been recently documented, suggesting that both natural and sexual selection may be involved in CP expression[106,107]. Nonetheless, the signal function of the ventral colouration is strongly supported by the morph assortative pairing[42,45,46], by the morph-specific male-male interactions[108,109], and by the lizard ability to discriminate colour morph[18]. Further, previous studies have already highlighted the occurrence of a chemical segregation of morphs[41]. Some lipophilic compounds, namely, tocopherol, are actually differentially allocated by morphs in the femoral pore secretions[32], and 1-D electrophoretic runs performed on proteins of different populations of this species have shown an among-individuals variability in the profiles in terms of occurrence and intensity of some distinct protein bands[73]. However, the comparison and characterization of the proteins from the three main colour morphs have never been attempted. Here, differentially expressed proteins were detected and tentatively identified for the first time.

RESULTS

Two-Dimensional Electrophoresis (2-DE)

The original gels from 2-DE are available as Supplementary Information. The master gels of W, Y and R morphs are shown in in the mid-line of Figure 1, left to right, respectively. The mean spot

126 number in the gels was 84, 53, and 55 for morphs W, Y, and R, respectively. The number of spots
127 in W morph was about 1.5 fold higher than in R and Y.
128 The comparison of master gel patterns allowed to generate three new virtual images indicated as
129 High Master Gels (HMG; Fig. 1) that evidenced these differences. In particular, the HMG generated
130 by matching Y against W (Fig. 1) revealed that 47 (68.6%) spots were common to both phenotypes;
131 37 (27.0%) were unique of W and 6 (4.4%) exclusive of Y. Likewise, the HMG produced when R
132 was matched against W (Fig 1) showed that 40 (57.6%) spots were common to both phenotypes; 44
133 (31.7%) were exclusive of W and 15 (10.8%) of R. Finally, the HMG obtained from the comparison
134 of Y and R master gels (Fig. 1) showed that these morphs had 32 (59.3%) spots in common; 21
135 (19.4%) were unique of Y and 23 (21.3%) of R. Taking advantage of the similarity among patterns,
136 the three HMGs were correlated to each other (Y *vs* W; R *vs* W and R *vs* Y) to understand which
137 were the spots common to all morphs and which unique to each of them. The same procedure
138 mentioned above allowed the creation of the final virtual image indicated as CHMG (Fig. 1),
139 comprehensive of all matched spots derived from the three HMGs.
140
141 *Mass spectrometry (MS) analysis of differential proteins*
142 As it can be seen from the magnified picture of CHMG (Fig. 2), a red, green, and blue colour was
143 assigned by the software to spots exclusive of morph W, Y, and R, respectively. Among the spots
144 peculiar of W morph, ten (numbered 1 to 10 in Fig. 2) were apparently not overlapping with others.
145 The same for six spots unique to morph Y (numbered 11 to 16 in Fig. 2) and four unique to morph
146 R (numbered 17 to 20 in Fig. 2). All these spots were carefully excised from the gel, destained,
147 digested with trypsin and peptides submitted to MS analysis.
148 A scheme illustrating the peptide-spectrum matching results on the MS data is shown in table 1.
149 The low abundance of proteinaceous material under spot 2, 7, 8, 9, and 16 most likely determined
150 the poor quality of their MS signals, which prevented any identification attempt. These spots were
151 then excluded from the subsequent spectrum-to-spectrum comparisons. Seven spots (3, 5, 6, 12-14,

17) did not produce any match, the remnants eight gave a total of 14 identified peptides, seven unique to W, three to Y, and four to R. Six identified peptides matched proteins known to be linked to skin colour (Tab. 1 and S3 in [36]). The lack of multiple peptide matches against a single protein prevented any identification at protein level.

The spectrum-to-spectrum comparison showed that there were no two identical spectra (105 pairwise comparison; Tab. 2) and highlighted the distinctness of the morph-specific spots (Fig. 3): the median "minimum non-self distance" was 0.963 (inter-quartile range = 0.567), while the median "self-distance" was 0.154 (inter-quartile range = 0.155). The difference is highly significant (Wilcoxon signed rank test: $W = 0.000$; $P < 3.052 \cdot 10^{-5}$; $n = 15$).

DISCUSSION

The proteinaceous content of the femoral pore secretions of *Podarcis muralis* appears well-differentiated among the three pure colour morphs, being W the richest in term of spot number (84 distinct spots), followed by R (55) and Y (53), which have similar scores. Out of 84, 15, 6, and 4 spots uniquely occur in W, Y and R, respectively. Even assuming these distinct spots could arise from protein under-expression rather than a true absence, the differences in the observed patterns are such as to allow easily discriminate each colour morph by 2-DE profiles alone (Fig. 1). Moreover, though not allowing protein identification, the MS data confirm that the differential spots hold unique peptides (Tab. 1; Fig. 3), making the 2-DE outcome further supported.

A similar match between chemical profile and colour morph has been already found in this species for the lipophilic part of the femoral gland secretions[32]. Whereas lipids are well-recognized chemical signals in lizard[83], and relatively few studies have explicitly related proteins to inter-individual chemical communication[93,110–114], the coherence between outcomes of the two studies on lipid and protein may be the result of a correlative effect: proteins simply form the non-informative matrix where lipids lie[62,115], and, accordingly, any variation in lipid composition will be indirectly reflected in the protein one. This interpretation has a weak experimental support, though. The

178 difference in lipid profiles is not as strong as that of proteins. Pellitteri-Rosa et al.[32] found R-morph
179 having relatively more tocopherol and less furanone than W, but only W showed a significant
180 difference in the overall profile, and the attempt to classify morph on the lipids basis did not score
181 well. This weakness can be explained considering that samples for the lipid study came from three
182 distinct populations (no information are available about the site × morph frequency in the sample)
183 over a period of two months (April to May). As both population and season can affect the
184 composition and amount of the lipid fraction[67,90,92,116–120], potentially in a morph-specific
185 way[43,44,121], an unbalanced sampling of morphs by period and population could have biased results.
186 On the opposite, the observed differences in the protein pattern cannot be imputed to population,
187 timing, or to sampling bias, since all sampled lizards came from the same site, were collected on the
188 same day, at the peak of the breeding season[43,86], and the pooled secretions were obtained by
189 balancing the contribution of each donor (see Material and Methods). So, the stronger and more
190 robust results from protein comparison are in contrast with what would be expected under a
191 correlative hypothesis, which, at most, would have predicted the opposite, i.e., a stronger relation
192 with lipids.
193 From a theoretical point of view, proteins look like a more probable candidate than lipids to convey
194 information about morph, given morphs to represent equally adapted traits combinations[5,7,9],
195 genetically hereditable[122], and unrelated to individual quality[8,11], i.e., individual quality is still part
196 of the story, but within each morph. Most lipids (or their precursors) from femoral glands cannot be
197 synthetized *ex-novo* by lizards[53,83,88]. Rather, they are acquired from the environment, and impose a
198 cost to their use in communication: this is exactly what a reliable quality signal does[123], and
199 evidences of such function have been already collected[53,81,90–92,124,125]. On the other side, proteins
200 own an undoubted morph-specific profile, have a direct link with genes, do not impose an actual
201 cost to the emitter (*sensu* Zahavi and Zahavi[123]), and can be detected by lizards[69,93,126] thanks to the
202 vomeronasal organ and taste. Altogether, these properties give the proteins the potential of being
203 used as proxy for colour morph, as a part of a more complex chemical badge[73,97,127]. Future studies

about the design of lizard chemical communication should hence adopt an integrated approach that simultaneously considers both chemical fractions of the signal, disentangle the unique information they carry, and investigate how they influence each other.

Finding a morph-specific pattern in proteins secreted by femoral gland has important consequences for the understanding of intra-specific interactions among free-ranging individuals of both sexes. Proteins are not volatile. When they are exploited as semiochemicals in terrestrial animals, they are usually in water solution (e.g., urine[110 128]) or directly transferred on the receiver chemoreceptive surfaces during close interactions (e.g., plethodontid salamanders[129]). In lizards, femoral gland secretions are typically left on dry substrates[62,130], and the only way they can be detected is through the direct inspection, i.e., tongue-flicking[54,59]. Nevertheless, proteins are long-lasting stable marks (1-d electrophoresis of three-years-old samples gave the same results as freshly collected ones; Mangiacotti et al., unpubl.), and are among the most suitable signals in territorial contests[131]. Indeed, typically territorial species are able to recognize familiars on a chemical basis[50,74–76,132], and also to build a spatial map of scent marking points[133]. In a CP system, assessing the morph identity of a potential rival or mate without (or before) seeing it (i.e., before the visual modality can be activated) may give a great advantage in decision-making and allows better tuning intraspecific interaction[12,39]. Indeed, non-random mating has been recognized as a key mechanism contributing to CP maintenance[9], and it has been reported also for the common wall lizard[42,45], where both male-male competition[108,109,134] and female flexible choice[45,135] seem to be at work. Combined with female preference for chemical rather than visual *stimuli*[135], the occurrence of a dual modality (visual and chemical) of morph-specific signals gains even more importance.

Unfortunately, the identification of the involved proteins has not been achieved, thus preventing us to shed light on the mechanism behind morph chemical signalling. The lack of a specific and targeted database to match against MS spectra and the absence of previous knowledge about the composition of proteins from lizard femoral glands[63,73] are probably the reasons for this trouble. The chosen database could have been hypothetically suitable, in that it pertained the skin gene

230 expressions of a polymorphic lizard[36], but retrieved sequences came from phylogenetic distant
231 species, maybe too distant to give better results. Nonetheless it allowed the identification of some
232 differential peptides, which, together with 2-DE and spectrum-to-spectrum comparison, is enough
233 to fix that morph-specific proteins are actually present, which was the primary study aim. Now,
234 more targeted work is needed to obtain a list of secreted proteins, to understand their role, also in
235 relation to the lipophilic fraction, and the underlying mechanisms, in order to attempt a more multi-
236 modal approach to animal communication.
237 The question of whether all the involved proteins (or only a few of them) have to do with
238 differences among morphs' chemical profiles rather than to other individual traits, as well as if
239 lizards are actually able to discriminate morphs based on the protein fraction alone need to be
240 proven by further molecular investigations and behavioural tests. The results of this pilot study just
241 add a further step towards the comprehension of the mechanisms by which chemical and visual
242 signalling cooperate in driving lizards' communication and CP maintenance.
243
244
245 MATERIALS AND METHODS
246 *Sample collection*
247 A total of 30 adult males (snout-to-vent length: mean = 64.7 mm; range: 59.0 – 71.0 mm[136]) of the
248 common wall lizard *Podarcis muralis* have been considered in this study. Lizards were captured by
249 noosing, which did not cause the animal avoidable pain, suffering, distress or lasting harm[137]. To
250 minimize sample heterogeneity, all lizards were captured at the same site (Castelseprio, Lombardy,
251 Italy: 45.73°N, 8.86°E, 358 m a.s.l.). Further, to avoid uncontrolled seasonal effects[86], captures
252 were concentrated on a single day (3rd April 2017), at the beginning of the breeding season, when
253 glandular activity is at its maximum[86] and males of the three morphs show comparable testosterone
254 levels[43]. According to the differences in their ventral coloration (see Fig. 1 in [99]), lizards were

assigned to one of the three pure morphs: white (W), yellow (Y), and red (R). Only lizards showing pure morphs were considered[99]. The final sample included ten individuals for each morph.

Femoral gland secretions were obtained from each individual by applying a gentle pressure around the thighs with the help of a small steel spatula, and collecting the protruding plugs directly into glass vials[73]. Lizards were then released at the capture point. Vials were transferred to the laboratory and samples preserved at -20 °C until analyses[73].

No lizards were killed or injured during the study. Permits for capturing and handling lizards were granted by the Italian Ministry of Environment (Prot. Aut. PNM-2015-0010423; PNM-2016-0002154), who also approved sampling collection (which was not invasive and did not cause damage to any animal tissues).

Extraction and quantification of proteins

Secretions of male lizards femoral glands were pooled according to the morph. Proteins were extracted from waxy secretions through a defatting procedure[73]. In brief, 200 µL of n-hexane were added to samples (an average of 1-2 mg of proteins), incubated at room temperature for 2 h and, after centrifugation (14,000 rpm for 10 min), proteins were isolated as a pellet. The procedure was repeated three times and proteins were finally air-dried. Protein pellets were then dissolved in 200 µL of 10 mM PBS buffer pH 7.4, containing 137 mM NaCl and 2.7 mM KCl. Their exact quantification was achieved by applying the Bicinchoninic Acid (BCA) assay using bovine serum albumin (BSA) as the standard protein for the production of the calibration curve (in the range of concentration between 5 and 25 µg/mL). At this point, aliquots belonging to the individuals of the same group and containing a similar quantity of proteins were pooled, according to the morph. The protein concentration was about 2,5 mg/mL for each group of individuals and the total amount of proteins was about 1.0 mg/group.

281 *Two-Dimensional Electrophoresis*

282 *Protocol set up.* Samples were prepared by dissolving about 150 μg of proteins in 125 μL of
283 rehydration buffer (8 M urea, 4% CHAPS (w/v), 65 mM DTE, 0.8% carrier ampholytes (v/v), 0.5%
284 bromophenol blue). As 2-DE was never carried out before on proteins from lizard femoral glands,
285 some preliminary attempts were made in order to attain a satisfactory outcome. Notably, the first
286 dimension (isoelectric focusing - IEF) was run with linear and non-linear IPG strips, having the
287 same pH range (pH 3–10; Amersham Biosciences, UK); for the second dimension the porosity of
288 the SDS polyacrylamide gel was alternatively set to 12.5% or 15%.

289 Samples were first loaded onto 7 cm IPG strips, which were rehydrated without applying voltage
290 for 1 h at 20°C. IEF was carried out at 15°C using an Ettan IPGphor system (Amersham
291 Biosciences), programmed with the following voltage gradient: 30 V for 8 h, 120 V for 1 h, 500 V
292 for 0.5 h, 1000 V for 0.5 h and 5000 V until a total of 25–27 kV/h was reached.
293 Reduction/alkylation steps were applied between the first and the second dimension. The focused
294 IPG strips were incubated for 15 min at room temperature in 6 M urea, 2% (w/v) SDS, 50 mM Tris
295 pH 6.8, glycerol 30%, containing 2% (w/v) DTE, followed by a second incubation of 15 min in the
296 same buffer containing 2.5% (w/v) iodoacetamide and 0.5% bromophenol blue. At the end of the
297 IEF step, strips were hold in place with 0.4% low melting temperature agarose and loaded onto 8 x
298 6 cm slabs, 12.5% or 15% SDS polyacrylamide gels[73]. Electrophoresis was carried out at a constant
299 current of 10 mA per gel in a PROTEAN II xi 2-D Cell equipment Bio-Rad (Berkeley, California),
300 until the buffer frontline was 1 mm from the bottom of the gels. The 2-DE gels were stained with
301 ‘‘Blue silver” (colloidal Coomassie G-250 staining)[138]. To minimize the technical mistakes
302 connected with sample manipulation, experimental steps concerning sample preparation and
303 electrophoretic runs were performed ‘‘in parallel” on all samples.

304 The visual inspection of the preliminary gels highlighted: i) an unexpected overcrowding of spots
305 being evident at the bottom of the slabs when using 12.5% porosity in second dimension; ii) a
306 lateral compression of spots, leaving a poorly coloured central area, when IEF used non-linear IPG

307 strips. The best outcome, which minimized spot overlap and blank areas, was attained with linear
308 strip and 15% porosity. Given the good resolution of spots, 2-DE analyses were performed in
309 quadruplicate for each group (W, Y, R) using the above settings, to produce the 12 gels used in the
310 final comparison (Fig. 1).
311 *Gel analysis.* Digital images of stained gels were acquired using the VersaDoc Imaging Model 3000
312 (BioRad) and then subjected to quali/quantitative analysis using the PD Quest (BioRad) version
313 8.0.1 software. Spot detection was achieved using the spot detection wizard tool after defining and
314 saving a set of detection parameters. After spot detection, the original gel scans were filtered and
315 smoothed to clarify spots, remove vertical and horizontal streaks and remove speckles. Three
316 images were created from the process: the original raw 2-D scan, the Filtered image and the
317 Gaussian image. A match set for each group was then created for comparison after the gel images
318 had been aligned and automatically overlaid. If a spot was saturated, irregularly shaped, or
319 otherwise of poor quality, then the Gaussian modelling was unable to accurately determine quantity.
320 In these cases, the spot was defined in the filtered image using the spot boundary tools. Thus, for
321 each group, a virtual image was produced which included protein spots only if present at least in
322 two out of the three best gels. This is indicated as "master gel".
323
324 *Mass spectrometry analysis*
325 *In situ enzymatic digestion.* The selected spots (Fig. 2) were carefully excised from the gel, placed
326 into Eppendorf tubes and broken into small pieces. This material was then washed twice with
327 aliquots (200 µL) of 100 mM ammonium bicarbonate buffer pH 7.8, 50% acetonitrile (ACN) and
328 kept under stirring overnight, until complete destaining. Gels were dehydrated by addition of ACN
329 (100µL). After removal of the organic solvent, reduction was performed by addition of 50 µL of
330 10mM Dithiothreitol (DTT) solution (40 min at 37°C). DTT was replaced with 50 µL of 55 mM
331 iodoacetamide for 45 min at 56°C. This solution was removed and the gel pieces were washed twice

with 200 µL of 100 mM ammonium bicarbonate for 10 min, while vortexing. The wash solution was removed and gel dehydrated by addition of 200 µL of ACN until the gel pieces became an opaque-white color. ACN was finally removed and gel pieces were dried under vacuum. Gels were rehydrated by addition of 75 µL of 100 mM ammonium bicarbonate buffer pH 7.8, containing 20 ng/µL sequencing grade trypsin (Promega, Madison, WI, USA) and digestion was performed overnight at 37 °C. Following enzymatic digestion, the resultant peptides were extracted sequentially from gel matrix by a three-step treatment (each step at 37 °C for 15 min) with 50µL of 50% ACN in water, 5% trifluoroacetic acid (TFA) and finally with 50 µL of 100% ACN. Each extraction involved 10 min of stirring followed by centrifugation and removal of the supernatant. The original supernatant and those obtained from sequential extractions were pooled, dried and stored at -80°C until mass spectrometric analysis. At the moment of use, the peptide mixture was solubilized in 100µL of 0.1% formic acid (FA) for MS analyses.

LC-MS/MS. All analyses were carried out with a liquid chromatography-mass spectrometry (LC-MS, Thermo Finnigan, San Jose, CA, USA) system consisting of a thermostated column oven Surveyor autosampler controlled at 25°C, a quaternary gradient Surveyor MS pump equipped with a diode array detector, and an Linear Trap Quadrupole (LTQ) mass spectrometer with electrospray ionization ion source controlled by Xcalibur software 1.4. Analytes were separated by reverse phase high performance liquid chromatography (RP-HPLC) on a Jupiter (Phenomenex, Torrance, CA,USA) C_{18} column (150 × 2 mm, 4µm, 90 Å particle size) using a linear gradient (2–60% solvent B in 60 min) in which solvent A consisted of 0.1% aqueous FA and solvent B consisted of ACN containing 0.1% FA. Flow-rate was 0.2 mL/min. Mass spectra were generated in positive ion mode under constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, sheath gas flow 40 (arbitrary units), auxiliary gas flow 10 (arbitrary units), sweep gas flow 1 (arbitrary units), capillary temperature 200°C, tube lens voltage–105 V. MS/MS spectra, obtained by CID studies in the linear ion trap, were performed with an isolation width of 3 Th m/z, the activation amplitude was 35% of ejection RF amplitude that corresponds to 1.58 V[139].

358 *Protein identification.* Protein identification was attempted using a peptide-spectrum matching
359 (PSM) approach[140,141], as implemented in the MS-GF+ v2018.07.17 software[142–145]. According to
360 the instrument sensibility, digestion protocols[140,141], and general guidelines[142], the algorithm settings
361 were as follows: tolerance, 0.5 Da; charge range, 1 – 6+; range of peptide length, 6 – 35; isotope
362 error 0 – 2 Da; cleavage, semi-tryptic; post translational modification, fix carbamidomethylation of
363 cysteine[140,146,147]. The database choice is a crucial step in PSM, and, unfortunately the study species
364 and the peculiarity of the protein samples prevented the extraction of an actually reliable dataset
365 from the usual repositories[148]. So, an *ad hoc* database was built by taking advantage from the paper
366 by McLean et al.[36], where a list of differentially expressed genes at the skin level was made
367 available for the colour morphs of the tawny dragon, *Ctenophorus decresii* (table S3 in [36]). Even if
368 the tawny dragon (Order Squamata, Fam. Agamidae) is not phylogenetically close linked to the
369 common wall lizard (Order Squamata, Fam. Lacertidae), McLean's and our study share these
370 common main points: i) they both involve polymorphic lizards; ii) they both involve tissues with an
371 epidermal origin; iv) proteins conveying information about colour could derive from, or be related
372 to, the same set of genes involved in skin colouration. The UniProt Knowledgebase release
373 2018_07[149] was then surveyed for the 458 unique gene names available in table S3[36], and the so-
374 obtained entries were filtered out to match the vertebrate taxon. Further, to account for any
375 contamination[147], mammalian trypsin and human keratin sequences, also retrieved from UniProt,
376 were added to the previous database. The final dataset counted 59,622 unique sequences.
377 To maximize power, PSM was run as a two-stage process[150] with target-decoy approach. All the
378 candidate proteins identified in the first stage (target or decoy) were used in the second stage to
379 refine identification[151], adjusting the proportion of target/decoy sequences to reach an unbiased
380 estimation of false detection rate (FDR)[151–153]. Decoy sequences were obtained by reversing the
381 target ones in both stages. FDR was calculated at the peptide level as n_{decoy}/n_{target} for a given
382 spectrum E-value, which was used as score[151]. Before FDR computation, the list of identified
383 spectra was purged from all the spectra i) simultaneously matching target and decoy sequences, ii)

384 corresponding to peptides with semi-tryptic cleavage, and iii) having more than two irregular
385 cleavage[151]. Only spectra with FDR ≤ 0.01 were considered. A protein was considered identified if
386 more than two different peptides match the same protein.
387 To further assess the effective distinctness of morph-specific spots, a pairwise spectrum-to-
388 spectrum comparison was performed[154–156]. The set of spectra from each MS run was compared to
389 all the others belonging to a different morph, and the cosine distance computed[155]. The minimum of
390 this distances for each spot (minimum non-self-distance) was retained and compared to the one
391 computed between each spot and itself (self-distance). A Wilcoxon signed rank test (one tail, with
392 exact P estimation) was then used to assess if self-distance was significantly smaller than minimum
393 non-self distance[157], and to exclude spots identity.
394 All the above operations were implemented in R v3.5.0[158], using the packages mzID[159],
395 Biostrings[160], stringr[161], functions by Rieder et al.[155], and *ad hoc* functions (available upon request)
396 to prepare database and call external software (MSGF+).
397
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406
407

COMPETING INTERESTS

The authors declare no competing interests.

DATA AVAILABILITY

The scans of the best three 2-DE gels and MS raw data used in this study are available in Zenodo archive (doi: 10.5281/zenodo.1460606).

AUTHORS' CONTRIBUTIONS

MM, SS, and RS conceived the ideas, collected the samples, and lead the writing of the manuscript; MF, MC, SV, AMB developed the lab protocols and performed lab analyses; MM, MF and MC analyzed data. All authors critically supported the drafts and gave final approval for publication.

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morph	Spot	peptide	error	score	FDR	accession	gene	description	Colour link
W	1	R.RCRCFR.R	-0.063	9.160	0.000	O75443	TECTA2	tectorin alpha gene 2	
		R.FQGNLWK.T	0.057	9.082	0.000	G1SEM4	ADA	adenosine deaminase	Purine metabolism, Tyrosine
		K.DYVNDLKDSYGQEWTR.Y	-0.085	9.053	0.000	P54707	ATP12A	ATPase H+/K+ transporting nongastric alpha polypeptide	Purine metabolism
	3					Unknown			
	4	K.YNIEEEGTWR.R	-0.030	8.901	0.000	F6TWE8	OBSCN	obscurin cytoskeletal calmodulin and titin-interacting	Purine binding, Tyrosine
	5					Unknown			
	6					Unknown			
	10	K.TPEGTLPR.L	0.228	8.640	0.000	A0A2R9A5X2	AXDND1	axonemal dynein light chain domain containing 1	
		K.RQMHKPIK.V	-0.447	7.622	0.000	W5UKP0	CYLD	cylindromatosis	
		K.GTDPQVR.Y	0.249	7.618	0.000	I3J9Y8	PARP9	poly (ADP-ribose) polymerase family member 9	
Y	11	K.VLSVHPWNRPSLQDCLAHPWLQDAYLMKLR.R	-0.454	9.182	0.000	G3TQN9	SPEG	SPEG complex locus	Purine binding
	12					Unknown			
	13					Unknown			
	14					Unknown			
	15	R.LTVGTRPDGLPDERWCFR.V	0.143	7.593	0.000	A0A2U4C2P6	TRPV2	transient receptor potential cation channel subfamily V member 2	
		K.TWTSFLSGVNIQIVGDDLTVTNLK.R	-0.262	7.512	0.000	Q1KYT0|ENO3	ENO3	enolase 3	Iridophore, Purine
R	17					Unknown			
	18	R.DIPKGIR.Q	0.167	7.609	0.000	A0A096NX44	WFDC3	WAP four-disulfide core domain 3	
	19	K.DINTFVHGNRHHITAICGDENGSPYGGNLR.I	-0.321	8.038	0.000	Q8WN63	ANG	angiogenin ribonuclease RNase A family 5	
	20	K.LSASSEASEVDKKEK.S	-0.384	8.373	0.000	A0A2K6EX08	DTX3L	deltex 3-like	
		K.GGGAPK.T	-0.348	7.987	0.000	A0A2K5QEN8	MYO18B	myosin XVIIIB	Purine binding

Tab. 1. List of the identified peptides using the database from tab. S3 in [36]. Error = difference between the measured and calculated parental ion mass (Da); score = MSGF+ spectrum E-value (-$\log_{10}$ transformed); FDR = false detection rate at the peptide level; accession = uniprotKB accession; gene = gene name as reported in tab. S3 [36]; description = protein description as reported in tab. S3 [36]; colour link = previous link to colour as reported in tab. S3 [36]. Spots 2, 7,8,9, and 16 are not shown due to poor quality spectra.

spot	#01	#03	#04	#05	#06	#10	#11	#12	#13	#14	#15	#17	#18	#19	#20
#01	*0.06*	**0.95**	0.96	0.99	1.00	0.96	0.98	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00
#03	**0.95**	*0.13*	**0.95**	0.98	1.00	0.96	0.99	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00
#04	0.96	**0.95**	*0.15*	0.98	1.00	**0.95**	0.99	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00
#05	0.99	0.98	0.98	*0.10*	1.00	**0.97**	0.99	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00
#06	1.00	1.00	1.00	1.00	*0.17*	1.00	1.00	1.00	1.00	1.00	1.00	**0.40**	0.41	0.41	0.43
#10	0.96	0.96	**0.95**	0.97	1.00	*0.17*	0.96	0.97	0.99	1.00	1.00	1.00	1.00	1.00	1.00
#11	0.98	0.99	0.99	0.99	1.00	**0.96**	*0.21*	0.98	0.99	1.00	1.00	1.00	1.00	1.00	1.00
#12	**0.96**	0.99	0.99	0.99	1.00	0.97	0.98	*0.38*	0.99	1.00	1.00	1.00	1.00	1.00	1.00
#13	1.00	**0.99**	**0.99**	**0.99**	1.00	**0.99**	**0.99**	**0.99**	*0.55*	1.00	**0.99**	1.00	1.00	1.00	1.00
#14	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.62*	**0.99**	1.00	1.00	1.00	1.00
#15	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	**0.99**	**0.99**	*0.56*	1.00	1.00	1.00	1.00
#17	1.00	1.00	1.00	1.00	0.40	1.00	1.00	1.00	1.00	1.00	1.00	*0.14*	**0.41**	0.42	0.42
#18	1.00	1.00	1.00	1.00	0.41	1.00	1.00	1.00	1.00	1.00	1.00	0.41	*0.15*	**0.40**	0.41
#19	1.00	1.00	1.00	1.00	0.41	1.00	1.00	1.00	1.00	1.00	1.00	0.42	**0.40**	*0.14*	0.42
#20	1.00	1.00	1.00	1.00	0.43	1.00	1.00	1.00	1.00	1.00	1.00	0.42	**0.41**	0.42	*0.15*

Tab. 2. Pairwise distance matrix obtained from the spectrum-to-spectrum comparison of the spots that gave reliable spectra. Values are cosine distance between spectra from a spot pair. The diagonal represents the "self-distance" values for each spot (shadowed and italicized); in each row, the values corresponding to the "minimum non-self distance" for each spot are bolded.

CAPTIONS TO FIGURES

Fig. 1. Scheme of the two-dimensional electrophoresis analysis. Top: scanned images of the four gel obtained from each morph sample (original images are available as Supplementary Information); MG: master gel after PD Quest (Biorad) elaboration, representing the virtual gel associated to each morph (from left to right: white, yellow, red); HMG: high master gel obtained by the comparison of each MG pair (from left to right: W vs Y, W vs R, and Y vs R); bottom: combined high master gel (CHMG) obtained by superimposing the three HMGs to highlight those spots unique to each morph: red = W, green = Y, and blue = R.

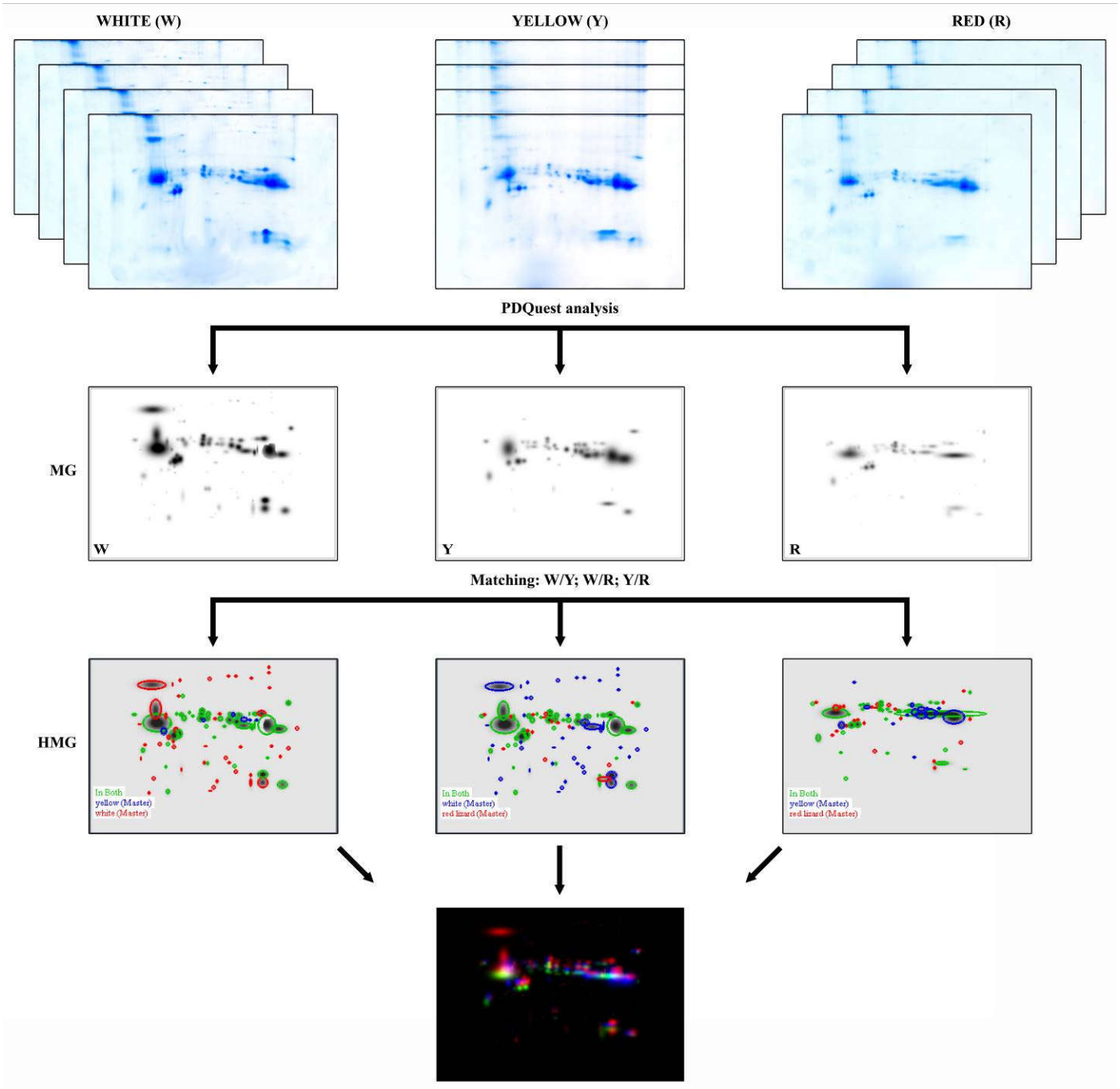


Fig. 2. Position on the CHMG of the 20 excised spots finally used in mass spectrometry analysis. Numbers 1-10 belong to W, 11-16 to Y, 17-20 to R.

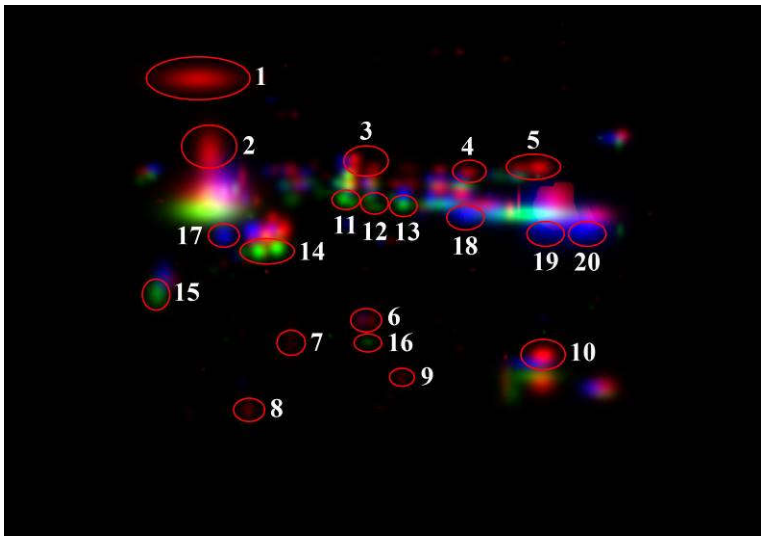
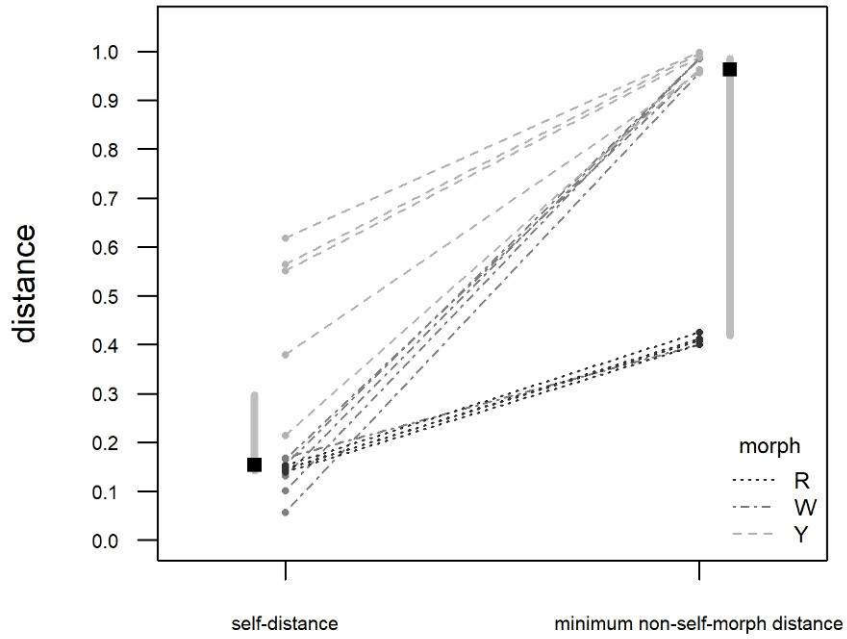
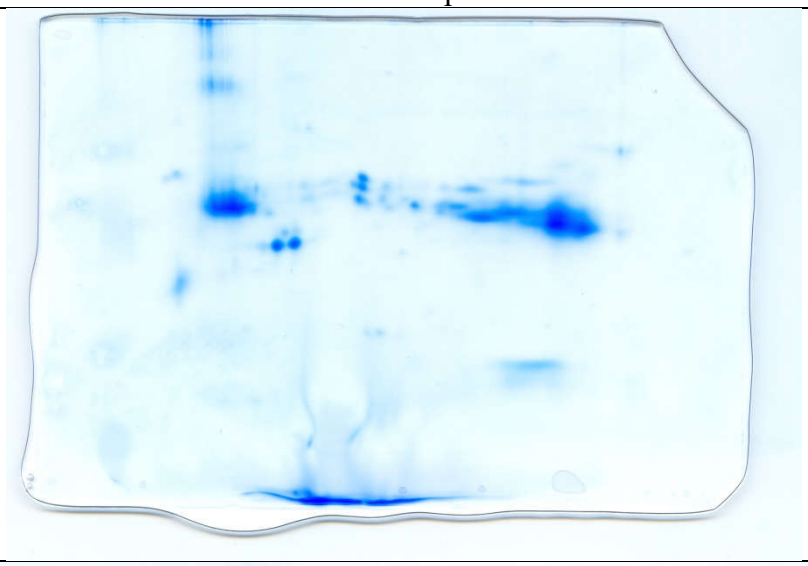
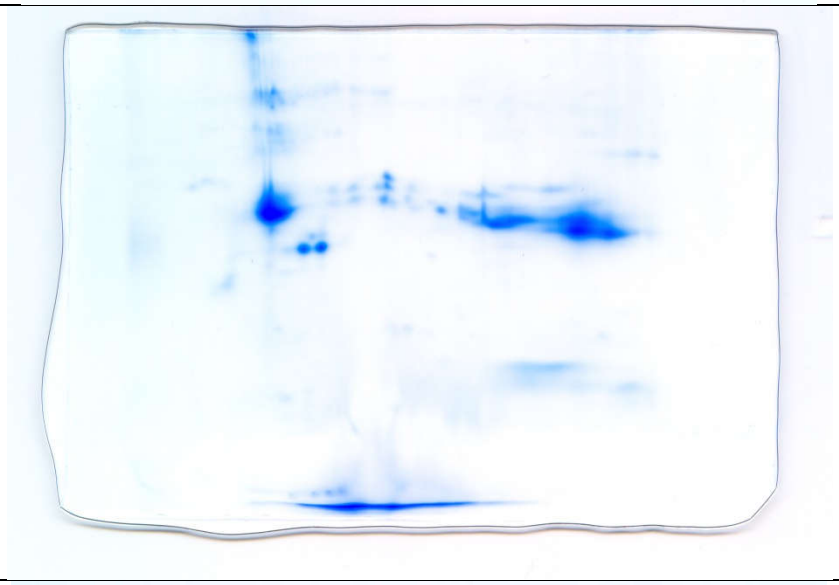
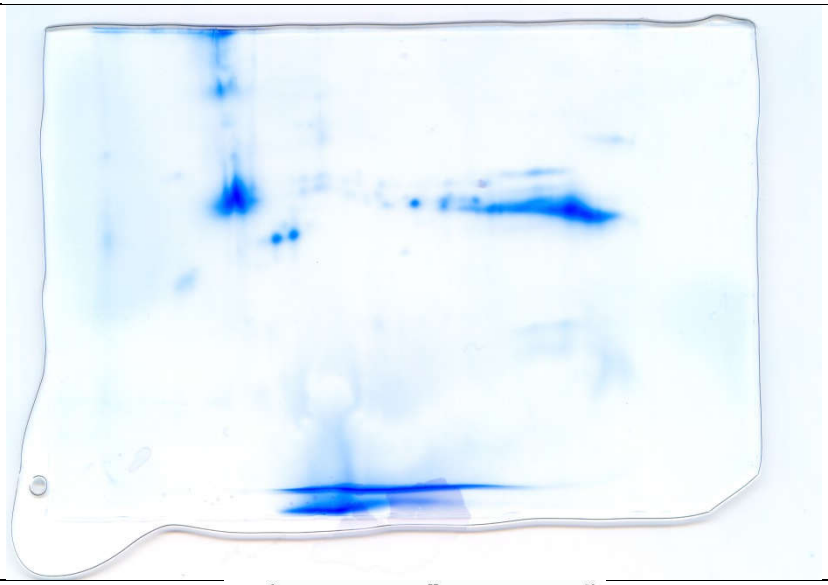
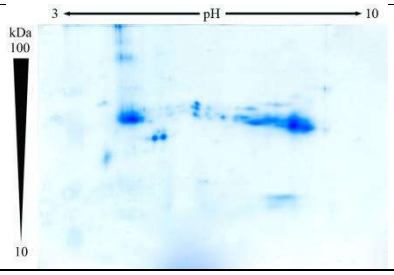


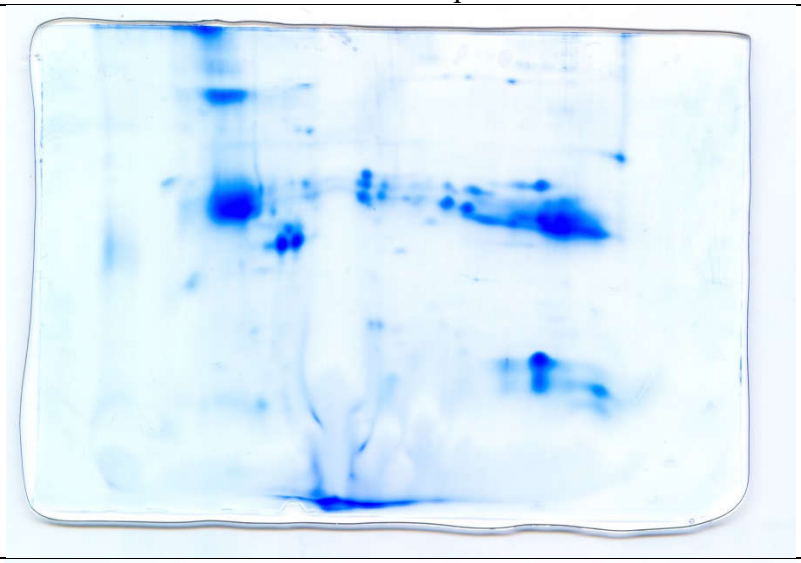
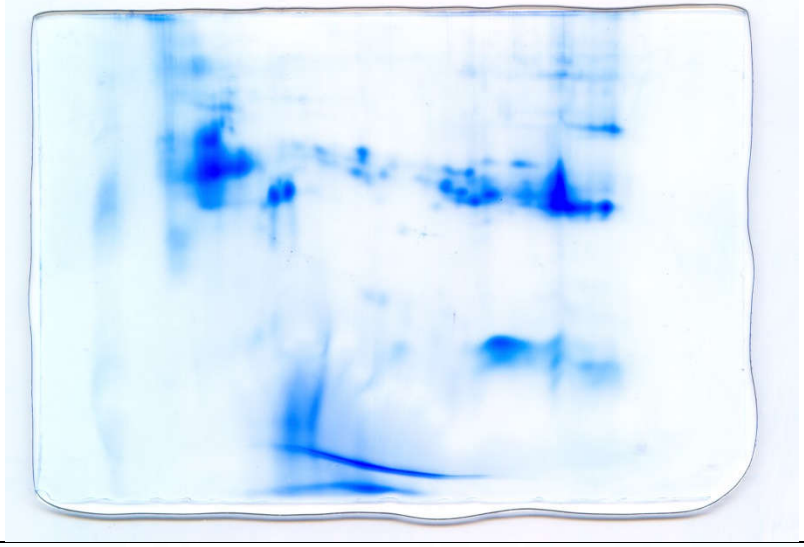
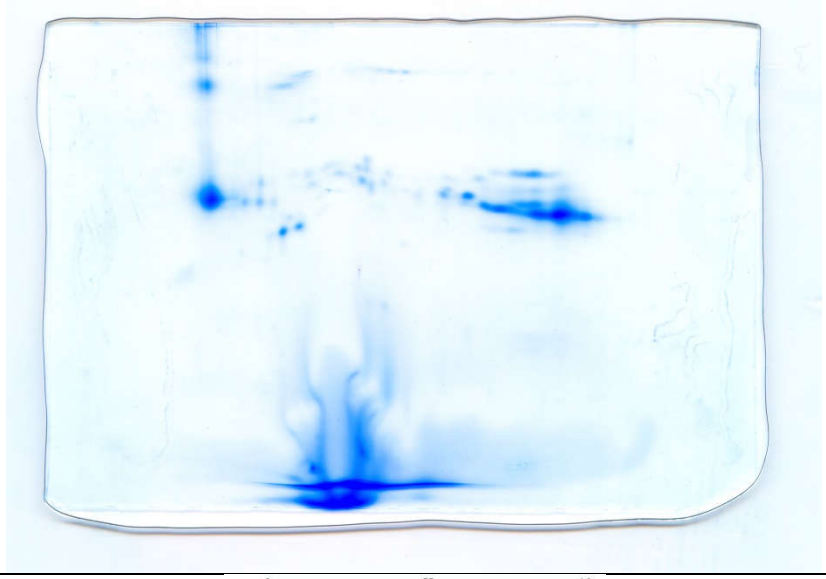
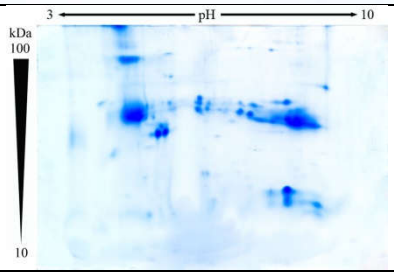
Fig. 3. Comparison between the spectrum-to-spectrum distance of each analysed spot from itself (self-distance) and from the most similar spot among the ones belonging to a different morph (minimum non-self-morph distance). Values on the ordinate are cosine distance. Grey dots = observed distance value; dashed lines are used to link each self-distance to the corresponding non-self-morph. Black squares represent the medians of self- and non-self-morph distances; vertical grey bars show the interquartile range for each distance group.

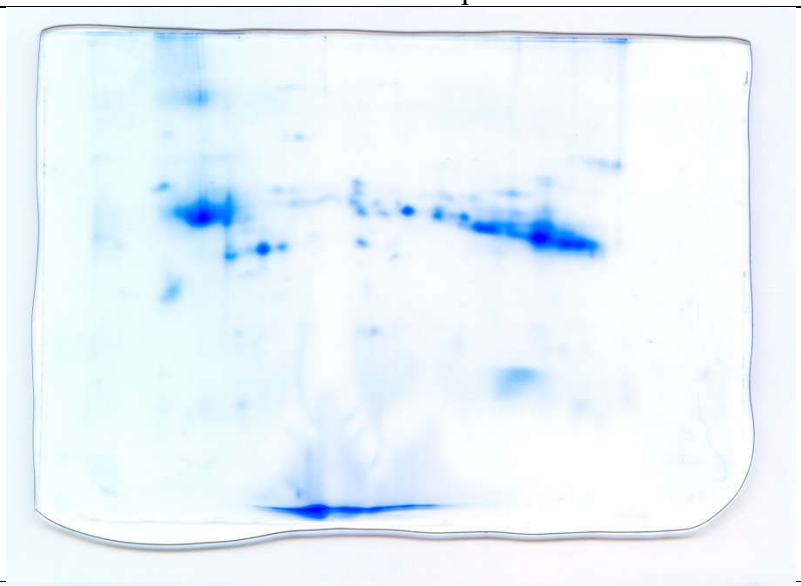
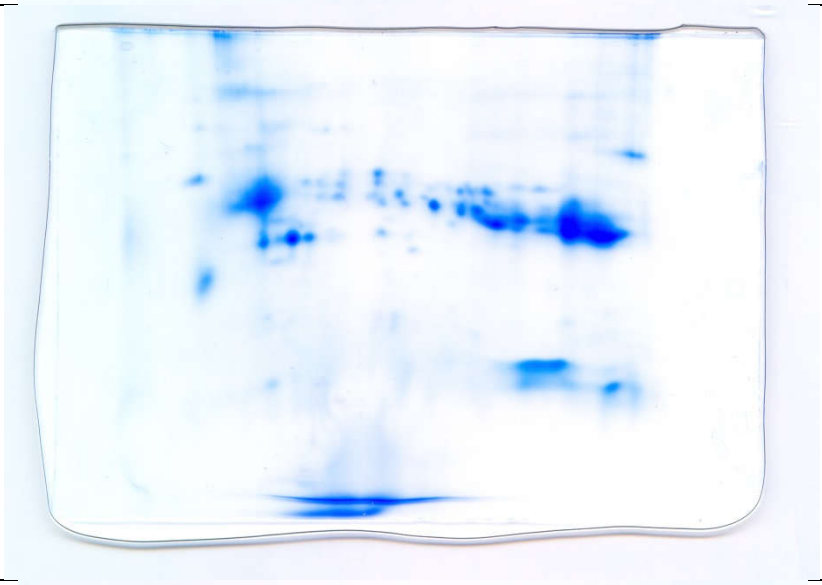
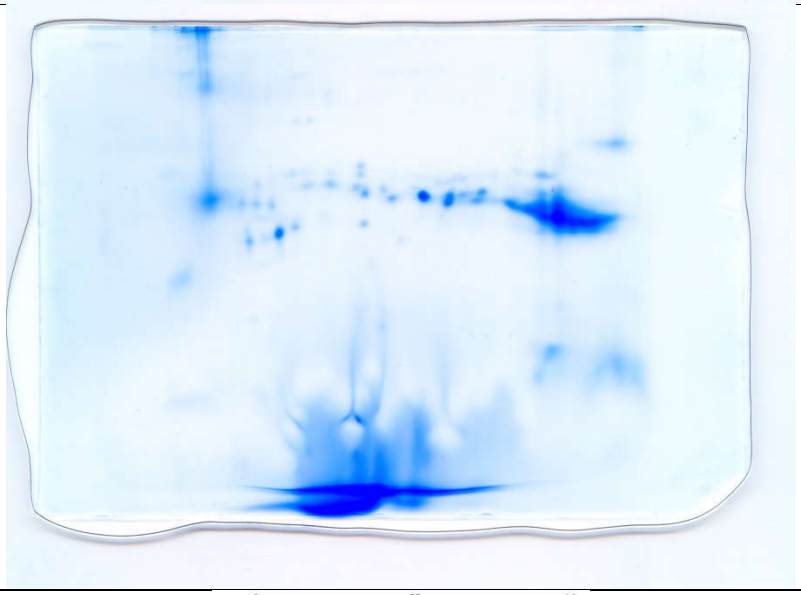
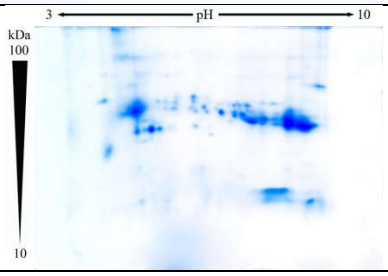


SUPPLEMENTARY INFORMATION

Original 2DE gels. Here it follows the image list of the gels from the two-dimensional electrophoresis as they were originally acquired by VersaDoc Imaging Model 3000 (BioRad). Images were not manipulated, nor contrast and luminosity were altered. The same settings were used in each acquisition. For each colour-morph, the three best replicates actually used in the analysis are reported. Further, for each morph series, an inset with pH and weight scales has been added.

Red morph	replicate #
	1
	2
	3
	scale

White morph	replicate #
	1
	2
	3
	scale

Yellow morph	replicate #
	1
	2
	3
	scale

CHAPTER 4

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

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

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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); 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 displays 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 conspecifics 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). 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 (20^{th} March – 20^{th} May): one half were noosed in the botanic garden of Pavia (Northern Italy), and constituted the experimental focal sample. The other half were 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 were 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 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 x 30 x 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-steps 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 two hours 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.

Experimental setup

The experimental protocol resembles those typically used to investigate the response to chemical scent of predators (e.g., Thoen et al. 1986; Mencía 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 side 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 thirty regularly spaced points where to release 50 µL of the stimulus solution (a total of 1500 µL); 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 five minutes 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, Indiana, USA; sensitivity: 0.1°C; precision: ± 2%). Then, the lizard was transferred to the experimental box, and maintained for five more minutes inside an opaque plastic tube laid in the middle of the octagon, in order to reset the escaping 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 x 600 pixels and 15 frames/s. Recording duration was set to 20 minutes (18,000 frames), starting 5 seconds after the tube removal (Mencía et al. 2016). Room temperature was set to 28 °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); 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 minutes 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); 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 (Δt) 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 Δt = 0.067 s (the inverse of the frame/s), R resulted 1.47 cm. The proportion of each category within a trajectory describe 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 were differentially affected by the stimuli. 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_{50}; HDI_{95}) (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 ten minutes even repeating the trial. Consequently, the analysis is based on the 28 lizards, for a total of 84 videos (one for each treatment for focal lizard). On average, 54.90% of trajectories 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_{95}.

According to model I, TDIM was positively affected by NON-SELF (Tab. 1: model I), but not by SELF treatment, which did not differ from CTRL (Tab. 1: model I). NON-SELF treatment predicted larger value for TDIM than CTRL (Fig. 3; $P_{NON\text{-}SELF>CTRL} = 0.992$) and SELF (Fig. 3;

206 $P_{NON\text{-}SELF>SELF}$ = 0.968). Body temperature at trial start had no any effect (Tab. 1: model I), as well
207 as the proteinaceous concentration and the focal lizard size in NON-SELF treatment (Tab. 1: model
208 II; Fig. 3).
209
210 **DISCUSSION**
211 We showed that male common wall lizards responded differently to the proteins secreted by an
212 unfamiliar conspecific compared to a neutral stimulus and to their own proteins. Notably, when
213 proteins from femoral gland secretions of a never-met male were used to mark the substratum,
214 TDIM increased by 1.23 times the CTRL, and 1.16 the SELF value (Fig. 3). The observed outcome
215 was not affected by experimental contingency (i.e., body temperature, proteinaceous concentration,
216 or lizard size), as their respective effects are not credible (Tab. 1).
217 In the present study, TDIM corresponds to escaping attempts (climbing and scratching the box
218 walls and corners, jumping) or exploratory activity (slow movements along the perimeter often
219 accompanied by tongue-flicking). A TDIM intensification in the NON-SELF treatment can reflect a
220 situation where an intruder enters the territory marked by the scent of an unfamiliar male:
221 perceiving the odour of the unknown rival without being able to see it may trigger more explorative,
222 and "nervous" movement patterns. Most studies having used a comparable experimental setup
223 (Labra and Niemeyer 1999; Aragón et al. 2003; Van Dyk and Evans 2007; Aguilar et al. 2009)
224 consistently found non-self (or unfamiliar) cues to elicit an increase of the intruder's movements,
225 with few exceptions: (Aragón et al. 2001), who found no significant difference, but the same trend;
226 and (Font and Desfilis 2002), who found a significant opposite trend (familiar > unfamiliar), but
227 working with juveniles (see discussion therein for interpretation). Further, in agonistic contests
228 staged to test the occurrence of a residence effect in lizards, intruders typically increase avoidance
229 behaviours (e.g., running, climbing, scratching the cage walls; López and Martín 2001; Aragón et
230 al. 2006; Sacchi et al. 2009; Titone et al. 2018). All the above responses require some abilities for
231 rival recognition (Glinski and Krekorian 1985; Whiting 1999; López and Martín 2001; Thom and

232 Hurst 2004; Tibbetts and Dale 2007; Carazo et al. 2008) and, therefore, imply a IRI transferring.
233 Applied to the present study case, this is equivalent to say that proteins from femoral glands are able
234 to convey IRI, as they were the only available cue to identify the conspecific as a stranger.
235 A circumstantial evidence supporting the previous conclusion may come from combining model I
236 and II outcomes. Within the NON-SELF treatment (i.e., the treatment level giving the maximum
237 response to chemicals), the proteinaceous concentration in the solution did not affect the focal lizard
238 response (Tab. 1: model II). Hence, the increase in TDIM did not depend upon the amount of
239 proteins (model II), but only by their occurrence at a perceivable level (model I). Such all-or-
240 nothing response is expected for an IRI signal, since it has not to be related to signaller quality or
241 condition (Dale et al. 2001; Tibbetts et al. 2017). Indeed a response proportional to the
242 concentration of specific compounds has been already observed in lizards (e.g., López and Martín
243 2005; Martín and López 2006, 2007; Martín et al. 2007), but only when lipophilic substances or the
244 complete (proteins and lipids) secretions were used. Coherently with the properties of a quality
245 signal (Dale et al. 2001; Tibbetts et al. 2017), the abundance of such elements was found to
246 correlate to qualitative traits (size, fighting ability, immune-response level, parasites load; Martín
247 and López 2015). This progressive effect in the response has disappeared when the lipophilic
248 fraction was removed, still preserving the ability to inform about the secretion provenience (NON-
249 SELF vs SELF) to the proteinaceous remain. The lack of correlation may then suggests proteins to
250 inform about discrete traits (like identity or strategy, *sensu* (Tibbetts et al. 2017). We do admit that
251 alternative explanations might be considered, such as an artefact due to the reduced sample size (28
252 lizards with one replicate), or more complex effects: e.g., protein concentration is proportional to
253 donors' size, which may affect the behaviour of focal lizards in a non-linear way, depending on the
254 opponent size (Sacchi et al. 2009; Titone et al. 2018); or lack of lipids may have reduced the
255 detectability or the efficacy of the signal (Alberts and Werner 1993), thus masking the relation.
256 In conclusion, the present study provides for the first time (as far as we are aware) experimental
257 support to the hypothesis that proteins from lizard femoral glands can be used as intraspecific

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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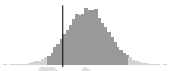
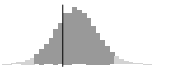
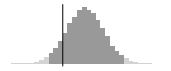
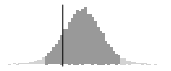
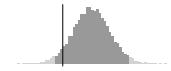
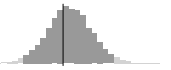
466 **TABLES**

467

Model	Parameter	β	HDI_{95} *lower*	HDI_{95} *upper*	
0	Proteinaceous concentration difference (NON-SELF minus SELF)	0.735	-0.530	2.113	
I	Intercept	0.499	0.437	0.570	
	$Treatment_{NON\text{-}SELF}$	0.110	0.023	0.196	
	$Treatment_{SELF}$	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	

468 **Tab. 1**. Parameter estimates for model 0, I, and II. The half sample mode (β) and the 95% highest
469 density intervals (HDI_{95}) are given for each parameter; the graphical representation of the posterior
470 distribution of the estimates is also reported (dark grey areas = HDI_{95}) and compared with the null
471 value (black vertical line). SVL (model II) is the lizard snout-to-vent length, proxy for its size.

472

473

FIGURES

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

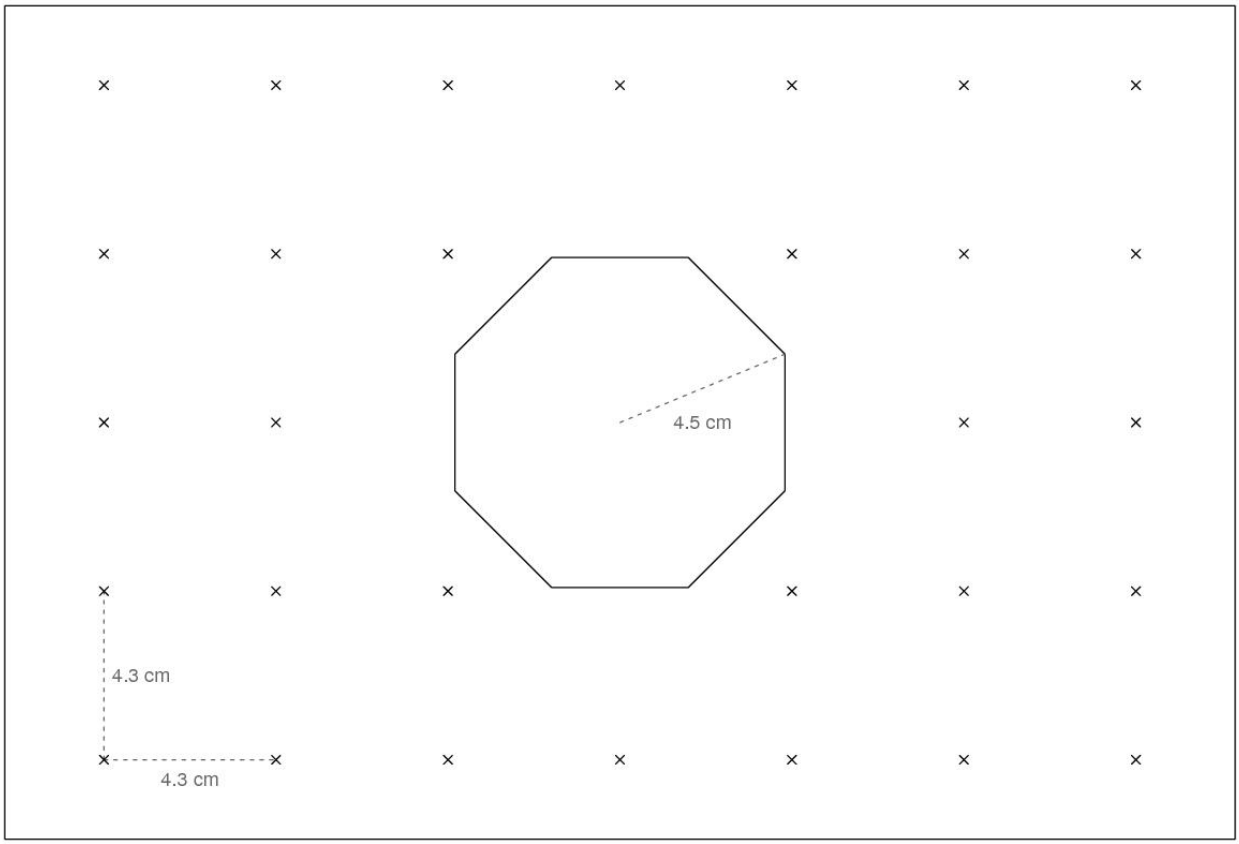


Fig. 2. Exemplificative RST analyses of NON-SELF (top), SELF (centre), and CTRL (bottom) trajectories obtained for the focal lizard ORT107. For each panel: on the left is reported the recorded trajectory (grey line) with the corresponding RST point classification (grey "×" = TDIM; black dots = TIM); 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.

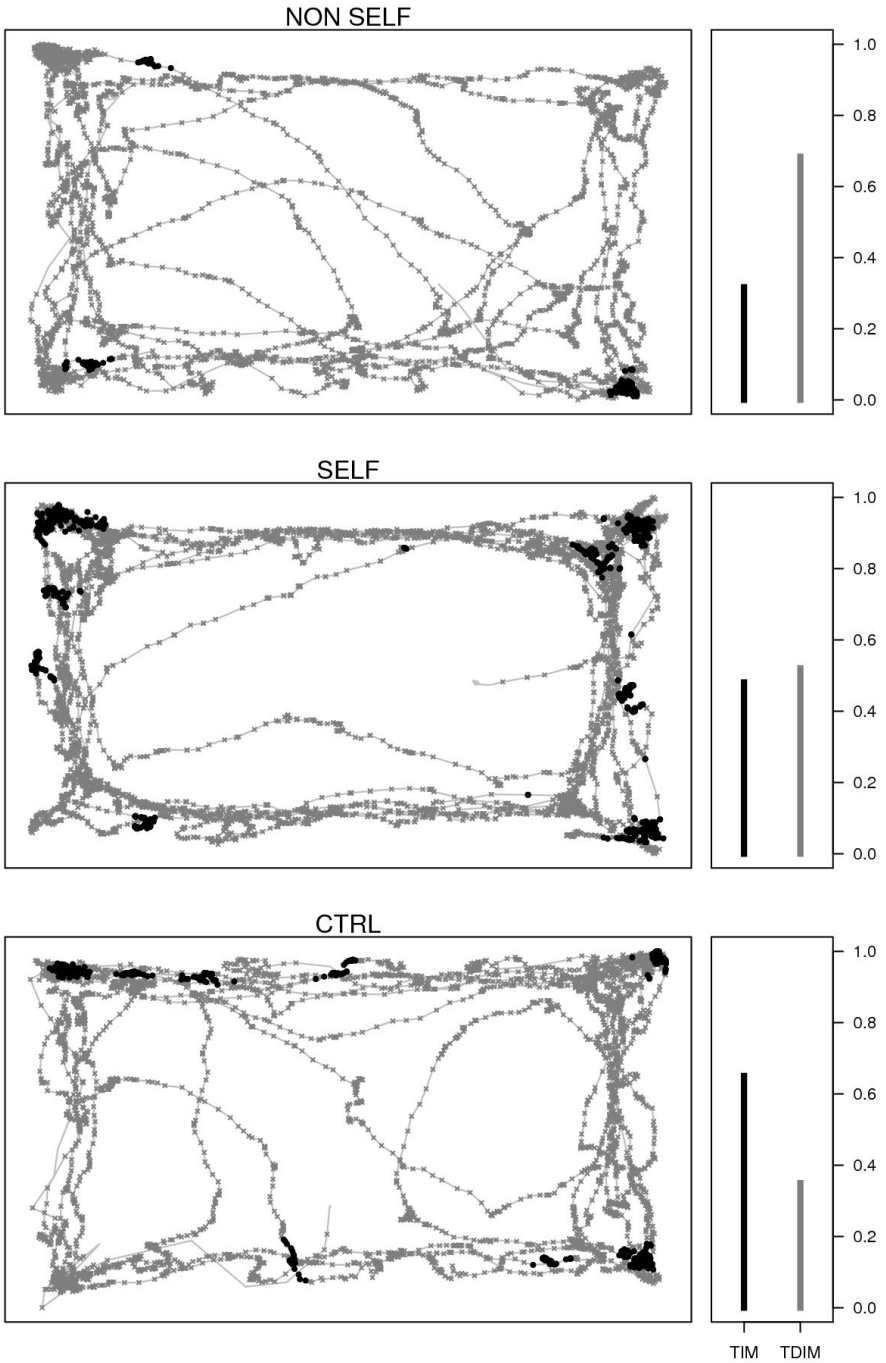
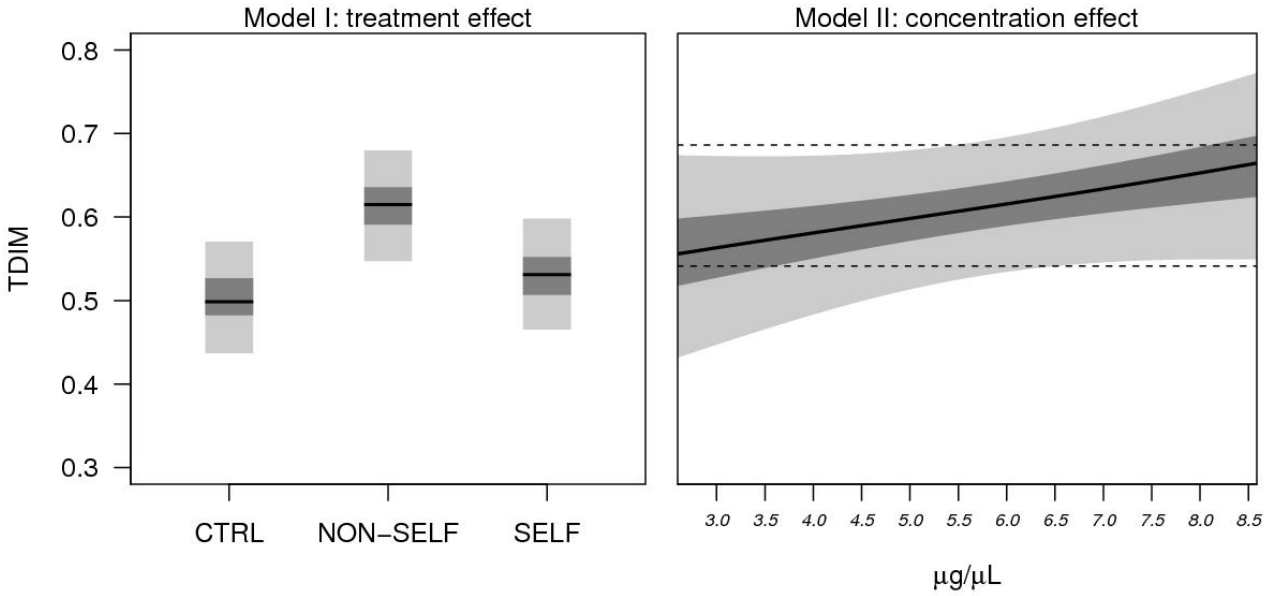


Fig. 3. Posterior predictions of the effect of treatment (left) and protein concentration (right) on the response variable (TDIM). Black solid lines = mode of the posterior distribution; dark grey areas = HDI_{50}; light grey areas = HDI_{95}; dashed lines = HDI_{95} of the model II intercept (i.e., the most probable values of the response in the absence of a concentration effect).



CHAPTER 5

Seasonality of complex chemical language in lizards: a protein story

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In submission

Seasonality of complex chemical language in lizards: a protein story

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ABSTRACT

Animals modulate intraspecific signal's shape and intensity, notably during reproductive periods. Thus, signal variability typically follows a seasonal scheme, traceable through the expression of visual, acoustic, chemical and behavioural patterns. The chemical channel is particularly important in lizards, as demonstrated by well-developed epidermal glands in the cloacal region that secrete lipids and proteins recognized by conspecifics. In males, the seasonal pattern of glands activity is underpinned by variation of circulating androgens. Changes in the composition of lipid secretion

convey information about the signaller's quality (e.g., size, immunity). Presumably, individual identity is associated with a protein signature present in the femoral secretions, but this has been poorly investigated. For the first time, we assessed the seasonal variability of the protein signal in relation to plasma testosterone level (T), glandular activity and the expression of lipid signal. We sampled 174 male lizards (common wall lizard, *Podarcis muralis*) over the whole activity season. An elevation of T was observed one-two months before the secretion peak of lipids during the mating season, when males attempt to attract females; such expected delay between hormonal fluctuation and maximal physiological response fits well with the assumption that lipids indicate individual quality. The proteins 1-dimensional electrophoretic analysis showed that gel bands were preserved over the season with an invariant region; a result in agreement with the hypothesis that proteins are stable identity signals. However, the relative intensity of bands varied markedly, synchronously with that of lipid secretion pattern. These variations of protein secretion suggest additional roles of proteins, an issue that requires further studies.

Key words. Chemical communication; season; testosterone; quality; identity; femoral glands; cosinor models; lizards; *Podarcis muralis*

INTRODUCTION

Seasonality affects many biological functions of vertebrates and invertebrates, notably in temperate and polar zones (Crews, 1984; Paul, Zucker & Schwartz, 2008; McGuire *et al.*, 2010; Stroeymeyt *et al.*, 2014; Follett, 2015). One of the most apparent effects is the time constraint to reproduction, which is usually restricted to the part of the year matching the most suitable environmental conditions (Paul *et al.*, 2008; McGuire *et al.*, 2010; Follett, 2015). Consequently, the whole set of physiological, behavioural, and ecological traits involved in reproduction shows a synchronous co-variation (Crews, 1984).

Seasonality largely influences intraspecific communication, since both intra- and inter-sexual interactions play central roles in reproduction (West-Eberhard, 1979; Endler, 1992). Complex, often multimodal, signals are costly to produce and to maintain (Johnstone, 1996, 1997a; Bradbury & Vehrencamp, 2011), and they entail predation risks (Magnhagen, 1991). Therefore, signallers that could modulate signal production, save resources, and reduce risks have been favoured by selection (Johnstone, 1997a). For instance, shape and intensity of signals are typically reduced outside the mating season (Schwabl & Kriner, 1991; Alberts *et al.*, 1992b; Smith & John-Alder, 1999; Gonzalez *et al.*, 2001; Örnborg *et al.*, 2002; McGraw & Hill, 2004; Irschick *et al.*, 2006; Lucas *et al.*, 2007), losing their ability to trigger receiver's response (Ferkin & Seamon, 1987; Labra & Niemeyer, 1999; Smith & John-Alder, 1999; Labra *et al.*, 2001; Aguilar, Labra & Niemeyer, 2009).

Lizards offer suitable models to study intraspecific communication plasticity associated to reproductive cycles (Edwards & Jones, 2017; Jones, 2017). Most species breed “seasonally” (Crews, 1984; Lovern, 2011; Jones, 2017) and use multimodal signals of various complexity (Schwenk, 1995; Olsson, Stuart-Fox & Ballen, 2013; Pérez i de Lanuza & Font, 2014; Robinson *et al.*, 2015; Baeckens *et al.*, 2017c). The chemical modality is particularly important in lizards (Baeckens *et al.*, 2017c, 2017a; García-Roa *et al.*, 2017), and it is associated with the development of peculiar traits: i) the vomeronasal organ combined to tongue-flicking behaviour (Schwenk, 1995), and ii) specialized epidermal glands in the cloacal region used for intraspecific communication (Cole, 1966; Mayerl, Baeckens & Van Damme, 2015). The femoral (or pre-cloacal) glands are more developed in males than in females (Alberts, Pratt & Phillips, 1992a; Baeckens *et al.*, 2015; García-Roa *et al.*, 2017), and their activity is stimulated by an increase of androgen levels (Padoa, 1933; Fergusson, Bradshaw & Cannon, 1985; Martín *et al.*, 2007a; Baeckens *et al.*, 2017b), peaking during the breeding season (van Wyk, 1990; Alberts *et al.*, 1992a).

The gland secretion is a complex waxy mixture of lipids and proteins (Cole, 1966; Alberts, 1990; Mangiacotti *et al.*, 2017), which may be used by conspecifics to retrieve information about various signaller's features, like size (López, Amo & Martín, 2006), fighting abilities (Martín & López,

2007), parasites load (Martín, Amo & López, 2008), immunity (Martín & López, 2006), but also familiarity (Alberts & Werner, 1993), and individual identity (Alberts, 1992; Carazo, Font & Desfilis, 2008). Therefore, lizards can use femoral secretions to deliver sophisticated messages. Even though they can detect both lipids and proteins (Cooper, 1991; Alberts & Werner, 1993), only the formers have been thoroughly studied, and mainly associated to condition- and quality-traits of the signaller (Martín & López, 2011, 2015). Proteins have received far less attention (Font *et al.*, 2012; Mayerl *et al.*, 2015; Mangiacotti *et al.*, 2017). Preliminary data from iguanas suggest that they can be used in IC in general, and, more specifically that they may convey information about signaller's identity (Alberts, 1990; Alberts & Werner, 1993): a role recently confirmed in a lacertid lizard (Mangiacotti *et al.*, 2018). Individual identity signals are expected to evolve when the signaller pays the cost of being misidentified (Johnstone, 1997b; Tibbetts & Dale, 2007), which is quite common in those social context where individuals may interact repeatedly (Tibbetts & Dale, 2007). Lizards are often territorial and poorly mobile species (Fox, McCoy & Baird, 2003). Hence, they may benefit from an individual recognition system which helps modulating neighbourhood dynamics (Aragón, López & Martín, 2001; Carazo *et al.*, 2008), or establishing dominance relationships (López & Martín, 2001), thus reducing the cost of aggressive interactions (Dale, Lank & Reeve, 2001; Tibbetts & Dale, 2007). So, it could be hypothesized that lipid and protein components may be used as parallel channels to simultaneously inform the receiver about the quality (lipids) and identity (proteins) of the signaller (Alberts & Werner, 1993; Mangiacotti *et al.*, 2018).

The importance of delivering a comprehensive message is maximal during the breeding season, when efficient IC pays off. Coherently, glandular activity (i.e., gland size and secretion production) peaks during the breeding season (van Wyk, 1990; Alberts *et al.*, 1992a; Martins *et al.*, 2006). The lipophilic fraction shows also a qualitative change: the proportion of more volatile unsaturated fatty acids increases during breeding season, thereby enhancing signal detectability (Alberts *et al.*, 1992b). Knowledge about the protein content of femoral secretion is far more fragmentary, notably

104 regarding seasonal fluctuations. To tackle this issue, we used the common wall lizards (*Podarcis*
105 *muralis*), a medium-sized lacertid widespread in central and southern Europe (Sillero *et al.*, 2014).
106 This species is well-suited because reproductive cycle, chemical communication, and hormonal
107 profile have been accurately investigated (Oppliger *et al.*, 2004; Martín *et al.*, 2008; Sacchi *et al.*,
108 2012, 2017; Heathcote *et al.*, 2014; Pellitteri-Rosa *et al.*, 2014; MacGregor *et al.*, 2017; Mangiacotti
109 *et al.*, 2017). In this study we focused on protein femoral secretion of males during the whole
110 activity cycle. We also examined changes in plasma testosterone and lipid femoral secretion. Our
111 objective was to assess if protein secretion exhibits a seasonal pattern. A lack of variation may
112 suggest a role limited to individual identity while marked variations may suggest additional
113 functions.
114
115 MATERIALS AND METHODS
116 *Sampling lizards*
117 From March to October 2016, during the activity season of the common wall lizard (*Podarcis*
118 *muralis*) in Northern Italy (Sacchi *et al.*, 2012), adult males were captured by noosing in two nearby
119 sites, in the city of Pavia (45.18° N, 9.15°E; Botanic garden and Castle; about 500 m apart).
120 Sampling effort was equally spanned, on a monthly base, across the study period. Lizards were
121 transferred to the University lab within two hours from capture, measured for their snout-to-vent
122 length (SVL; to the nearest mm), weighed (± 0.01 g), and photographed for individual recognition
123 (Sacchi *et al.*, 2010, 2016; Sannolo *et al.*, 2016). Then, the secretions from the femoral glands were
124 collected by applying a gently pressure along the thighs, with the help of a steel spatula, until no
125 more material was gettable. Secretions were weighed using a semi-micro balance (ORMA
126 BCA625SM; sensitivity = 0.01 mg), and stored into glass vials at -20 °C until chemical analyses
127 (Mangiacotti *et al.*, 2017). A blood sample (75-100 µl) for each lizard was gathered from the retro-
128 orbital plexus using heparinized capillary tube (McLean, Lee & Wilson, 1973). Tubes were
129 centrifuged (6,700g for 5 minutes) to retrieve the plasma fraction, which was stored at -25 °C until

assay (Sacchi *et al.*, 2017). Plasma samples were shipped to the Centre d'Etudes Biologiques of Chizé, where testosterone assays were performed using a highly sensitive radioimmunoassay method, following Sacchi *et al.* (2017).

At the end of lab procedures, all lizards were kept under observation for two hours and then released, healthy, at their capture point.

Lipids

The lipophilic fraction of the secretion was analysed using gas-chromatography coupled to mass spectrometry (GC-MS at Laboratoire d'Ethologie Expérimentale et Comparée, Université Paris 13). Lipids were extracted using *n*-pentane (≥99%, HPLC grade, Sigma-Aldrich) and then analysed with an Agilent Technologies 7890A gas chromatograph equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) with helium as carrier gas at 1mL/min. The oven temperature was programmed at 50°C for 1 min, increased to 180°C at 30°C/min, then to 250°C at 10°C/min and finally to 320°C at 3°C/min and kept at 320°C for 5 min. The above settings were similar to (Heathcote *et al.*, 2014), and (MacGregor *et al.*, 2017). The GC was coupled with an Agilent 5975 C mass spectrometer with 70 eV electron impact ionization.

As chromatograms appeared more and more simplified along the season (loosing most peaks), and the aim of the analysis was not the compilation of the full list of lipids from *P. muralis* secretions (already described in: Martín *et al.*, 2008; Heathcote *et al.*, 2014; Pellitteri-Rosa *et al.*, 2014; MacGregor *et al.*, 2017), only two conspicuous lipids were quantified: i) provitamin D_3 (retention time = 24.4 min), known to convey quality-related information (López & Martín, 2005; López *et al.*, 2006; López, Gabirot & Martín, 2009; Martín & López, 2006; Martín *et al.*, 2007a); ii) and cholesterol (retention time = 23.9 min), the most abundant lipophilic component of *P. muralis* secretions (Martín *et al.*, 2008; Heathcote *et al.*, 2014; Pellitteri-Rosa *et al.*, 2014; MacGregor *et al.*, 2017), and which can be considered an "unreactive apolar matrix that aids in the delivery of other truly semiochemicals" (López *et al.*, 2009). The amount of provitamin D_3 was expressed as the log-

156 ratio between the area under the peaks of provitamin, and cholesterol (Aitchison, 1982), which was
157 used as reference. The identification of compounds was made by comparison to the mass spectral
158 library in NIST 2008, and checked against previously published spectral data (Heathcote *et al.*,
159 2014; MacGregor *et al.*, 2017). Peaks identification and integration were performed using
160 OpenChrom v1.1.0 (Wenig & Odermatt, 2010).
161
162 *Proteins*
163 After GC-MS, samples underwent three steps: protein extraction; protein assay, and one
164 dimensional electrophoresis (Mangiacotti *et al.*, 2017). Extraction was achieved by first adding 200
165 µL of n-hexane to complete defatting, vortexing for two minutes, and then centrifuging at 13,000g
166 for other two minutes. The supernatant was removed, and the pellet air-dried. This procedure was
167 repeated two times. Afterwards, 200 µL of 10 mM (pH 7.4) phosphate-buffered saline (PBS) were
168 added to the dry pellet. After vortexing and centrifuging, the supernatant containing the soluble
169 proteins was recovered and stored in freezer (-20 °C). The concentration of the extracted proteins
170 was assessed by the bicinchoninic acid assay (Smith *et al.*, 1985), using bovine serum albumin as
171 the standard for the calibration curve. The calibration curve and the concentration estimates were
172 computed using the R-package chemCal v0.2.1 (Ranke, 2018).
173 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to obtain
174 individual protein patterns (proxy for protein composition). Aliquots containing a maximum of 10
175 µg of proteins were used from each sample and added to 10 µL of loading buffer solution (50 mM
176 Tris–HCl pH 6.8, 2% sodium dodecyl sulphate SDS, 0.1% bromophenol blue, and 10% glycerol).
177 Prepared samples were denatured by incubating at 95 °C for five minutes. Electrophoretic runs were
178 performed in a discontinuous mode (5% stacking gel and 15% running gel) by applying a constant
179 voltage of 180 V for 2 h (Garfin, 2009). Gels were stained with a 0.12% (w/v) Coomassie Blue G-
180 250 solution, containing 10% (v/v) orthophosphoric acid, 10% (w/v) ammonium sulphate and 20%

(v/v) methanol. After achieving discoloration using a solution of 5% (v/v) acetic acid, gels were finally scanned, obtaining one image for each one.

To allow the comparison of the different gel images, an *ad hoc* procedure was set up, starting from gel images and counting six main steps: i) gel images were converted into greyscale using the luma formula (Poynton, 2012); ii) an electrophoretogram (EPG) for each lane was extracted using a vertical line through the middle of each lane; iii) the EPGs were aligned by fitting a cubic spline on the positions of the standard molecular weights of the gels they belonged to; iv) a baseline detection algorithm independently identified and removed the basal noise from each EPG (Gan, Ruan & Mo, 2006); v) the aligned and de-noised EPGs were cropped to the same molecular weight extent (8 - 80 kDa), and divided into 238 equal intervals, each bearing the mean luma value of about 10 adjacent pixels; vi) the binned EPGs were normalized, to account for not exactly identical amount of proteins loaded by each lane. All these operations were implemented in R v3.5.0 (R Core Team, 2018) by specifically designed functions (available upon request).

A principal component analysis was conducted on the refined EPGs, and the first component, explaining 29.5% of the total variance, was used as a proxy for the main structure of the proteinaceous signal.

Statistical analysis

Five parameters monitored along the whole season were examined: plasma testosterone level (T; $\log_{10}$-transformed), secretion mass (SM; $\log_{10}$-transformed), provitamin D_3 relative abundance ($proD_3$; see lipids section), protein proportion (PP; protein mass/secretion mass; not transformed), and protein signal (PS; the score of the first component of the PCA on EPGs). To account for the expected circannual rhythm of T and glandular activity (Padoa, 1933; Lofts, 1969; Alberts *et al.*, 1992a; Amey & Whittier, 2000; Edwards & Jones, 2017; Sacchi *et al.*, 2017), single component cosinor models (Bingham *et al.*, 1982; Refinetti, Cornélissen & Halberg, 2007; Cornelissen, 2014) were fitted. Cosinor models are typically used in chronobiology (Refinetti *et al.*, 2007), when the

value of a response variable (Y) is assumed to depend on time (t) following a regular cycle. Therefore, a cosine function is incorporated into a linear model:

$$Y(t) = M + \mathrm{A}\cos\left({}^{2\pi t}/_{\tau} + \varphi\right) + e(t),$$

where M is the MESOR (Midline Statistic Of Rhytm, i.e., the time-corrected mean of the response), A is the amplitude (maximum absolute deviation from MESOR), τ the period of the cycle (365 days for the circannual case), φ the acrophase (i.e., the timing of highest values), and e(t) the error term (Cornelissen, 2014). The model can be linearized by rewriting the formula:

$$Y(t) = M + \beta x + \gamma z + \mathrm{e(t)};$$

being $x = \cos\left({}^{2\pi t}/_{\tau}\right)$ and $z = \sin\left({}^{2\pi t}/_{\tau}\right)$ the cosinor terms, and $\beta = A\cos\varphi$ and $\gamma = -A\sin\varphi$ the cosinor coefficients (Cornelissen, 2014). From the latter A and φ can be recovered (Bingham *et al.*, 1982). To control for possible effect of lizard size, SVL was always added as a main effect covariate in cosinor models. The reliability of each cosinor model was assessed by comparing it to the corresponding linear model without cosinor terms (i.e., the model with only SVL as predictor), using the penalized deviance information criterion (Plummer, 2008).

Both cosinor and linear models were implemented in JAGS 4.3.0 (http://mcmc-jags.sourceforge.net/), using flat priors for coefficients and intercept (μ = 0 and σ = 0.001), and uninformative gamma priors for errors (a = 0.001 and b = 0.001). For all models, Markov Chain Monte Carlo parameters were set as follows: number of independent chains = four; number of iterations = 32,000; burning = 2,000; thinning = 5 (Kéry, 2010). Convergence was checked and results from the posterior distribution are reported as the half sample mode (HSM) (Bickel & Frühwirth, 2006) plus 95% (or 50%) highest density intervals (HDI_{95}; HDI_{50}) (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 & Yajima, 2015), modeest (Poncet, 2012), and HDInterval (Meredith & Kruschke, 2018). R scripts and datasets are available upon request.

RESULTS

A total of 174 adult male lizards were captured during the study period (~22 lizards/month; range: 14 - 27). Nine recaptured individuals were excluded from the analyses to avoid pseudoreplication. Due to various technical difficulties (e.g., insufficient quantity of femoral secretion material), the total sample size for each parameter varied from 86 ($proD_3$) to 158 (SM; Tab. 1).

T was positively correlated with SM, $proD_3$, and PS, and negatively with PP (Tab. 1). In general, all correlation coefficients were below 0.60 (mean absolute value = 0.44), suggesting that the relation among variables was weak (Tab. 1).

Cosinor models outperformed corresponding linear models (Tab. 2): penalized deviance of the former was always lower than the latter, and the difference was always larger than its standard error (Plummer, 2008). Together, these results supported the occurrence of a seasonal component in the observed variation of all the response variables (Fig. 1).

A slight positive effect of lizard size (SVL) was found on SM (Tab. 3), while the HDI_{95} for the other responses always encompassed the null value, thus not supporting any relationship.

The amplitude of the seasonal oscillation was quite large for all parameters, except PP, where it was rather small (Table 3; Fig. 1). T peaked by mid-February (HSM = 18.40 ng/mL; Tab. 3; Fig. 1A, F), while glandular productivity (SM) reached its maximum more than two months later (HSM = 1.79 mg; Tab. 3; Fig. 1B, F). $ProD_3$ and PS were synchronous, with acrophase in mid-March, one month later than T (Fig. 1D, E, F). PP was maximum in late season, at the beginning of September (HSM = 0.59; Tab. 3; Fig. 1C, F), which means that the bathyphase (the minimum) occurred in early March, when $proD_3$ and PS were peaking.

Focusing on the seasonal variation of the protein pattern, the comparison of the predicted EPGs for the acrophase, mesor, and bathyphase (obtained by back-projecting the predicted score of the first principal component; Fig. 2) showed that the ensemble of protein clusters remained constant along the season, while changing its relative expression. Notably, the upper region slightly increased in colour (proxy for relative amount), the central part did not vary, and the two distinct bands in the

low-molecular weight region sharply decreased. The same general trend was also visible comparing the observed gels from early and late season (Fig. 2, right panel).

DISCUSSION

This study, which combined investigations on hormonal, femoral lipid and protein secretions, indicates that common wall lizards use a more complex chemical language than previously assumed. As expected, all the parameters examined exhibited a strong seasonal pattern. Following a peak of T at the onset of the activity season, femoral gland activity increased and was maximal during the period of intensive courtship (Fig. 1F). These results fit well with the role of femoral secretions in intraspecific communication (Alberts, 1993; Martín & López, 2015), and with the central regulatory role of androgen levels (Fergusson *et al.*, 1985; Alberts *et al.*, 1992a; Baeckens *et al.*, 2017b). Body size did not influence femoral secretions, with the exception of the total amount of SM that slightly increased with increasing SVL, as already found in this species (Baeckens *et al.*, 2017b).

The delay between the peak of T and femoral gland activity was broadly of one-two months, depending on the parameter considered. A comparable time decoupling between T elevation and femoral secretion has been documented in the green iguana (Alberts *et al.*, 1992a). Moreover, more than one month elapsed between the experimental administration of exogenous testosterone and the stimulation of glandular secretions in different lizard species (Fergusson *et al.*, 1985; Martín *et al.*, 2007a; Baeckens *et al.*, 2017b). Both the possible functional role and the underlying physiological mechanisms of the delay for high T to induce physiological effects remain poorly understood (Randall, Burggren & French, 1997); it has been proposed that such delay could allow synchronizing sexual signalling and spermatogenesis (Gribbins & Gist, 2003; Carretero, 2006). More generally, a peak of T that precedes the expression of male sexual behaviours has been documented in different squamate species (e.g., (Bonnet & Naulleau, 1996; Schuett *et al.*, 1997; Edwards & Jones, 2001; Graham *et al.*, 2008; Chamut *et al.*, 2012)).

283 The relative abundance of the protein fraction in the overall femoral secretion was quite variable
284 among lizards throughout the year. This variability may explain the scattered data and poorly
285 discernible oscillation of PP over time (Fig. 1C). Nevertheless, the protein fraction was higher in
286 early September, and reached the minimum in March. Being the complementary fraction, the lipid
287 component followed an opposite pattern compared to proteins, reaching the maximum (about 57%
288 of mass) in spring, just after lizards emerged from hibernation. The predominance of lipid secretion
289 matches the period when males start fighting to define territories and intensively court females
290 (Edsman, 1986; Sacchi *et al.*, 2009; Font *et al.*, 2012); thus, when the quality and intensity of sexual
291 signalling is expected to be maximized. The same trends for protein and lipid secretions were also
292 found in the green iguana (Alberts *et al.*, 1992b), albeit less pronounced (seasonal range relative
293 lipid content: 13 – 35% of secretion mass) compared to *P. muralis*. Consistently with these
294 findings, the (relative to cholesterol) provitamin D_3 abundance drops more than one hundred times
295 from early spring to early autumn. It has been experimentally shown that provitamin D_3 is involved
296 in the trade-off between sexual signalling and immune-system regulation in lizards (López &
297 Martín, 2005; Martín & López, 2007; López *et al.*, 2009): only healthy males are able to allocate
298 vitamins to femoral secretions without paying the cost of a reduced immune response. The
299 maintenance of a high content of $ProD_3$ in femoral secretions is physiologically demanding, thus
300 providing to males a mean to signal their quality to females during courtship (Zahavi, 1975; Grafen,
301 1990; Westneat & Birkhead, 1998). Taken together, these outcomes support the hypothesis that
302 lipids convey quality-related information (Martín & López, 2015).
303 Our results on protein secretion patterns suggest that they also contribute to the seasonal modulation
304 of sexual signalling. Femoral secretions contained approximately 17 bands (Fig. 2) that were
305 constantly expressed throughout the whole activity season. This band expression steadiness
306 supports the notion that proteins deposited in femoral secretions convey identity-related information
307 as shown in green iguana and wall lizard (Alberts & Werner, 1993; Mangiacotti *et al.*, 2017, 2018).
308 Because individual identity is not supposed to vary over time, individual protein signature is

309 expected to be stable (Dale *et al.*, 2001). Yet, beside this stability in terms of band occurrence, we
310 observed time variations in the relative expression of those bands characterized by a molecular
311 weight below 18 kDa or above 45 kDa. This variation correlates with lipid signalling: time
312 variations of relative protein expression were in phase with that of the lipophilic fraction (Fig. 1E,
313 F). Principal component analysis of EPGs (explaining 29.5% of variation) emphasizes the
314 seasonality of the relative expression of gel bands, not their mere occurrence. In other words, all
315 bands are expressed along the season, but their relative intensity changes markedly. This suggests
316 that protein signalling is not restricted to a simple and stable individual identity message.
317 From backward projection of predicted lanes (Fig. 2, left panel), seasonal EPGs reveal three distinct
318 regions subjected to different expression trends: the intensity of the bands below 18 kDa decreases
319 with season, while the intensity of bands above 45 kDa does the opposite; the intensity of bands in-
320 between does not vary over time. Therefore, the invariant component of EPG that codes for
321 identity-related information might be contained within the 18-45 kDa spectrum. Conversely, the
322 two variable regions of EPG cannot carry stable individual identity information. Instead, as their
323 variability parallels lipid variability, they may be involved in individual quality (or status)
324 signalling. For example, these proteins may constitute a suitable matrix enhancing the stability of
325 the lipophilic fraction (e.g., by preventing oxidation, or reducing their volatility; Gabirot *et al.*,
326 2008; Heathcote *et al.*, 2014; Martín *et al.*, 2016)). Alternatively (or additionally), some proteins
327 may carry their own informative function, and may be used to advise conspecifics about signaller
328 characteristics other than its identity and health status, i.e., the reproductive status or
329 aggressiveness. Like many lacertid lizards, males *Podarcis muralis* display a prenuptial
330 spermatogenetic cycle (Gribbins & Gist, 2003), and they are not able to produce fertile spermatozoa
331 after the breeding season (late June; Carretero, 2006). The switch between fertile and non-fertile
332 status may be signalled by the proteins in the gland secretions, and could be used in IC to modulate
333 interaction with rivals (e.g., territorialism, aggressiveness) or with females (e.g., attractiveness)
334 (Martín, Moreira & López, 2007b; Lattanzio, Metro & Miles, 2014). In this case, the protein-lipid

correlation would be an inevitable side effect of reproductive seasonality without involving any functional molecular relationship between lipids and proteins. Our results demonstrate for the first time that femoral protein patterns vary seasonally, bringing more questions than responses, but they reveal that the chemical language of lizards is more complex than previously known (Alberts, 1990; Alberts, Phillips & Werner, 1993; Font *et al.*, 2012; Mayerl *et al.*, 2015; Baeckens *et al.*, 2017c; Mangiacotti *et al.*, 2017).

Alternative, but not exclusive, hypotheses offer a framework to better understand how male lizards secrete complex and varying mixture of lipids and proteins (at least) to communicate with conspecifics of both sexes during the mating season. Experiments are needed to disentangle the respective roles of the different proteins secreted by femoral glands, and to assess their possible interplay with lipids. Lipids and proteins may act in synergy or not, and differentially on their targets (e.g., deterring rivals *versus* attracting coveted females). The physiological mechanisms that control seasonal changes of complex secretions are demanding in terms of chemical substrate and functioning (e.g., cascading hormonal regulations underpinned by specific alleles); their maintenance thus results from strong selective pressures. Overall, further studies combining laboratory and field investigations should focus on the protein part of the lizard chemical sexual language.

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TABLES

Variable	n	mean (*range*)	Pearson correlation coefficients					
			T	**SM**	**PP**	**proD$_3$**	**PS**	**SVL**
T	153	4.23 (*0.04, 38.93*)	–	0.43	-0.33	0.44	0.49	0.22
SM	158	1.27 (*0.08, 4.35*)	*153*	–	-0.42	0.50	0.59	0.36
PP	147	0.52 (*0.25, 0.96*)	*142*	*147*	–	-0.17	-0.46	-0.02
proD$_3$	86	-5.19 (*-10.70, -1.81*)	*83*	*86*	*82*	–	0.57	0.23
PS	155	0.00 (*-0.02, 0.02*)	*150*	*155*	*144*	*86*	–	0.15
SVL	165	62.63 (*50.00,70.00*)	*153*	*158*	*147*	*86*	*155*	–

Table 1. Descriptive statistics and Pearson bivariate correlation matrix of the monitored variables, and lizard size: n = sample size; mean (*range*) = mean and range of the observed values. Correlation matrix: upper triangle = correlation coefficients; lower triangle = bivariate sample size (italicized). T = hematic testosterone level; SM = secretion mass; PP = protein proportion; proD$_3$ = provitamin D$_3$ relative abundance; PS = protein signal; SVL = snout-to-vent length (mm).

Response	model	components	$\bar{D}$	$\bar{D}_p$	Δ	SE(Δ)
T	cosinor	4	223.7	228.7	0	
	linear	2	332.6	335.4	106.7	16.2
SM	cosinor	4	4.7	9.7	0	
	linear	2	113.0	116.0	106.3	15.3
PP	cosinor	4	-154.7	-149.6	0	
	linear	2	-134.1	-131.0	18.6	9.2
$proD_3$	cosinor	4	355.0	360.1	0	
	linear	2	408.5	411.4	51.3	8.4
PS	cosinor	4	1299.0	1304.1	0	
	linear	2	1377.7	1380.8	76.7	18.3

Table 2. Model comparison to assess the occurrence of a seasonal cycle in the monitored response variable. Cosinor (seasonal) model was compared to a simple linear model: components = no. of predictors in the model; $\bar{D}$ = mean expected deviance; $\bar{D}_p$ = mean penalized expected deviance (accounting for model complexity); Δ = difference between the largest and the smallest $\bar{D}_p$; SE(Δ) = standard error of the difference.

Response	M	A	φ	β_{SVL}
T	0.410	0.855	41.598	0.069
	(0.284, 0.529)	(0.703, 1.006)	(31.107, 51.440)	(-0.014, 0.148)
SM	-0.107	0.359	117.653	0.099
	(-0.168, -0.050)	(0.297, 0.420)	(103.396, 130.103)	(0.057, 0.135)
PP	0.509	0.082	244.653	0.008
	(0.473, 0.543)	(0.050, 0.120)	(216.114, 284.926)	(-0.016,0.030)
$proD_3$	-4.985	2.412	78.345	0.321
	(-5.599, -4.366)	(1.889, 3.088)	(60.907, 104.305)	(-0.058, 0.668)
PS	0.001	0.018	81.110	0.051
	(-0.002, 0.005)	(0.015, 0.022)	(63.752, 100.349)	(-1.774, 3.246)

Table 3. Cosinor parameter estimations for all the monitored response variables: M = mesor; A = amplitude (expressed in the variable scale); φ = acrophase; β_{SVL} = coefficient for the SVL term, added to each model to control for lizard size. For each parameter, the HSM (above), and HDI_{95} (below) are reported. For T and SM, M and A are log_{10}-transformed; φ is expressed as the Julian date (days from the 1st January).

FIGURES

Fig. 1. Graphical comparison of the cosinor models. A-E) Models predictions for the five response variables. In each plot: the thick black line joins HSM of the predictions for each date; the grey shaded area and the black dashed lines highlight HDI_{95} of the predictions; horizontal and vertical grey dashed lines represent HSM and HDI_{95} of the mesor and the acrophase, respectively; the small grey dots stand for the observed values. F) Acrophases comparison for the five models: thick grey and black segments represent HDI_{95} and HDI_{50}, respectively; grey shaded area highlights the HDI_{95} extent of the acrophase for the testosterone model. T stands for plasma testosterone level; SM for the overall secretion mass; PP for the protein proportion; $proD_3$ for the provitamin D_3 abundance; PS for the protein signal (score along the first principal axis of the principal component analysis of EPGs).

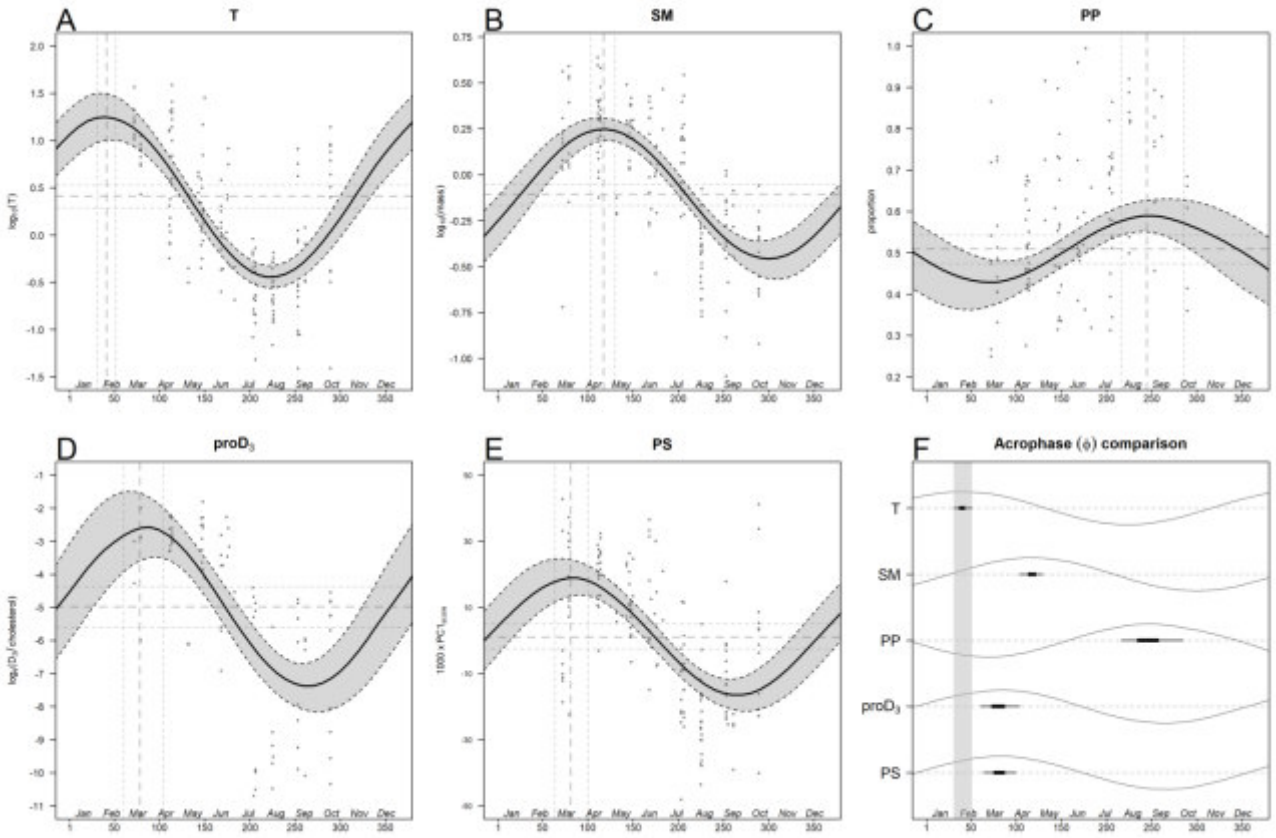
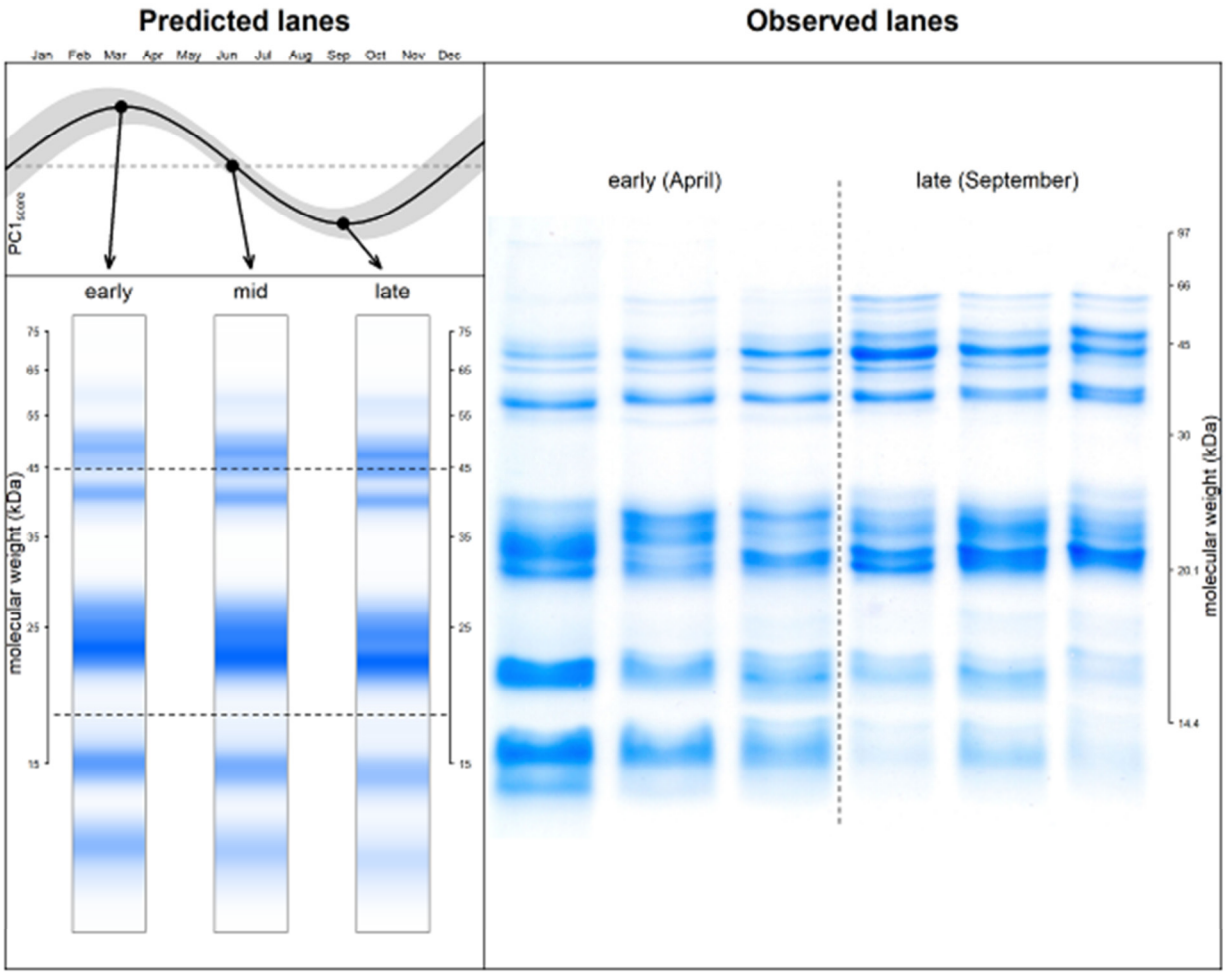


Fig. 2. Predicted and observed gel patterns throughout the season. Left panel: predicted lanes corresponding to the acrophase, mesor, and bathyphase of the protein signal (first principal component; $PC1_{score}$) as predicted by the cosinor model; horizontal dashed lines separate the upper and lower regions of higher variability. Right panel: six observed lanes chosen to represent the pattern of variation between the early (April), and late season (September); the vertical dashed line separates the lanes from each period; molecular weights are drawn on the right.



CHAPTER 6

Conclusions and remarks

General discussion

My thesis aimed at investigating the role of the proteins from femoral gland secretions in lizard intraspecific communication. The general framework was that proteins are able to convey information about signaller's identity or strategy, thus complementing the quality cues carried by lipids. I used both experimental and correlative approaches to test this hypothesis in the Common Wall lizard, a colour polymorphic species.

In **Chapter 2**, the one-dimensional electrophoretic patterns of Common Wall lizards secretions were proved to be high variable, with more than 13 clusters of proteins of different molecular weights. The occurrence of a large variability in a trait is the basis for it to potentially evolve as a signal (Beecher, 1989), and allows coding unique or discrete information as required to signal identity or strategy (Dale *et al.*, 2001; Tibbetts & Dale, 2007; Tibbetts *et al.*, 2017). A main outcome of this analysis was the occurrence of a large intra-population variation in bands presence/absence, which points out the individual as one of the main source of variability of the protein assemblage. Further, no correlation were found among protein patterns and any proxies for individual quality. These findings are coherent with the predictions of the identity-signal hypothesis.

However, some of the variability responded also to geographical origin of individuals. This could be explained by the genetic divergence among populations, or, alternatively, by the environmental differences among sites. Even if the two interpretations cannot be disentangled, the within-clade nesting of the among-populations variability suggests the genetic interpretation to be more likely: a similar investigation of the variability in protein patterns of two subfamilies of iguanas *s.l.* led to an analogous conclusion (Alberts, 1991; Alberts *et al.*, 1993).

An additional support to the hypothesis of the genetic basis of the observed variability came also from the comparison of the protein composition among the three main colour morphs (**Chapter 3**). Firstly, the two-dimensional electrophoresis increased the estimate of the number of proteins (or

protein fragments) potentially involved by more than three times, from less than 20 to more than 60. So, the amount of variability had been even underestimated by the first analysis (**Chapter 2**). Secondly, and more interestingly, the protein maps of the three colour morphs were sharply different, with spots varying in number and position: the white morph showed the richest pattern, while the red and yellow ones had a similar count, but with some unique spots each one. They shared near 60% of the proteins, with the remnant 40% making the difference. Unfortunately, the LC-MS analysis did not allow the identification of the proteins involved, due to the lack of an effective database against which matching the mass spectra. Nevertheless, the identified peptides confirmed the actual distinctness of the analysed proteins, supporting the potentiality for the proteinaceous signal to convey information about the strategy associated to each colour morph. Further, the occurrence of a multimodal signal (visual and chemical) for the strategy is expected (Johnstone, 1996), especially in territorial and polymorphic species, where mechanisms like assortative mating and morph-specific intrasexual competition occur (Pérez i de Lanuza *et al.*, 2013; Abalos *et al.*, 2016; Sacchi *et al.*, 2018a).

Together, **Chapter 2** and **3** showed that the variability of the proteinaceous component fulfilled the prerequisites for identity and strategy signals. But, are proteins actually used by lizards in chemical communication? The experiments using the isolated protein fraction from femoral glands (**Chapter 4**) gave a positive answer. Common Wall lizard males increased escaping attempts and peripheral exploration of the terrarium when proteins from femoral glands of unfamiliar males were used to scent-mark the substrate, proving they are not only able to detect proteins, but also to discriminate on this basis the scent owner (self or a never-met lizard). Furthermore, the degree of the response was independent from the concentration of the stimulus, demonstrating the absence of the proportionality in the response expected for a quality signal. Though many studies have already highlighted lizards ability to discriminate familiar and unfamiliar conspecifics using chemical cues alone (Alberts & Werner, 1993; Aragón, López & Martín, 2001; Font & Desfilis, 2002; Ladage, Ferkin & Ladagel, 2006; Aguilar, Labra & Niemeyer, 2009), and supported the hypothesis that

femoral gland secretions convey also identity-related information, none of them have investigated if specific component of the secretions were responsible for such a skill. Here, proteins seem to be the favourite candidate, and results are again coherent with the identity- or strategy-signal hypothesis.

So far, so good. Results from **Chapter 5** complicate the scenario. The signal composition changed over the activity season: notably, just a portion of the protein component was stably expressed, while some proteins increased or decreased their relative proportion. The seasonal variation of the protein was synchronous to that of quality cues, and delayed compared to testosterone oscillation. Given the relation between the glandular activity and the reproductive cycle, a quantitative drop in the expression of the whole signal should be expected only outside the mating period. It should also expected that quality components of the signal, being the most costly part, will pay more: indeed, the lipid fraction decreased. On the contrary, no qualitative variation should occur inside those parts which code identity or strategy. This was found to be true only for the stable slice of the electrophoretogram. Therefore, the part of the protein signal showing seasonality might be associated to other functions than identity- or strategy-signalling. A possible explanation is that some proteins may serve to increase lipids stability, and consequently they will vary according to the lipid abundance. Alternatively or additionally, some proteins may be still work as signals *per se*, but conveying other kinds of information, which depend upon time. In any case, the scenario goes complicating.

Concluding remarks

This thesis provided concrete basis to sustain the semiochemical role of proteins in lizard intraspecific communication: (1) the proteinaceous fraction of femoral gland secretions shows a well-structured variability, with distinct patterns at different levels, from the individual, to the morph, population, and clade; (2) lizards are able to use proteins to gain information about

conspecifics. Therefore, there seems to be no actual reasons to continue restricting the study of lizard chemical communication exclusively on lipids any more, assuming proteins to be less important or unable to contribute to the signal (Font *et al.*, 2012; Mayerl *et al.*, 2015). So far, a huge bias exists between the knowledge of the two components, lipids being far more studied and described than proteins.

The main limit of this thesis could be also seen as the starting point for future studies: I have not been able to identify the proteins occurring in Common wall lizards secretions. The lack of a good database to compare spectrometry data is probably the main cause of such failure; but it immediately suggests the aim of further researches: identifying the proteins may mean validating or widening the hypotheses on proteins role, decoding the signal, and shed light to the evolution of lizard chemical communication. Indeed, having found a meaningful seasonal variation in the protein signal, demonstrates that the starting hypothesis (proteins = identity; lipids = quality) is probably over-simplistic, and needs to be better tuned to reality. Protein identification might be the key, and may open other, even more interesting, questions.

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