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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

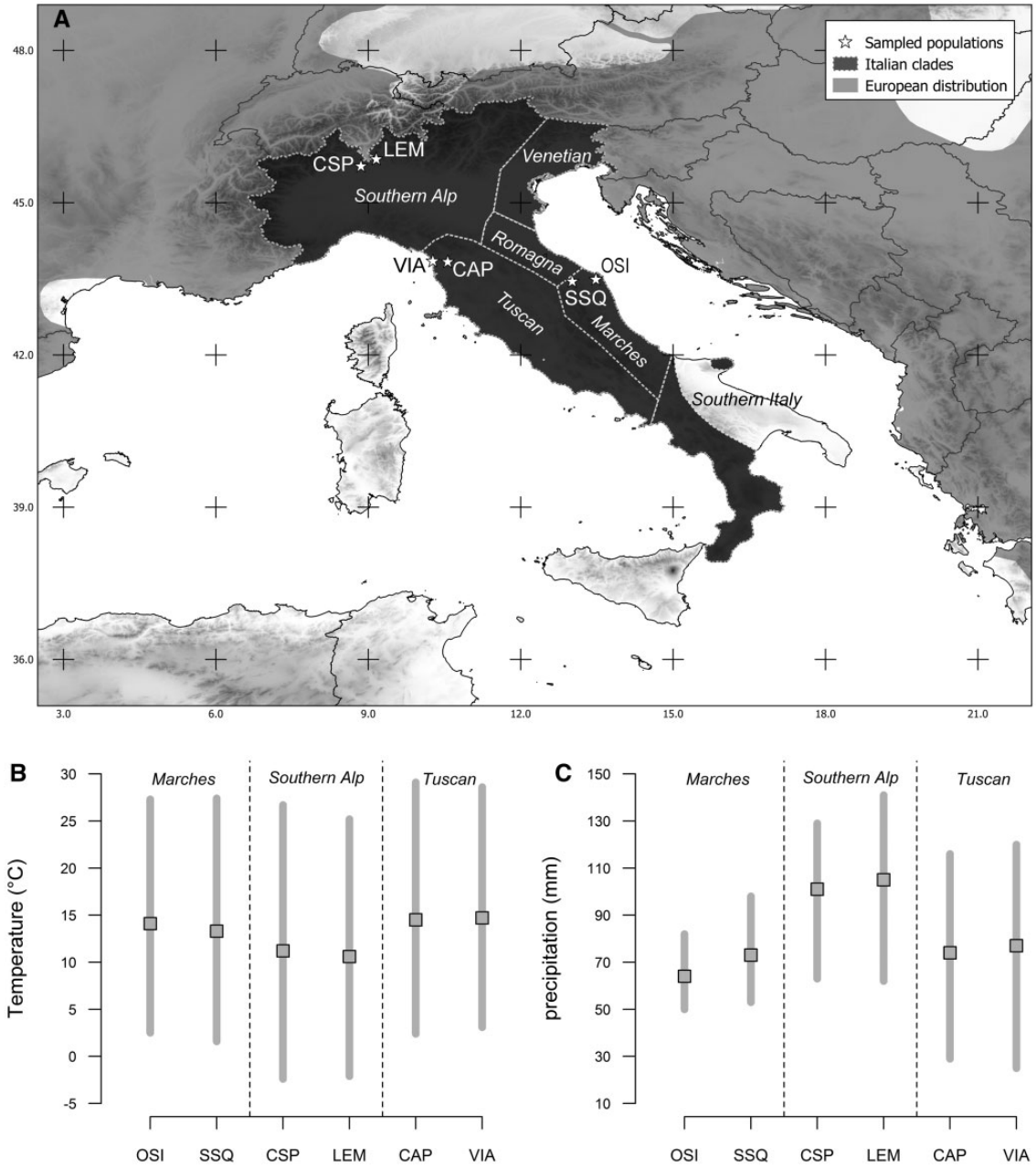

Figure 1. (**A**) Distribution map of Italian clades of the common wall lizard. The geographic delimitations of the clades follow Salvi et al. (2013). Stars represent the 6 considered populations, from North-West to South-East: Castelseprio (CSP), Lemna (LEM), Viareggio (VIA), Capannori (CAP), Serra 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.

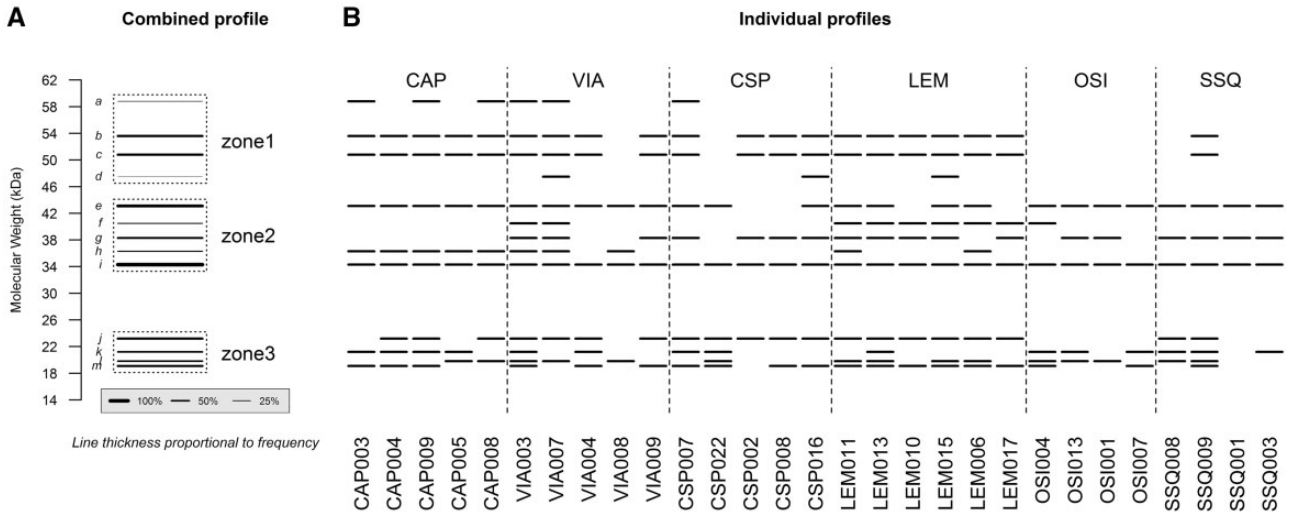

Figure 2. Schematized protein profiles after gel alignment and band detection. (**A**) Overall profile obtained by combining all the individual profiles: line thickness is proportional to the frequency of a band in the whole sample. (**B**) Individual profiles sorted by population of origin.

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

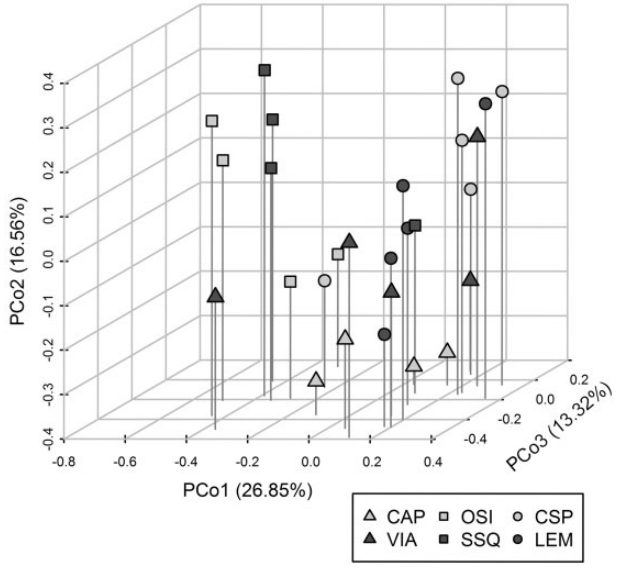

Figure 3. Principal coordinates analysis of the distance matrix computed on the individual protein patterns. The scores of the first 3 axes are used and the explained variance associated to each axis was reported as percentage. The shape of the symbols is clade-specific: triangles for Tuscan, squares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

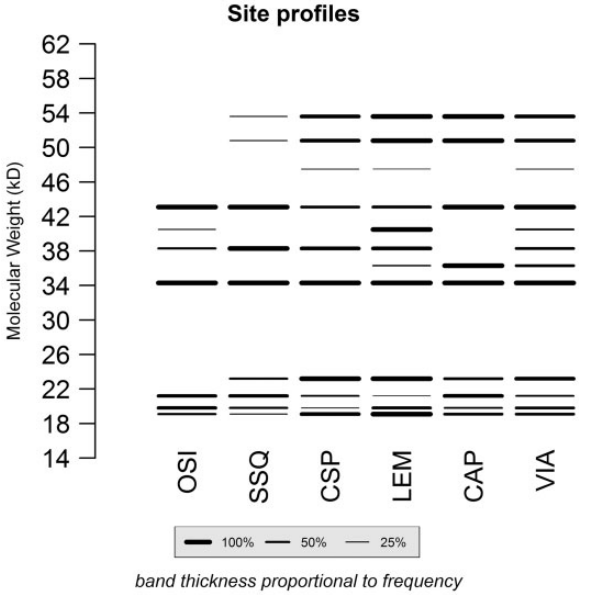

Figure 4. Weighted within-site protein profiles. The thickness of the bands is proportional to their frequency in each population.

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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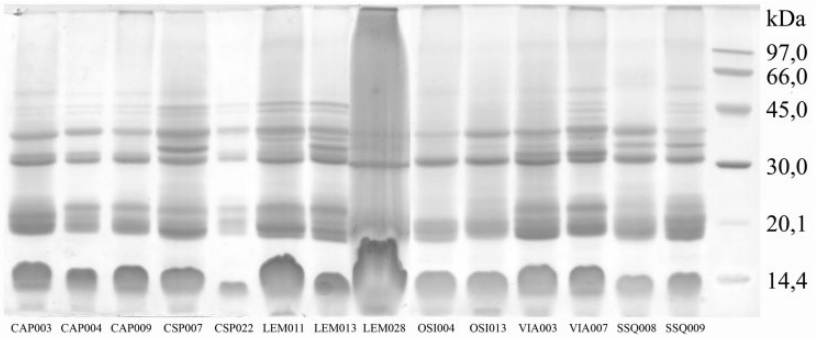
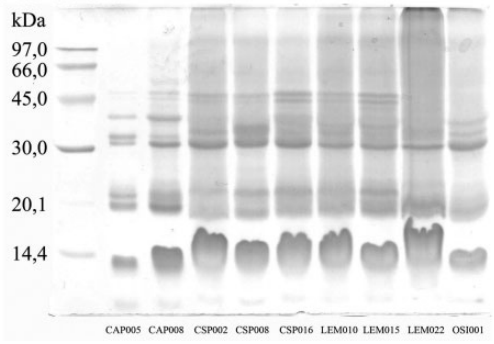
Appendix

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.

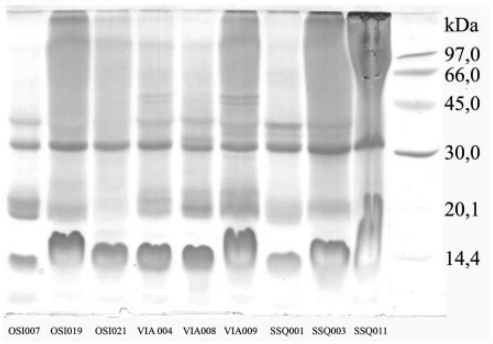
Gel #1



Gel #2A



Gel #2B



Gel #3