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Sexual Selection and Proteinaceous Diversity in the Femoral Gland Secretions of Lacertid Lizards

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Abstract: Sexual selection contributes to the diversity of chemical signals in various animal groups. Lizards are good model species to study how sexual selection shapes signal diversity, as they are a chemically oriented taxonomic group with different levels of social interactions. Many lizard species bear epidermal glands secreting a waxy mixture of lipids and proteins, which are used in intraspecific communication. Previous among-species comparative analyses failed to find a relationship between the strength of sexual selection with the composition of the lipid blend in lizards. Here, we extend the investigation to the proteinaceous fraction. By using a phylogenetically informed approach, we correlated the average electrophoretic profiles of the protein from the femoral glands of 36 lacertid lizard species with the level of sexual dimorphism in size and shape, which are proxies for the strength of sexual selection. We found that as sexual size dimorphism advances, five distinct molecular weight regions in the protein profile increased their expression. Using tandem mass spectrometry, we successfully identified one of these five proteins: a carbonic anhydrase—an enzyme catalyzing the reversible hydration of carbon dioxide. Our findings suggest that proteins may be the target of sexual selection, as an active semiochemicals or as a dynamic support to other molecules: sexual selection may act indirectly on semiochemicals (namely lipids) by modifying the matrix (namely proteins).

Keywords: chemical communication; sexual selection; sexual dimorphism; proteins; carbonic anhydrase; femoral glands; Lacertidae

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1. Introduction

While less conspicuous to humans than other sensory modalities, chemical signals are the most ubiquitous form of information exchange in the natural world and their diversity is probably comparable to or even greater than that of visual and acoustic signals [1–4]. As such, the same questions about the mechanisms responsible for the origin and evolution of signal diversity in the visual and acoustic sensory channels also apply to the chemical modality [5–7], and sexual selection should be considered a key driver [8–11]. Both experimental and correlative studies have indeed shown how sexual selection can influence the evolution and design of chemical signals involved in both mate choice as well as intrasexual competition [9–11].

Lizards are a well-known chemically oriented vertebrate group [12,13], and in the last decade, they have increasingly been used as a model system to study chemical communication [14,15]. Recently, the occurrence and degrees of sociality in lizards have been

linked to the species' investment in chemical signaling [16], highlighting the importance of chemical communication in mediating intraspecific interactions. Lizards, indeed, use chemical signals to inform conspecifics about a variety of "socially interesting" features (e.g., territory ownership, individual identity, familiarity, size, parasite loads, health status, fighting ability, immune-response [17–27]). Further, specialized secretory structures show a male-biased sexual dimorphism in most species [28,29], combined with an activity that peaks during the reproductive season [30–32]. Together, all these observations lead to the hypothesis that sexual selection may play a pivotal role in the evolution of lizard chemical signals [33].

A first attempt to test whether and how sexual selection has contributed to shaping lizard chemical signal diversity focused on the Lacertidae family [33]. Lacertids are distributed over much of Eurasia and Africa and include about 350 species of typically small-to-medium-sized lizards [34–36]. Chemical communication has been extensively studied in lacertids over the last 15 years or so, notably concerning the femoral glands (FG) [14,15,29,37]. FG secretions consist of waxy protein–lipid mixtures [29,38], both components being active in communication [26,37,39,40]. Lipids, being more volatile and easily associated with male quality- and condition-related traits, have been historically considered of superior importance in functioning as semiochemicals compared to proteins [2,29,37,41]. On this basis, Baeckens and colleagues [33] reconstruct the evolutionary history of the lipid fraction of the secretions across the Lacertidae phylogeny but were not able to detect any significant effect of sexual selection on trait evolution: lipid composition, richness, and diversity did not covary with the expression of sexual dimorphism in shape or size, used as proxy for the strength of sexual selection [33]. Among the many possible interpretations of this unexpected outcome proposed by the same authors, it was pointed out that the indirect measures of signal structure (proportion of the main lipid categories) and complexity (number and diversity of compounds) might not have captured the signal features actually targeted by sexual selection [33]. Here, we put forward the possibility that the inclusion of the protein fraction in the analysis may add valuable information to the above-mentioned approach, at least because of three confirmed qualities of FG proteins, i.e.: (i) the occurrence of intraspecific variability at individual level in protein profiles [38,42,43]; (ii) the capability of proteins alone to convey socially relevant information to conspecifics [26,39,40]; and (iii) the seasonal variation in the relative abundance of the protein components, which follows the reproductive cycle and is synchronous to that of lipids [30]. The synchronized variation in protein abundances with lipids leads us to hypothesize that proteins may be involved in sexual signaling [44], therefore also being a potential target for sexual selection to act on, which eventually contributes to the observed diversity of FG proteins [43,45].

With the present study, we aimed to assess whether sexual selection may have contributed to the variability of the protein fraction of FG secretions. Following [33], we applied a phylogenetically informed generalized least square models to the protein profiles of 36 lacertid species [46], using the level of sexual dimorphism as a proxy for the strength of sexual selection [33]. We then used tandem mass spectrometry to identify the potentially involved proteins.

2. Materials and Methods

2.1. Data Collection

We assembled three distinct datasets at the species level for the Lacertidae family, notably concerning: (i) the proteinaceous composition of the femoral gland secretions; (ii) the average body and head measures of males and females, upon which to build sexual dimorphism indexes; and (iii) the phylogenetic tree of the species. Overall, the datasets included complete information about 36 lacertid species.

The information about the composition of the femoral gland proteins were obtained from the already published data [46]. Data consisted of species-level one-dimensional normalized electrophoretic profiles (electrophoretograms, EPGs) from 36 lacertid species. Data came only from males, since they were not available for females, which, at least in the

considered species, bear almost vestigial glands [29,47] or produce very little amounts of secretion [48,49]. Each specific EPG corresponds to a sequence of 300 values representing the relative amount of protein clusters ordered by their molecular weight and averaged across individuals from the same species [50]. Although some degree of within-species variability can occur [38,46], such variation is less than the among-species one [46] and typically affects the relative height of some peaks rather than the overall band pattern [46]. Therefore, we can reasonably assume EPGs to represent a raw proxy of species FG protein composition [30,42,46,51]. Indeed, protein identification is still challenging [45,52], and, as far as we are aware, proteins from femoral gland secretions have been characterized in only two lizards [43,45], one of which does not belong to the lacertid family (but see [42]). For the same reason, as a working hypothesis, we assumed that each electrophoretic band corresponded to a single protein.

Following Baeckens et al. [33], for each species, we computed two indexes of sexual dimorphism (SD), which may be used as proxy for the strength of sexual selection [33]. The first is a sexual size dimorphism index (SSD) obtained as the ratio of the snout-to-vent length (SVL) of males compared to females ($SVL_{♂}/SVL_{♀}$) when males were larger than females, and 2-($SVL_{♀}/SVL_{♂}$) if the opposite occurred [53,54]. This way, values larger than one indicate male-biased SSD, while values lower than one are for female-biased SSD. In lizards, male-biased SSD is usually associated with the increase in male–male intrasexual competition [55–57], since larger males are favored in male–male combats [58,59]. Besides overall size, head size also affects male fighting abilities [60,61]. Consequently, male-biased SD in relative head dimensions can also be informative about the strength of intrasexual competition and therefore sexual selection [33,57]. We computed the sexual head dimorphism index (SHD) using the same formulas as for SSD, by substituting SVL with the relative head size (head length/trunk length). The morphometric measures needed for the computation of SSD and SHD indexes were obtained from the literature (Table S1).

Finally, the phylogenetic tree of the 36 considered species was obtained by pruning the lacertid phylogenetic available in [36]. We used the keep.tip function of the ape R package [62] to subsample it to match the 36 species included in the study.

2.2. Statistical Analysis

To assess the occurrence of any effect of the strength of sexual dimorphism on the composition of the protein fraction of the femoral gland secretions, we used multivariate phylogenetic generalized least squares (pGLS) models, adapted to high-dimensional datasets [63]. In such a model, the matrix of normalized EPG (proxy for protein composition) was the response variable, while SSD and SHD entered the model as predictors. Being compositional in their nature, EPGs were centered-log–ratio transformed before entering the analysis [64,65].

To account for the phylogenetic dependency of the data, the correlation structure of the error was set according to five different models of continuous trait evolution: Brownian motion (BM), Ornstein–Uhlenbeck (OU), early burst (EB), late burst (LB), and Pagel's λ (PA). The best model was then selected according to the Extended Information Criterion (EIC), an equivalent of the Akaike Information Criterion suited for models fitted using penalized likelihood [63]. The significance of the SSD and SHD terms were assessed for the best model using a permutational MANOVA [66]. All the analysis were implemented in R v.4.1.2 [67], using the package mvMORPH [68].

2.3. SSD-Related Protein Identification

Phylogenetic GLS highlighted a significant effect of SSD on EPGs (see results). To identify the potentially involved proteins, we ran novel electrophoretic shots for those samples (*Acanthodactylus scutellatus*, *Gallotia stehlini*, see results) showing the highest EPGs peaks just in the molecular region associated with the increase in SSD. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the selected samples followed the methodology described in [30]. From the fresh gels, we excised the bands of the regions

of interest (Figure S1) and individually prepared them for mass spectrometry analysis adapting procedures from [69] (see Supplementary Material M1). Mass spectrometry (MS) analyses were carried out with a LC unit (ExionLCTM AD) equipped with a column oven thermostated at 40 °C, an autosampler cooled at 10 °C, and a binary gradient pump system. The MS instrument consists of a high resolution QTOF mass spectrometer (X500B, AB Sciex LLC, Framingham, MA, USA) equipped with a Turbo V Ion source and a Twin Sprayer ESI (electrospray ionization) probe, controlled by SCIEX OS v2.1 software (AB Sciex LLC, Framingham, MA, USA) (see Supplementary Materials M1 for setting details).

We performed protein identification via peptide-spectrum matching [52,70] using MS-GF+ v2022.01.17 [71,72]. We set the algorithm as follows: tolerance, 20 ppm; charge range, 1–6+; range of peptide length, 6–70; isotope error 0–1 Da; cleavage, tryptic; and post-translational modification, fix carbamidomethylation of cysteine [52,73,74]. Since no ad hoc database was available for the study species, searching was performed against UniProt dataset [75], after filtering for the taxonomic family "Lacertidae" (taxonID = 8522). To control for contaminants, we added pig trypsin sequence (accession: sp | P00761) to the database. The final dataset included 46,935 unique sequences. Only spectra with false detection rate (FDR) ≤ 0.01 were considered. All the above operations were implemented in R v4.1.2 [67], using the packages mzID v 1.28.0 [76], Biostrings v2.46.0 [77] and ad hoc functions to prepare database and call external software (MSGF+).

3. Results

The expression of sexual dimorphism is quite variable across the 36 considered species (Figure 1), both considering body size (SSD) and head size (SHD), with nine cases and one case of female-biased SSD and SHD, respectively. Furthermore, SSD and SHD did not behave coherently, almost showing a negative correlation, which highlights that the two indexes might be related with different behavioral traits (as discussed in [33]).

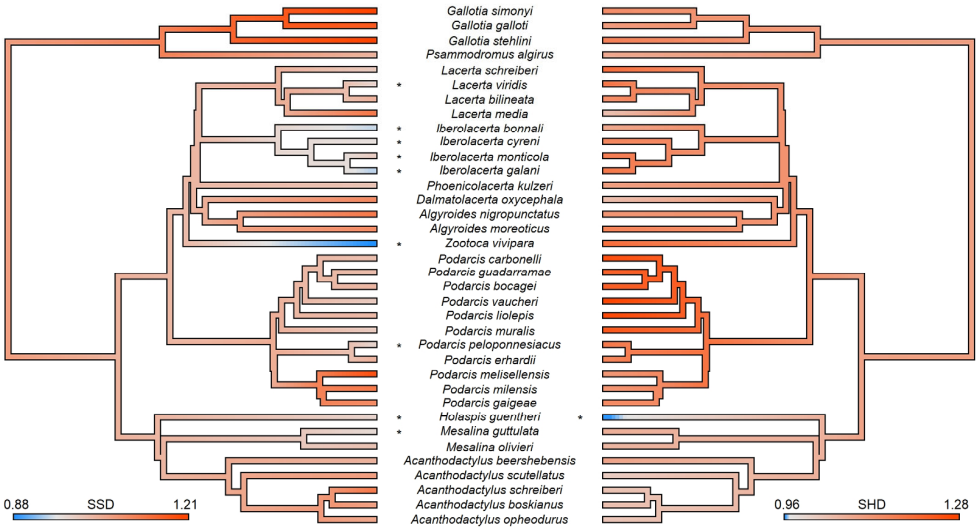

Figure 1. Phylogenetic map of the expression of sexual size dimorphism (SSD; left tree) and sexual head dimorphism (SHD; right tree) in the 36 lacertid species included in this study. Light blue and asterisk indicate female-biased dimorphism; the orange gradient represents the intensity of male-biased dimorphism.

According to EIC, the best evolutionary model explaining SD-EPG relations was LB, which performed slightly better than OU and much better than EB, PA, and BM (Figure 2).

Permutational MANOVA of the best model highlighted a significant effect of SSD (Pillai's trace = 0.613, $p \leq 0.046$; 9999 permutations), while SHD had no effect (Pillai's trace = 0.523, $p \leq 0.403$; 9999 permutations). Keeping constant SHD, an increase in SSD corresponded to a raise in the concentration of proteins from five main molecular regions of the predicted EPG, approximately at 9.0, 13.5, 17.9, 35.4, and 66.3 kDa (Figure 3).

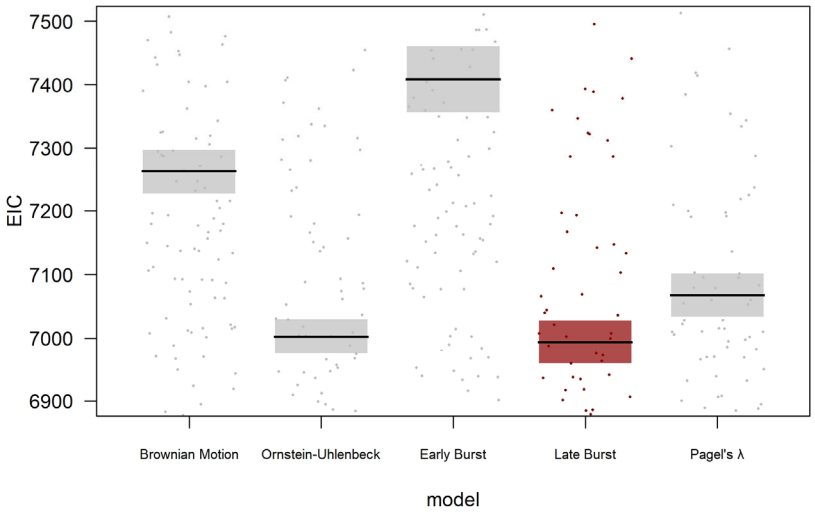

Figure 2. Bootstrapped extended information criterion (EIC) values for the five evolutionary models used to account for the phylogenetic dependences between protein electrophoretic profiles and sexual dimorphism in the phylogenetic generalized least squares model. Solid lines = mean value; shadowed rectangles = ±1 SE interval around the mean; grey points = observed bootstrapped value for each model (100 in total). The best model (lowest EIC) is dark-red colored.

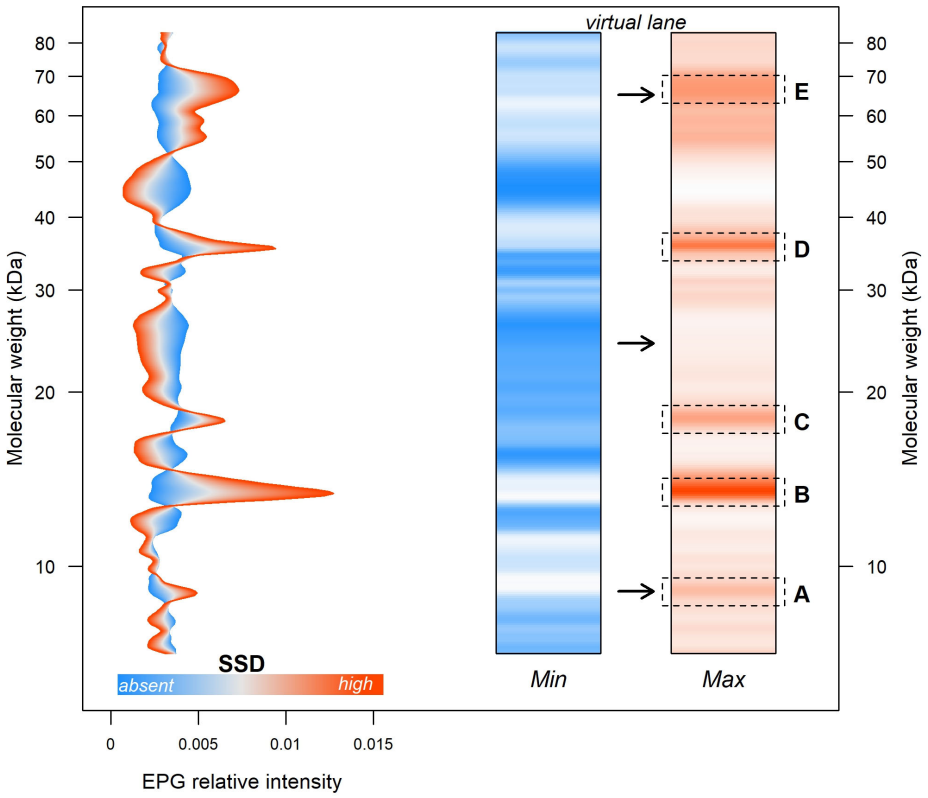

Figure 3. Predicted protein profiles following male-biased sexual size dimorphism (SSD) increase. **Left side**: predicted electrophoretic profile (EPG) spanning from the absence of SSD (unit value; light blue) to the maximum (orange) observed SSD. **Right side**: virtual gel lane corresponding to the predicted EPG at the unit (light blue) and maximum (orange) SSD value, respectively: these are a translation of the relative peak heights of the profiles reported in the left side into a color intensity strip resembling a gel lane theoretically obtainable in a virtual electrophoretic run. Dotted boxes A–E denote the molecular region most associated with the SSD increase, which were the focus of the MS analysis.

SSD-Related Protein Identification

The species showing the highest EPG peaks in the region associated by pGLS with SSD were *Acanthodactylus scutellatus*, for the lower bands, at 9.0 and 13.5 kDa (bands A, B; Figure 3), and *Gallotia stehlini*, for the upper ones at 17.9, 35.4, 66.3 kDa (bands C, D, E; Figure 3). The novel SDS-PAGE allowed identifying the target bands (Figure S1), which were excised and analyzed by MS.

Peptide-spectrum matching against Lacertidae database allowed identifying peptide sequences from all samples (Table S2). After filtering by contaminants (i.e., pig trypsin) and FDR < 0.01, only bands B, C, and D produced reliable identifications at peptide level (Table 1): two spectra matched three different database entries for band B; a single peptide was selected for band C and D. The above results prevent any identification at protein level. Nonetheless, considering the high scores, the relative coverage, and the approximate correspondence of predicted molecular weights with band positions in the gel, two peptide identifications may be also credible at protein level (Table 1): UPAR/Ly6 domain-containing protein (band B) and carbonic anhydrase (band D). Since the lack of specific databases for the considered species may have lowered the power of peptide-matching search [52,78], we tried refining the analysis of MS data from these bands by building ad hoc protein datasets basing on the outcomes of the first analysis [79]. Notably, we extracted from UniProtKB [75] all those protein sequences sharing at least one third of the amino acids with the two potentially identified proteins (uniprotID: A0A670IP55; A0A670JE51). We used BLASTP v.2.12.0 [80,81], available as an online tool on the uniport.org platform, to retrieve the target sequences. If the protein was correctly identified, we expected peptide-matching to improve its performance and to match more entries than against the Lacertidae database. Therefore, we re-ran the peptide-matching search against the above-obtained UPAR/Ly6 database (372 sequences) for band B, and Carbonic anhydrase database (990 entries) for band D. The approach was revealed as useful for band D (carbonic anhydrase; Table 2), for which three more peptides were identified, matching fish sequences, and making the protein identification credible. The performance was poor for band B (UPAR/Ly6 domain-containing protein; Table 2), with only a single peptide added to the list, thus not providing enough support to protein identification.

Table 1. List of the identified peptides with a false detection rate below 0.01. For the complete list, see Table S2. Band = gel region considered in the MS analysis as in Figure 3 and Figure S1; ID = spectrum ID; score = MSGF+ spectrum E-value (−log10 transformed); error = difference between measured and calculated parental ion mass (Da); UniProtID = unique uniprotKB identifier; description = uniprotKB description; coverage = percent protein sequence covered by the identified peptide; MW = predicted molecular weight (kDa) of the corresponding protein.

Band	ID	Peptide	Score	Error	UniProtID	Description	Coverage (%)	MW
B	3544	SCIDTELCDVGYGSASITSSMYIQSK	9.374	0.036	A0A670IP55	UPAR/Ly6 domain-containing protein	17.81	15.8
	2948	AHDGIR	8.026	−0.009	A0A670JC88	Zinc finger protein 436-like	1.16	58.8
	2948	AHDGLR	8.026	−0.009	A0A670JYQ1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1	0.61	109.1
C	3168	QMIKINFK	8.341	−0.005	A0A670KI84	COP9 signalosome complex subunit 2	1.78	52.4
D	3157	YSMELHIVHTK	14.856	0.005	A0A670JE51	Carbonic anhydrase	4.25	28.4

Table 2. List of the identified peptides with false detection rate below 0.01 using ad hoc databases for UPAR/Ly6 and Carbonic anhydrase. Headings as in Table 1; already identified peptide sequences are italicized; identity = % sequence identity with the query protein in BLASTP.

Band	ID	Peptide	Score	Error	UniProtID	Identity (%)	Description	Organism	Coverage (%)	MW
B	3544	*SCIDTELCDVGYGSASITSSMYIQSK*	9.434	0.036	A0A670IP55	100.0	UPAR/Ly6 domain-containing protein	*Podarcis muralis*	17.81	15.8
	2908	REERPR	5.558	−0.008	A0A8C5QXT2	37.4		*Leptobrachium leishanense*	2.76	23.1
D	3157	*YSMELHIVHTK*	14.836	0.005	A0A670JE51	100.0	Carbonic anhydrase	*Podarcis muralis*	4.25	28.4
	3184	EPITHYIPACRQVNR	6.191	0.970	A0A8C1L806	41.4		*Cyprinus carpio*	4.44	36.9
	2893	MELHVVNK	6.191	1.020	A0A6P6QLI1	38.2		*Carassius auratus*	2.48	35.8
	3816	ANDSSALAVLGFFIEGTDEADK	5.667	0.982	Q08C20	38.6		*Danio rerio*	6.79	35.1

4. Discussion

Sexual size dimorphism correlates with the protein electrophoretic profiles of femoral glands secretions across the Lacertidae family. Five distinct molecular weight regions in the profile increased their expression with increasing SSD level. Out of these five proteins, one, at 35.4 kDa (band D, Figure 3), was successfully identified via tandem mass spectrometry, namely carbonic anhydrase, an enzyme catalyzing the reversible conversion of carbon dioxide into bicarbonate [82].

The significant relationship between SSD and EPGs suggests that sexual selection contributes to shaping the chemical signal design in lizards. Previous studies that focused on single species found FG secretions to impact both mate choice [18,83–87] and male intrasexual competition [20,23,88–91]. Further, specific compounds have shown to convey information about male quality-related traits, such as immunity, parasite load, and fighting abilities [17,19,20,27], implying that FG secretions act as sexual signals and, consequently, should be governed by sexual selection. In this light, our findings, based on a multi-species, phylogenetic approach, provide general support to this hypothesis, and bring along more specific underlying questions: i.e., how are proteins involved in sexual signaling, and how does sexual selection act on them. Unfortunately, the lack of a complete identification of the target proteins allows us to just delineate some possible interpretative scenarios.

On the one hand, being active in communication [26,39,40], FG proteins may convey information about male quality, which can be used to drive decision making (i.e., mate selection, rival assessment [14,33,85,90]), thus offering the basis for sexual selection to act on [8,37,92]. Aside from the semiochemical function of proteins, there is weak support for such interpretation: the occurrence of variation in EPGs at inter-individual level [38,42,43]; the EPGs changes between the reproductive and non-reproductive season [30]. Unfortunately, in the few studies investigating this aspect, no direct correlation was found between protein composition and traits linked to the signaler's quality (e.g., size or body condition [30,38]). Furthermore, in the two lizard species (i.e., the Galapagos marine iguana, *Amblyrhynchus cristatus*, and the sand lizard, *Lacerta agilis*) for which FG proteins have been preliminarily characterized, no proteins easily relatable to such functions have been identified [43,45].

Although peptides and proteins may be actually used in sexual communication in other vertebrates (e.g., [44,93–95]), in lizards they are more probably involved in conveying identity-related, rather than quality-related, information [26,40,46,69]. From this perspective, it could be argued that proteins associated with SSD increase may allow for a more accurate individual discrimination: it could indeed be expected that distinguishing among very similar individuals may become pivotal when sexual selection is stronger and the cost for inaccuracy higher [25,90,96,97].

An alternative interpretation is suggested by the only identified protein among those associated with SSD increase. Carbonic anhydrase is a basic and ubiquitous enzyme [98], also found in vertebrate secretions (e.g., milk, saliva, tears [99–101]). The same enzyme has also been identified in the FG secretions of Galapagos marine iguana [45] and sand lizard [43], making our identification in *Gallotia stehlini* even more robust and reliable. By catalyzing the hydration of carbonic dioxide, carbonic anhydrase fundamentally acts on system homeostasis [99], conferring FG secretion the potential ability to react to the chemo-

physical changes caused by the environmental conditions where it is left [45]. By extension, FG proteins may provide dynamic support to the other components of the mixture, eventually determining the overall chemo-physical characteristics of the signal or influencing the semiochemical properties of specific compounds. In the Asian Elephant [102], for example, female urine is enriched with a serum albumin, which serves for transport, to extend lifespan, and to improve detection by males via the sexual pheromone, (*Z*)-7-dodecenyl acetate. This way, sexual selection has favored the recruitment of an already available protein for a novel function, supporting that of the semiochemical [102]. Similarly, in mice, major urinary proteins (MUPs), besides working as semiochemicals conveying individual identity information themselves, bind volatile molecules (e.g., dehydro-exo-brevicomin and 2-sec-butyl-4,5-dihydrothiazole) used by females to assess the signaler status and for mate choice [102]. MUPs are able to extend and enhance the effectiveness of the airborne small molecules [103]. In this case, sexual selection has promoted the evolution of proteins with both direct and indirect functions. Applied to lizard FG secretions, sexual selection may have acted indirectly on the semiochemical portion of the signal (namely lipids), by modifying the matrix (namely proteins) of the blend, and our analysis may therefore have detected such an indirect effect.

Although speculative, the above rationale provides a testable prediction. We indeed expect to find a non-random association between the expression in the SSD region of EPG and some lipophilic compounds occurring in the mixture (which should represent the semiochemical counterpart); more precisely, those same lipids showing correlation with mate choice or rival assessment [14,41]. One can argue that such prediction has been already disregarded by the previous analysis performed on FG lipids [33], which failed to track any effect of sexual selection. Yet, the analysis on lipids grouped them into a priori chemical classes. According to our hypothesis, they should be specific semiochemical molecules to undergo sexual selection, and consequently, the class-level categorization of the lipophilic fraction may have masked the relation with SSD. Indeed, different lipids, even from the same chemical category, can follow independent evolutionary trajectories [104]. The same issue probably did not occur in the analysis of the protein counterpart because it has not been a priori reduced into categories, and it is probably less complex than the lipophilic mixture [38,69] (but see [43,45]). At the time of writing, a new study [105] found that the abundance of provitamin D_3 (a lipophilic component) in FG secretions is associated with the same increase in the relative expression of two FG proteins: carbonic anhydrase and protein disulfide isomerases, the former corresponding to the one we have identified in the current study, the latter matching the molecular region predicted by our model (band B; Figure 3). High proportions of provitamin D_3 in FG secretions are associated with a high-quality immune system in male wall lizards [18] and increases attractiveness towards females [106]. These findings corroborate our predictions and support our hypothesis concerning possible protein roles.

Finally, we found that sexual dimorphism in size and shape showed no apparent correlation. This makes the interpretation of the relation between SSD and SHD with sexual selection more complex. While male-biased sexual size dimorphism is typically linked to male intrasexual competition [55–57,107], sexual dimorphism in the relative head/trunk proportion may also possibly reflect other selective forces [55,107], which may eventually weaken or mask the relationship with sexual selection. Baeckens et al. [33], using a larger sample of lacertid species, found SSD and SHD to be negatively correlated, suggesting that a trade-off may occur between the two traits (size and shape). As the same author suggested, this is a noteworthy point which requires further investigation.

In conclusion, we acknowledge the preliminary and correlative nature of our results and interpretations, as well as the simplification we made in assuming a one-to-one relation between protein profiles and protein composition (by corresponding each band to a single protein). Nonetheless, we are cumulating evidence that proteins constitute more than a passive matrix for more volatile compounds, and they may be an important component

of the signal. This indicates that further efforts are required to characterize them both chemically and functionally.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15060777/s1, Methods M1: Protocol details for mass spectrometry analysis; Figure S1: SDS-PAGE of the samples used to excise the bands associated with the SSD increase and analyzed by mass spectrometry; Table S1: Morphometrics measures used for the computation of the sexual dimorphism indexes; Table S2: Complete list of the identified peptides from tandem MS.

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