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Seasonal Variations in Femoral Gland Secretions Reveals some Unexpected Correlations Between Protein and Lipid Components in a Lacertid Lizard

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Abstract

Animals modulate intraspecific signal shape and intensity, notably during reproductive periods. Signal variability typically follows a seasonal scheme, traceable through the expression of visual, acoustic, chemical and behavioral 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 gland activity is underpinned by variation of circulating androgens. Changes in the composition of lipid secretions convey information about the signaler'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 concentration of provitamin D_3 in the lipid fraction. We sampled 174 male common wall lizards (*Podarcis muralis*) over the entire activity season. An elevation of T was observed one to two months before the secretion peak of lipids during the mating season; such expected delay between hormonal fluctuation and maximal physiological response fits well with the assumption that provitamin D_3 indicates individual quality. One-dimensional electrophoretic analysis of proteins 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.

Keywords Chemical communication · Season · Testosterone · Quality · Identity · Femoral glands · Cosinor models · Lizards · *Podarcis muralis*

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Introduction

Seasonality affects many biological functions of vertebrates and invertebrates, notably in temperate and polar zones (Crews 1984; Paul et al. 2008). 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). Consequently, the whole set of physiological, behavioral, 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). Complex, often multimodal, signals are costly to produce and to maintain (Johnstone 1997), and they entail predation risks (Magnhagen 1991). Therefore, signalers that could modulate signal production, save resources, and reduce risks have been

favored by selection (Johnstone 1997). For instance, shape and intensity of signals are typically reduced outside the mating season (McGraw and Hill 2004), losing their ability to trigger a receiver's response (Aguilar et al. 2009). Similarly, the receiver sensitivity to conspecific signal also decreased outside the breeding period (Dawley and Crowder 1995). Lizards offer suitable models to study intraspecific communication plasticity associated with reproductive cycles (Baeckens 2019; Whiting and While 2017). Most species breed "seasonally" and use multimodal signals of various complexity (Whiting and While 2017). The chemical modality is particularly important in lizards (Baeckens 2019), and it is associated with the development of peculiar traits: i) the vomeronasal organ combined to tongue-flicking behavior (Schwenk 1995), and ii) specialized epidermal glands in the cloacal region used for intraspecific communication (Mayerl et al. 2015). The femoral (or pre-cloacal) glands are more developed in males than in females (Mayerl et al. 2015), and their activity is stimulated by an increase of androgen levels (Baeckens et al. 2017), peaking during the breeding season (Alberts et al. 1992a).

The gland secretion is a complex waxy mixture of lipids and proteins (Alberts 1990; Mangiacotti et al. 2017), which may be used by conspecifics to retrieve information about various signaler's features, like size, fighting abilities, parasites load, immunity (Martín and López 2015), but also familiarity (Alberts and Werner 1993), and individual identity (Carazo et al. 2008). Therefore, lizards can use femoral secretions to deliver sophisticated messages. Even though they can detect both lipids and proteins (Alberts and Werner 1993), only the formers have been thoroughly studied, and partly associated to condition- and quality-traits of the signaler (Martín and López 2015). Proteins have received far less attention (Mangiacotti et al. 2017; Mayerl et al. 2015). Preliminary data suggest that they can be used in intraspecific communication in general (Alberts 1990; Mangiacotti et al. 2017; Mangiacotti et al. 2019a), and, more specifically that they may convey information about signaler's identity (Alberts and Werner 1993; Mangiacotti et al. 2019b). Individual identity signals are expected to evolve when the signaler pays the cost of being misidentified, which is quite common in those social contexts where individuals may interact repeatedly (Tibbetts and Dale 2007). Lizards are often territorial and poorly mobile species (Whiting and While 2017). Hence, they may benefit from an individual recognition system which helps modulating neighborhood dynamics (Carazo et al. 2008), or establishing dominance relationships (López and Martín 2001), thus reducing the cost of aggressive interactions (Tibbetts and Dale 2007). So, it could be hypothesized that the complex lipid and protein mix allows the signaler to simultaneously inform the receiver about both its quality and identity. Notably, we may expect some lipids to be more suited to convey quality-related information, and some proteins to signal the identity-related counterpart (Alberts and Werner 1993; Dale et al. 2001; Mangiacotti et al. 2019b).

The importance of delivering a comprehensive message is maximal during the breeding season, when efficient communication pays off. Accordingly, 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). In the green iguana (*Iguana iguana*), the proportion of more volatile unsaturated fatty acids increases during the mating period, thereby enhancing signal detectability (Alberts et al. 1992b). This suggests that the lipophilic fraction may undergo a qualitative modification across the year. Knowledge about the seasonal variation of the protein content of femoral secretion is scanty and anecdotal. If proteins convey identity-related information, no temporal variation should be expected in this signal component (Dale et al. 2001). A partial support to such prediction comes again from iguanas (Alberts 1990; Alberts et al. 1993), where the protein electrophoretic pattern is reported not to change throughout the season. Unfortunately, no quantitative data about the relative abundance of the different protein clusters in the electrophoretic pattern is available, thus preventing to know whether proteins actually form stable cues across time or not.

In order to expand the knowledge of lizard intraspecific chemical communication and shed light on the role of the protein component of the signal, for the first time, we analyzed the seasonal variation of the femoral gland secretions focusing on the relative proportion of the proteinaceous and lipophilic fractions, the stability of the protein electrophoretic pattern, and the co-variation with hematic testosterone level, known to control glandular activity (Baeckens et al. 2017; Martín and López 2015). Our main objective was to assess if protein secretion exhibits a seasonal pattern. A lack of variation may suggest a role limited to individual identity while marked variations may suggest additional functions.

As model species, we used the common wall lizards (*Podarcis muralis*), a medium-sized lacertid widespread in central and southern Europe (Sillero et al. 2014). This species is well-suited because it shows a clear seasonal activity, with mating season spanning from April to June (Sacchi et al. 2012; Sacchi et al. 2017), and a preference for the chemical modality (Baeckens et al. 2017; Cooper 1991; Sacchi et al. 2015). The composition of the lipophilic fraction of the femoral gland secretions is known for different populations and clades (Heathcote et al. 2014; MacGregor et al. 2017; Martín et al. 2008; Pellitteri-Rosa et al. 2014), and has been partly related to male quality traits, such as immune-response and parasite loads (Martín et al. 2008). In the end, it is the only lacertid for which information about the proteinaceous counterpart is available, showing how: protein patterns vary according to some identity-related traits (individual, color morph, population, clade) (Mangiacotti et al. 2017;

Mangiacotti et al. 2019a); proteins are actually used in intra-specific communication (Mangiacotti et al. 2019b).

Methods and Materials

Sampling Lizards and Hormonal Assay From March to October 2016, during the activity season of the common wall lizard (*Podarcis muralis*) in Northern Italy (Sacchi et al. 2012), adult males were captured by noosing in two nearby sites, in the city of Pavia (45.18° N, 9.15°E; Botanic garden and Castle; about 500 m apart). Sampling effort was equally spanned, on a monthly base, across the study period. Since we knew from a previous study (Sacchi et al. 2017) and from personal experience that recapture rates of *P. muralis* in high density sites was too low for a longitudinal study on the same males, we opted for sampling a minimum of 20 "new" males/month. To avoid pseudo-replication, we photographed each capture lizard for individual recognition (Sacchi et al. 2010). Within two hours from capture, Lizards were transferred to the University lab, measured for their snout-to-vent length (SVL; to the nearest mm) and weighed (± 0.01 g). Then, the secretions from the femoral glands were collected by applying a gently pressure along the thighs, with the help of a steel spatula, until all the glands (both legs) were emptied. Secretions were weighed using a semi-micro balance (ORMA BCA625SM; sensitivity = 0.01 mg), and stored into glass vials at −20 °C until chemical analyses (Mangiacotti et al. 2017). A blood sample (75–100 μl) for each lizard was gathered from the retro-orbital plexus using heparinized capillary tube (McLean et al. 1973). Tubes were centrifuged (6700 g for 5 min) 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, widely used in reptiles (Bonnet and Naulleau 1996), including wall lizards (Sacchi et al. 2017). We used 50 μl of plasma for the assay, after di-ethyl-ether extraction (extraction efficiency 93 ± 10%, mean ± SD). The cross reactivity of the antibody (kindly provided by Dr. G. Picaper, Nuclear Medicine laboratory, CHU, 45900 La Source, France) with other steroids was low. Tritiated testosterone was supplied by Perkin Elmer. In the assays performed in this study, the detection limit was 0.30 ng/ml, the sensitivity 7.8 pg by tube, intra assay precision was 7.45%, and inter assay precision was 10.42%.

At the end of lab procedures, all lizards were kept under observation for two hours and then released, healthy, at their capture point. The study was performed in accordance with the European and Italian laws on animal use in scientific research, and all the protocols have been authorized by Italian Environmental Ministry (Aut. Prot. PNM-2015-0010423, PNM-2016-0002154).

Lipids The lipophilic fraction of the secretion was analyzed 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 analyzed 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 1 ml/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; Pellitteri-Rosa et al. 2014; Heathcote et al. 2014; MacGregor et al. 2017), only two conspicuous lipids were quantified: i) provitamin D_3 (Cholesta-5,7-dien-3-ol, (3β)-; retention time = 24.4 min), used as proxy for quality-related information (López and Martín 2005; López et al. 2006, 2009; Martín and 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; Pellitteri-Rosa et al. 2014; Heathcote et al. 2014; MacGregor et al. 2017), 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-ratio between the area under the peaks of provitamin, and cholesterol (Aitchison 1982), which was used as reference. The identification of compounds was made by comparison to the mass spectral library in NIST 2008, and checked against previously published spectral data (Heathcote et al. 2014; MacGregor et al. 2017). Peaks identification and integration were performed using OpenChrom v1.1.0 (Wenig and Odermatt 2010).

Proteins After GC-MS, samples underwent three steps: protein extraction; protein assay, and one dimensional electrophoresis (Mangiacotti et al. 2017). Extraction was achieved by first adding 200 μl of n-hexane to complete defatting, vortexing for two minutes, and then centrifuging at 13,000 g for other two minutes. The supernatant was removed, and the pellet air-dried. This procedure was repeated two times. Afterwards, 200 μl of 10 mM (pH 7.4) phosphate-buffered saline (PBS) were added to the dry pellet. After vortexing and centrifuging, the supernatant containing the soluble proteins was recovered and stored in freezer (−20 °C). The concentration of the extracted proteins was assessed by the bicinchoninic acid assay (Smith et al. 1985), using bovine serum albumin as the standard for the calibration curve. The calibration curve and the concentration

estimates were computed using the R-package chemCal v0.2.1 (Ranke 2018).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to obtain individual protein patterns (proxy for protein composition). Aliquots containing a maximum of 10 μg of proteins were used from each sample and added to 10 μl of loading buffer solution (50 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulphate SDS, 0.1% bromophenol blue, and 10% glycerol). Prepared samples were denatured by incubating at 95 °C for five minutes. Electrophoretic runs were performed in a discontinuous mode (5% stacking gel and 15% running gel) by applying a constant voltage of 180 V for 2 h (Garfin 2009). Gels were stained with a 0.12% (*w*/*v*) Coomassie Blue G-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 et al. 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 (see code in supplementary material).

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 (Alberts et al. 1992a; Sacchi et al. 2017), single component cosinor models (Cornelissen 2014; Refinetti et al. 2007) 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:

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

where M is the MESOR (Midline Statistic Of Rhythm, 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. To control for possible effect of lizard size and condition on hormonal level and gland activity (Carretero et al. 2006), the SVL and the Scaled Mass Index (SMI) (Peig and Green 2009) were always added as main effect covariates in the cosinor models. While SVL accounts for lizard size, SMI is a condition index obtained by standardizing the mass at a reference size (the SVL mean) according to the Thorpe-Lleonart scaling model and a standardized major axis regression between mass and SVL (see Peig and Green 2009 for details). 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 and SMI as predictors), 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 ($\mu = 0$ and $\sigma = 0.001$), 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 = 2000; thinning = 5 (Kéry 2010). Convergence was checked and results from the posterior distribution are reported as the half sample mode (HSM) (Bickel and 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 and Yajima 2015), modeest (Poncet 2012), and HDInterval (Meredith and Kruschke 2018). R scripts and datasets are available as supplementary material.

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; Table 1).

T was positively correlated with SM, $proD_3$, and PS, and negatively with PP (Table 1). In general, all absolute correlation coefficients were larger than zero, but below 0.60 (0.39 on average), suggesting that the linear relation among dependent variables was weak (Table 1).

Cosinor models outperformed corresponding linear models (Table 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) and condition (SMI) was found on T and SM (Table 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 = 16.83 ng/ml; Table 3; Fig. 1a, f), while glandular productivity (SM) reached its maximum more than two months later (HSM = 1.66 mg; Table 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 = 0.60; Table 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 (molecular weight > 45 kDa) slightly increased in color (proxy for relative amount), the central part did not vary, and the two distinct bands in the low-molecular weight region (< 18 kDa) 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). Further, a better body condition and larger size associated with higher T-level, which stimulates an increase in secretion amount. These results fit well with the role of femoral secretions in intraspecific communication (Alberts 1993; Martín and López 2015), and with the central regulatory role of androgen levels (Alberts et al. 1992a; Baeckens et al. 2017).

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 (Baeckens et al. 2017; Martín et al. 2007a). Both the possible functional role and the underlying physiological mechanisms of the delay for high T to induce physiological effects remains poorly understood (Randall et al. 1997). It has been proposed that such delay could allow synchronizing sexual signaling and spermatogenesis (Carretero 2006; Gribbins and Gist 2003;). Indeed, one-two

Table 1 Descriptive statistics and Pearson bivariate correlation matrix of the monitored variables

Variable	*n*	mean (*range*)	Pearson correlation coefficients				
			T	SM	PP	$proD_3$	PS
T	153	4.23 (*0.04, 38.93*)	–	**0.41**	**−0.21**	**0.32**	**0.37**
SM	158	1.27 (*0.08, 4.35*)	*153*	–	**−0.45**	**0.49**	**0.54**
PP	147	0.52 (*0.25, 0.96*)	*142*	*147*	–	−0.17	**−0.37**
$proD_3$	86	−5.19 (*−10.70, −1.81*)	*83*	*86*	*82*	–	**0.57**
PS	155	0.00 (*−0.02, 0.02*)	*150*	*155*	*144*	*86*	–

n = sample size; mean (*range*) = mean and range of the observed values. Correlation matrix: upper triangle = correlation coefficients (HSM estimation; bolded values are different from zero with $P \geq 0.95$); lower triangle = bivariate sample size (italicized). T = hematic testosterone level (ng/mL); SM = secretion mass (mg); PP = protein proportion (dimensionless); $proD_3$ = provitamin D_3 relative abundance (dimensionless); PS = protein signal (dimensionless)

t2.1 **Table 2** Model comparison to assess the occurrence of a seasonal cycle in the monitored response variable

t2.2 Response	Model	Components	$\overline{D}$	$\overline{D}_p$	Δ	SE(Δ)
t2.3 T	cosinor	4	215.3	221.5	0	15.7
t2.4	linear	2	314.4	318.5	97.0	
t2.5 SM	cosinor	4	−2.3	3.7	0	14.1
t2.6	linear	2	99.3	103.3	99.6	
t2.7 PP	cosinor	4	−155.9	−149.9	0	8.9
t2.8	linear	2	−135.9	−131.8	18.1	
t2.9 $proD_3$	cosinor	4	355.9	362.0	0	8.0
t2.10	linear	2	403.3	407.5	45.4	
t2.11 PS	cosinor	4	1297.0	1303.0	0	17.3
t2.12	linear	2	1368.0	1372.0	69.0	

Cosinor (seasonal) model was compared to a simple linear model: components = no. of predictors in the model; $\overline{D}$ = mean expected deviance; $\overline{D}_p$ = mean penalized expected deviance (accounting for model complexity); Δ = difference between the largest and the smallest $\overline{D}_p$; SE(Δ) = standard error of the difference

months corresponds to the delay between production of spermatozoa in the testis and their migration in the epididymis where they become available to be ejaculated during copulations. Before that transfer males are functionally sterile (Roig et al. 2000; Carretero et al. 2006), and sexual signaling would result pointless. More generally, a peak of T that

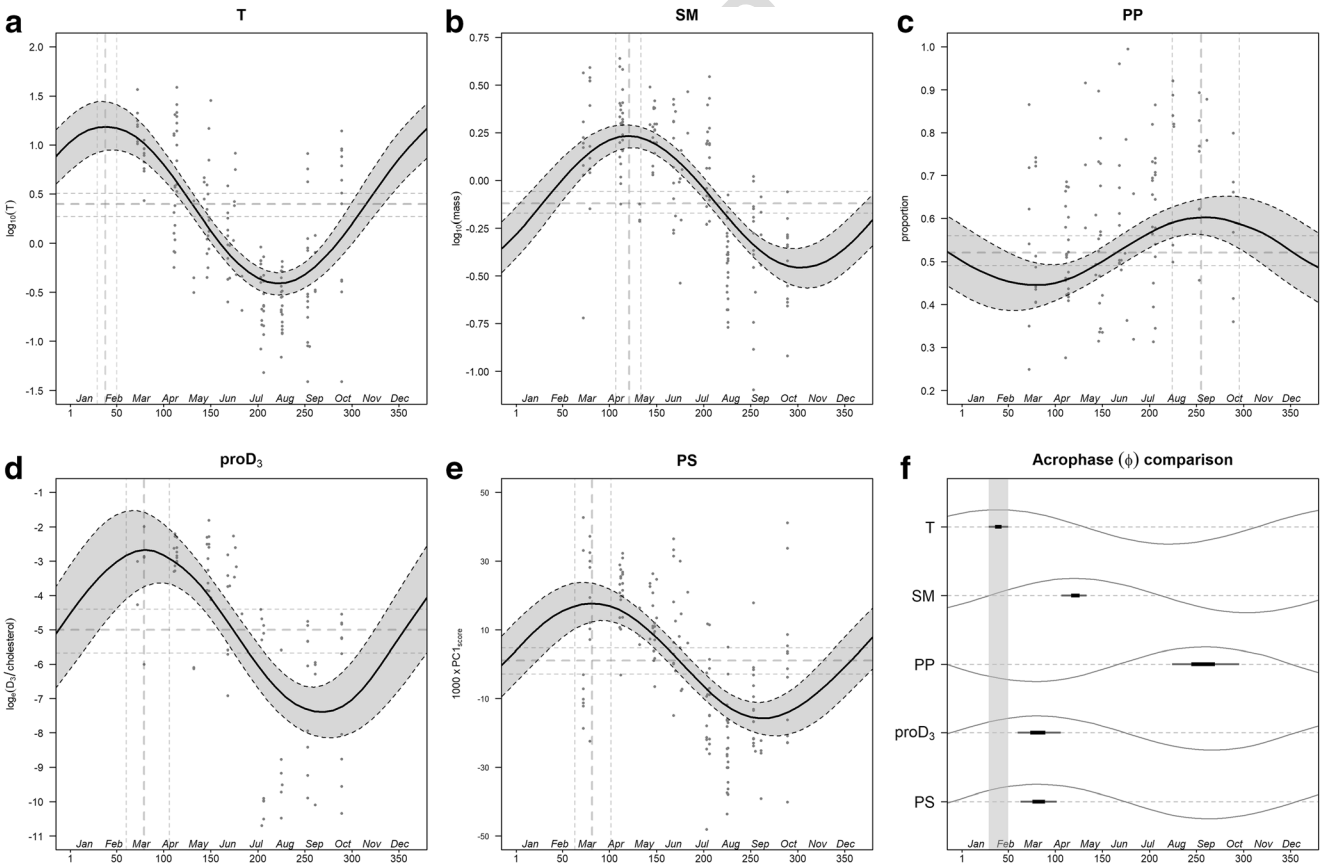

Fig. 1 Graphical comparison of the cosinor models. **a**-**e**) Models predictions for the five response variables. In each plot: the thick black line joins HSM of the predictions for each date; the grey shaded area and the black dashed lines highlight HDI_{95} of the predictions; horizontal and vertical 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) Q2

t3.1 **Table 3** Cosinor parameter
t3.2 estimations for all the monitored response variables

Response	M	A	φ	β_{SVL}	β_{SMI}
t3.3 T	**0.399**	**0.827**	**37.894**	**0.141**	**0.141**
	(0.268, 0.506)	(0.655, 0.961)	(29.265, 49.964)	(0.044, 0.231)	(0.050, 0.231)
t3.4 SM	**−0.121**	**0.341**	**120.995**	**0.130**	**0.059**
	(−0.172, −0.058)	(0.286, 0.410)	(106.487, 133.168)	(0.083, 0.170)	(0.019, 0.106)
t3.5 PP	**0.521**	**0.086**	**255.016**	0.007	0.002
	(0.491, 0.560)	(0.051, 0.119)	(224.367, 295.485)	(−0.016,0.037)	(−0.023, 0.029)
t3.6 $proD_3$	**−5.002**	**2.423**	**79.286**	0.381	0.151
	(−5.669, −4.394)	(1.799, 3.066)	(60.087, 106.052)	(−0.065, 0.825)	(−0.318, 0.558)
t3.7 PS	0.001	**0.017**	**81.368**	2.231	2.530
	(−0.003, 0.005)	(0.014, 0.021)	(63.143, 101.632)	(−0.914, 4.892)	(−0.171, 5.520)

M = mesor; A = amplitude (expressed in the variable scale); φ = acrophase; β_{SVL} = coefficient for the SVL term, proxy for lizard size; β_{SMI} = coefficient for the SMI term, proxy for lizard condition. 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). Coefficients different from zero with $P > 0.95$ are bolded

precedes the expression of male sexual behaviors has been documented in different squamate species (e.g., Bonnet and Naulleau 1996; Graham et al. 2008; Chamut et al. 2012).

The relative abundance of the protein fraction in the overall femoral secretion was quite variable among lizards throughout the year. This variability may explain the scattered data and poorly discernible oscillation of PP over time (Fig. 1c). Nevertheless, the protein fraction was higher in early September, and reached the minimum in March. Being the complementary fraction, the lipid component followed an opposite pattern compared to proteins, reaching the maximum (about 57% of mass) in spring, just after lizards emerged from hibernation. The predominance of lipid secretion matches the period when males start fighting to define territories and intensively court females (Sacchi et al. 2009; Font et al. 2012); thus, when the quality and intensity of sexual signaling is expected to be maximized. The same trends for protein and lipid secretions were also found in the green iguana (Alberts et al. 1992b), albeit less pronounced (seasonal range in relative lipid content: 13–35% of secretion mass) compared to *P. muralis*. Consistently with these findings, the (relative to cholesterol) provitamin D_3 abundance drops more than one hundred times from early spring to early autumn. It has been experimentally shown that provitamin D_3 is involved in the trade-off between sexual signaling and immune-system regulation in lizards (López and Martín 2005; López et al. 2009; Martín and López 2007): only healthy males are able to allocate vitamins to femoral secretions without paying the cost of a reduced immune response. The maintenance of a high content of $ProD_3$ in femoral secretions is physiologically demanding, thus providing to males a mean to signal their quality to females during courtship (Grafen 1990; Westneat and Birkhead 1998).

Our results on protein secretion patterns suggest that they also contribute to the seasonal modulation of sexual signaling. Femoral secretions contained approximately 17 bands (Fig. 2) that were constantly expressed throughout the whole activity season. This band expression steadiness supports the notion that proteins deposited in femoral secretions convey identity-related information as shown in green iguana and wall lizard (Alberts and Werner 1993; Mangiacotti et al. 2017, 2019b). Because individual identity is not supposed to vary over time, individual protein signature is expected to be stable (Tibbetts and Dale 2007). Yet, beside this stability in terms of band occurrence, we observed time variations in the relative expression of those bands characterized by a molecular weight below 18 kDa or above 45 kDa. This variation correlates with lipid signaling: time variations of relative protein expression were in phase with that of the lipophilic fraction (Fig. 1e, f). Principal component analysis of EPGs (explaining 29.5% of variation) emphasizes the seasonality of the relative expression of gel bands, not their mere occurrence. In other words, all bands are expressed along the season, but their relative intensity changes markedly. This suggests that protein signaling is not restricted to a simple and stable individual identity message.

From backward projection of predicted lanes (Fig. 2, left panel), seasonal EPGs reveal three distinct regions subjected to different expression trends: the intensity of the bands below 18 kDa decreases with season, while the intensity of bands above 45 kDa does the opposite; the intensity of bands in-between does not vary over time. Therefore, the invariant component of EPG that codes for identity-related information might be contained within the 18–45 kDa spectrum. Conversely, the two variable regions of EPG cannot carry stable individual identity information. Instead, as their variability parallels lipid variability, they may be involved in individual quality (or status) signaling. For example, these proteins may constitute a suitable matrix enhancing the stability of the lipophilic fraction (e.g., by preventing oxidation, or

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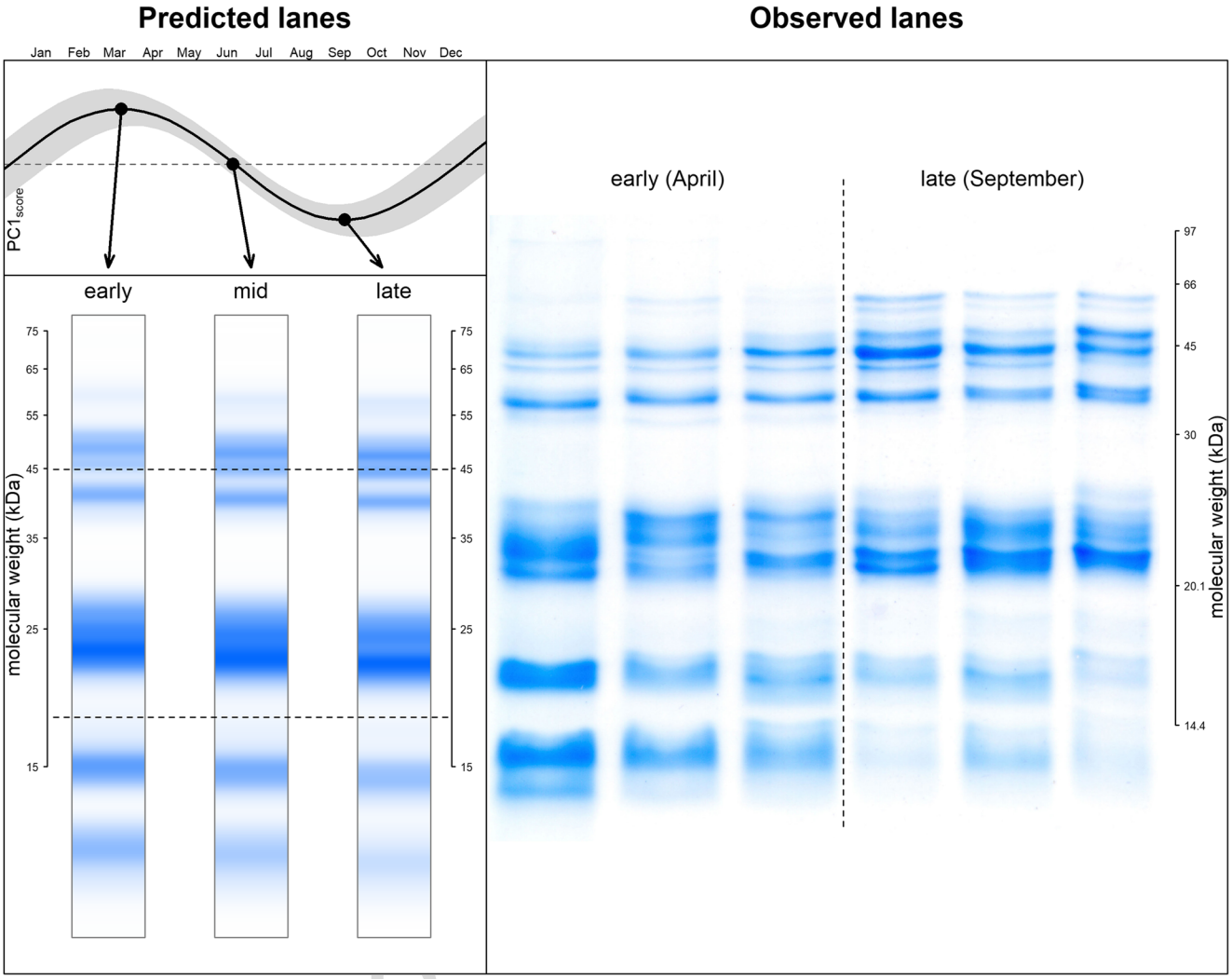

Fig. 2 Predicted and observed gel patterns throughout the season. Left panel: predicted lanes corresponding to the acrophase, mesor, and bathyphase of the protein signal (first principal component; $PC1_{score}$) as predicted by the cosinor model; horizontal dashed lines separate the upper and lower 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

reducing their volatility; Gabirot et al. 2008; Heathcote et al. 2014; Martín et al. 2016). Alternatively (or additionally), some proteins may carry their own informative function, and may be used to advise conspecifics about signaler characteristics other than its identity and health status, i.e., the reproductive status or aggressiveness. Like many lacertid lizards, males *Podarcis muralis* display a prenuptial spermatogenetic cycle (Gribbins and Gist 2003), and they are not able to produce fertile spermatozoa after the breeding season (late June; Carretero 2006). The switch between fertile and non-fertile status may be signaled by the proteins in the gland secretions, and could be used in intraspecific communication to modulate interaction with rivals (e.g., territorialism, aggressiveness) or with females (e.g., attractiveness) (Martín et al. 2007b). 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 answers, but they reveal that the chemical language of lizards is even more complex than previously known (Alberts 1990; Alberts et al. 1993; Baeckens et al. 2018; Font et al. 2012; Mangiacotti et al. 2017; Mayerl et al. 2015). 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 vs. 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 components of the lizard's chemical sexual language.

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