Chemical communication in the lacertid lizard **Podarcis muralis** : the functional significance of testosterone

TITLE: **Chemical communication in the lacertid lizard *Podarcis muralis*: the functional significance of testosterone**

RUNNING HEAD: **Testosterone, and lizard chemical communication**

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Abstract. Chemical signals are essential for intersexual communication in many animals, including lizards. While faeces have been suggested to contain socially relevant chemical stimuli, epidermal gland secretions are generally believed to be the leading source of chemosignals involved in lizard communication. Early research has shown that sex hormones affect epidermal gland activity, with androgens stimulating gland/pore size and/or gland productivity. However, the functional significance of hormone-induced glandular activity in lizard chemical communication remains unclear. In this study, we manipulated testosterone (T) concentrations in male *Podarcis muralis* lizards. While T supplementation did not change pore size, it did increase secretion production substantially. Chemosensory tests showed that female conspecifics tongue-flick at a higher rate and more quickly towards the secretion of males with experimentally-increased T levels than towards the secretion of control males, suggesting that females can discriminate between males with dissimilar T levels based on chemical cues of secretion alone. Based on the scent of faeces, however, females were unable to discriminate between males with differential T levels. Also, females reacted more quickly when offered larger amounts of secretion — irrespective of whether secretions were obtained from control or T-increased males. This result indicates that secretion quantity affects chemosignal detectability in *Podarcis muralis*.

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61 **Introduction**

62 Chemical signalling is the most widespread mode of communication between
63 organisms and plays a key role in intersexual communication in many vertebrate taxa
64 (Müller-Schwarze 2006; Wyatt 2014), including reptiles (Mason and Parker 2010). In
65 lizards, it is generally believed that epidermal gland secretions are the leading source
66 of chemosignals (Martín and López 2014; Mayerl *et al*. 2015), although there is some
67 evidence that faeces, cloacal secretions and skin lipids may also contain socially
68 relevant chemical stimuli (Cooper and Vitt 1984; Mason and Gutzke 1990; Cooper
69 1995; Labra 2008; Moreira *et al*. 2008). Epidermal glands of most lizards develop at
70 the onset of sexual maturity, and their activity is often greatest in male lizards and
71 during the reproductive season (Smith 1946; Cole 1966). These early observations
72 strongly suggest that the epidermal glands are controlled by androgens.

73 Experimental research indicates that castration of male lizards causes
74 glandular atrophy in *Lacerta agilis*, *Podarcis muralis* and *Cnemidophorus inornatus*
75 (Matthey 1929; Regamey 1932; Padoa 1933; Lindzey and Crews 1997). Testosterone
76 (T) supplementation restores gland activity in castrated *Goniurosaurus lichtenfelderi*
77 lizards (Golinski *et al*. 2015) and stimulates gland development in females of the
78 species *Gekko gecko* and *Urosaurus ornatus* (Chiu *et al*. 1970; Hews and Moore
79 1995). Oestrogen supplementation in males causes glandular atrophy in *Sceloporus*
80 sp. and *Hemidactylus bowringii*, a condition that can be reversed by exogenous
81 androgen administration (Forbes 1941; Chiu *et al*. 1975). Gland activity also
82 correlates strongly with testicular activity in *Sceloporus undulatus* and *Lacerta agilis*
83 (Regamey 1932; Altland 1941), and testis size in *Karusasaurus polyzonus* (Van Wyk
84 1990). Most of these studies have focused on proxies of glandular activity, such as
85 epidermal pore size (e.g. Hews and Moore 1995; Lindzey and Crews 1997; Rhen *et*

al. 2005); only a limited number of researchers scored secretion activity more directly by measuring glandular size on histological sections (e.g. Chiu *et al*. 1975; Van Wyk 1990) or by collecting and weighing the produced secretion (e.g. Fergusson *et al*. 1985; Alberts *et al*. 1992). In addition, the functional significance of increased glandular activity remains understudied. Martín *et al*. (2007b) established that T implants alter the chemical composition of the epidermal secretions of male Iberian wall lizards (*Podarcis hispanicus*) and that female conspecifics can discriminate between T-implanted and control males based on the scent of male secretions. Martins *et al*. (2006) found that seasonal increases in gland production enable male *Sceloporus graciosus* lizards to scent-mark more localities rather than to mark any single site more heavily, which may facilitate the expansion of a lizard's territory. We, however, propose an additional advantage of the production of large amounts of epidermal secretion in lizards. It is known that the absolute amount of volatile compounds determines the absolute amount of airborne chemicals in a specific environment (Apps *et al*. 2015). Based on the assumption that the proportion of volatile compounds in the epidermal gland secretion remains constant with increasing secretion quantity, we hypothesize that large amounts of secretion will increase the active space of airborne chemosignals and, therefore, that high secretion quantities will be detected more rapidly by lizard (vomer)olfaction.

Most research on chemical signalling in lizards has focused on chemosignals originating from either epidermal gland secretions or faeces, while studies integrating chemical cues of both origins are scant (but see e.g. Labra *et al*. 2002). Incorporating multiple chemosignal sources within a study may shed light on the importance of specific chemical sources, and on the multicomponent character of a message (Bro-Jørgensen 2010).

111 In this study, we investigate the effect of epidermal gland secretion quantity
112 on signal transmission in a lacertid lizard, and test if chemosignals originating from
113 epidermal gland secretions and faeces convey similar information. We manipulate T
114 concentrations in male *Podarcis muralis* lizards using a non-invasive technique
115 described by Knapp and Moore (1997). Our objectives are (i) to quantify the effect of
116 T on the secretory activity of male epidermal glands, (ii) to test the ability of *Podarcis*
117 *muralis* females to discriminate between control males and males with manipulated T
118 levels based only on chemical cues (originating from epidermal secretion and faeces),
119 and (iii) to determine if the amount of epidermal secretion affects signal transmission
120 and detectability. The potential benefits of high secretion production rates in lizards
121 will be discussed.
122
123 **Material and methods**
124 *Animals and their maintenance*
125 The common wall lizard *Podarcis muralis* is a diurnal saxicolous lacertid (adult
126 snout-vent length (SVL) 51 – 65 mm; this study), widely distributed throughout
127 central and southern Europe (Gassert *et al*. 2013; Michaelides *et al*. 2015). *Podarcis*
128 *muralis* lizards have on average 15 pore-bearing scales on each thigh, the so-called
129 'femoral pores' (Baeckens *et al*. 2015). Males of the species are highly territorial
130 (Stamps 1977; Edsman 1986) and use scent to mark their territory (Edsman 1990).
131 Based on chemical cues alone, female *Podarcis muralis* lizards are able to
132 discriminate between male conspecifics of different size (Heathcote *et al*. 2014).
133 Females of several lacertid species are known to exhibit chemically-mediated mate
134 choice (Martín and Lopéz 2015).

Early in their reproductive season (April 2015), 40 adult *Podarcis muralis* lizards (21 males; 19 females) were captured by noose at two localities in the north of Belgium ('Heverlee' and 'Muizen') and transported in cotton bags to our laboratory at the University of Antwerp. Females lacked ventral mating scars, indicating that females most probably had not mated yet. Males were individually housed in glass terraria (50 x 40 x 50 cm) to avoid physical or visual contact between them, whereas females were kept in group (maximum four females per 100 x 40 x 50 cm terrarium). Each terrarium contained a layer of sandy substrate, some vegetation and slate stones. A 60-watt bulb suspended above one end of the terrarium provided light (12L:12D) and heat that enabled the lizards to maintain a body temperature within their preferred range (i.e. between 30 °C – 35 °C; Bauwens *et al*. 1995). The larger terraria were equipped with two light bulbs. Lizards had access to drinking water at all times, and were fed moth larvae (*Galleria mellonella*) or crickets (*Acheta domesticus*) dusted with multivitamin powder thrice a week.

Hormonal treatments

In this study, male hormone levels were manipulated using a non-invasive technique based on the methodology described by Knapp and Moore (1997). Elevation of sustained T levels was obtained by transdermal administration of a mixture of the steroid hormone and sesame oil to the lizard. The lipid-rich character of the lizard skin enables lipophilic molecules to pass through the scales of the integument into the blood stream (Quay 1986). Unfortunately, protocolised T administration doses for *Podarcis muralis* are not available in the literature. Therefore, we based our T dose on a study by Belliure *et al*. (2004) ensuring that a chosen T dose increases T levels — while maintaining levels within the species' estimated physiological range. In Belliure

160 *et al*. (2004), T levels were manipulated in two lacertid species, i.e. *Acanthodactylus*
161 *erythrurus* and *Psammodromus algirus*, which are both comparable to the common
162 wall lizard in body size. In accordance with their study, we created a hormone
163 solution by diluting testosterone powder (4-androsten-17β-ol-3-one; Sigma #86500)
164 in commercial sesame oil (4 μg testosterone / μL sesame oil). Prior to the
165 experiments, we assigned males to one out of two treatment groups: a group with
166 experimentally manipulated T levels ('T-males', $n = 11$), or a control group ('C-
167 males', $n = 10$). Males from the two treatment groups did not differ in SVL
168 (ANOVA; $F_{1,20} = 0.090$; $P = 0.768$).
169 Males of the experimental T-group received 4 μL of the hormone dilution
170 every two days over a period of four weeks (June 2015), while C-males were
171 administered the same amount of sesame oil as a control. Without disturbing the
172 lizards, a droplet of the T mixture or control oil (depending on the treatment group)
173 was deposited onto the lizard's dorsum using a volumetric pipet. To minimize stress,
174 administration was carried out in the early evening when the lights were off and the
175 lizards had become inactive. To validate the hormonal manipulation, we measured
176 male testosterone levels before and after the treatment period using a non-invasive
177 steroid analysis based on faecal droppings. Following extraction of the droppings with
178 60% methanol (0.5 mL per 20 mg; Palme *et al*. 2013) testosterone (metabolites) were
179 analysed with a testosterone enzyme immunoassay (details see Palme and Möstl
180 1994). Dry droppings were collected twice for every individual: two weeks before the
181 start of the treatment, and three weeks after the start of the treatment. We used forceps
182 for faecal collection, which were rinsed in 70% ethanol, to avoid cross contamination.
183 Any visible sand and uric acid remains on the dry droppings were manually scraped

184 off, and individual faecal samples were placed in labelled plastic bags and stored at -
185 20 °C.
186
187 *Femoral pore size and secretion mass*
188 Snout-vent length was measured using digital callipers (Mitutuyo, CD-15CPX,
189 accuracy = 0.01 mm). Female and male pore size was estimated by digitising (ImageJ,
190 Abràmoff *et al*. 2004) the circumference of each of the three most proximal pores of
191 the left femur on images obtained with a stereomicroscope (Leica M165 C). Only
192 male *Podarcis muralis* lizards possess active epidermal glands, but merely for
193 quantitative comparative purposes, we measured the pore size of individuals of both
194 sexes.
195 Next, we collected epidermal gland secretions (from all the glands of the left
196 thigh) of male lizards, by gently squeezing the femoral pores with a forceps until each
197 gland was completely emptied and all secretion yielded. Secretions were directly
198 weighed on a microbalance (Mettler Toledo MT5, accuracy = 1 μg). The
199 measurements were conducted on the day preceding the hormonal treatment and
200 repeated 21 days after the start of the hormonal treatment. The obtained secretions
201 (after 21 days) were also used for the consecutive chemosensory tests.
202
203 *Chemosensory tests*
204 Squamates (amphisbaenians, lizards and snakes) react to a variety of chemical stimuli
205 with increased and differential rates of tongue extrusions. Tongue flick (TF) rates can,
206 therefore, be used as a quantitative bioassay for the degree of chemosensory
207 excitation (Burghardt 1970, 1980; Cooper 1994, 1998). To examine whether females
208 can distinguish between T- and C-males on the basis of their scent, we observed

female TF behaviour towards different stimuli presented on cotton-tipped applicators. This technique is a widely used method in studies on chemical detection in squamates (Cooper 1994, 1998, 2007). This particular part of the study was conducted 21 days after the start of the T- or C-administration.

Cotton swabs were presented to 19 females. These swabs were impregnated with (i) a single portion of male epidermal gland secretion (i.e. all secretion obtained from all glands of one thigh), (ii) a double portion of male secretion (i.e. all secretion obtained from all glands of both thighs) and (iii) male faeces. All three treatments were carried out in twofold, i.e. with excretions extracted from C-males and from T-males. Also, each female was subjected to an odourless control: a cotton swab impregnated with deionized water (Cooper and Burghardt 1990). In total, each female was subjected to seven different observation trials: one odourless control and six trials with stimuli derived from six different males. We did not implement a pungency control, as it is already well established that lacertid lizards can distinguish the scent of conspecifics from ecologically irrelevant scents (e.g. Cooper and Pèrez-Mellado 2002). As a default preparation, we dipped the cotton tip of the wooden applicator in deionized water before applying secretion or faeces onto the tip. Epidermal gland secretions were extracted by pressing gently with forceps around the pores, and were gathered on the swab after the secretions were weighed. Pending on the trial, we collected secretion from only one (single portion) or both (double portion) thighs. For the faecal trial, we made use of the fact that lizards often defecate when handled. As such, fresh male faeces were applied onto the cotton swab.

Prior to the experiments, female lizards were placed individually in a novel cage (50 x 25 x 30 cm), which contained slate stones under which the lizards could seek refuge. Each female was exposed to the scent stimuli in a randomized order, and

234 with a minimum interval of 30 minutes between subsequent trials. Females were
235 solely subjected to the scent of males of their own population. All observations were
236 performed during the lizards' peak activity hours (10:00-16:00).
237 After preparation of the swab, one of the researchers (SB) quietly approached
238 the female's cage and moved the cotton swab slowly in a straight-line towards the
239 lizard until it was approximately 2 cm anterior to the snout (Fig. 1). Subsequently,
240 two variables were scored: (i) the time period — or 'latency' — to the first elicit TF,
241 starting from the moment the cotton swab was in position; (ii) the total number of
242 elicited TFs during 60 seconds, starting from the first TF.
243
244 *Statistics*
245 Prior to analyses, all variables were $\log_{10}$-transformed to meet assumptions of
246 normality (Shapiro-Wilks test with $W \geq 0.95$). Within each lizard, femoral pore size –
247 determined as pore surface area – did not differ between pore number one, two and
248 three (repeated-measure ANOVA, Wilk's $\lambda = 0.973$, $F_{2,19} = 0.234$, $P = 0.794$).
249 Therefore, the average size of all three pores was calculated for each individual and
250 used in further analyses. Relationships between average femoral pore size, secretion
251 mass and SVL were explored using Pearson's correlations. To test for changes in pore
252 size, secretion mass and testosterone levels before and after the treatment period, we
253 used repeated-measure ANOVAs with 'time' (before vs. after) as within-subject
254 variable, and 'treatment' (C vs. T) as between-subject variable testing for differences
255 in changes between C- and T-males. When necessary, an ANCOVA was used with
256 SVL as covariate. Repeated-measures ANOVAs were used to examine differences in
257 TF behaviour with 'scent stimuli' as within-subject factor. Pairwise comparisons were
258 carried out using Tukey's honestly significant difference (HSD) tests. All statistical

analyses were conducted in IBM SPSS Statistics for Macintosh v. 22.0 (Chicago, IL, USA) and probabilities lower than 0.05 were considered statistically significant.

Results

Validation of the hormonal treatment

Prior to the administration period, T levels did not differ between C- and T-males (average faecal steroid concentration ± SE: 31.3 ± 10.1 ng/g for C-males versus 30.4 ± 6.0 ng/g for T-males; ANOVA, $F_{1,18}$= 0.224, P = 0.642). After three weeks of treatment, lizards from the T-group showed a significant increase in faecal testosterone concentration, whereas males from the control group exhibited even a minor decrease in testosterone levels (repeated-measure ANOVA, 'treatment' x 'time'; $F_{1,18}$ = 11.484, P = 0.001), with 10.7±5.6 ng/g for C-males versus 96.9±23.3 ng/g for T-males (Fig. 2). The small T-drop in the control group is most probably linked with an inevitable stress increase due to captive conditions: a common phenomenon, observed in various reptile taxa (e.g. Lance and Elsey 1986; Moore *et al*. 1991).

Femoral pore size and secretion mass

Average femoral pore size differed significantly between sexes, with males having larger pores than females (average pore size ± SE: 638±45 μm^2 for males versus 364±18 μm^2 for females; ANOVA, $F_{1,39}$ = 44.596, P < 0.001). Neither male nor female pore size correlated with SVL (male, r^2 = 0.033, P = 0.427; female, r^2 < 0.001, P = 0.982). Prior to the administration period, femoral pore size did not differ between C- and T-males (ANOVA; $F_{1,20}$= 0.729, P = 0.405). After an administration period of three weeks, pore size had not changed (repeated-measure ANOVA, 'time'-

284 effect; $F_{1,20}$= 1.325, P = 0.266), and did not differ between treatments (repeated-
285 measure ANOVA, 'treatment'-effect; $F_{1,20}$= 1.439, P = 0.247).
286 Larger males produced larger quantities of epidermal gland secretions (r^2 =
287 0.263, P = 0.009, Fig. 3). Prior to the administration period, the amount of secretion
288 that could be obtained from the males' glands did not differ between the C- and the T-
289 group (average secretion mass ± SE: 2.067±0.204 mg for C-males versus 2.156±0.302
290 mg for T-males; ANCOVA; $F_{1,20}$ = 0.001, P = 0.861). After three weeks of C- and T-
291 administration, lizards from the T-group showed an increase in secretion production,
292 whereas C-males showed a decrease (repeated-measure ANCOVA, 'treatment' x
293 'time'; $F_{1,20}$ = 13.325, P = 0.002; Fig 4). Compared to the sampling prior to the
294 administration period, the amount of secretion that could be obtained from the glands
295 was lower in C-males (-9.51%), and higher in T-males (+9,76%). Larger males did
296 not show a higher increase in secretion production (correlation of 'difference in
297 secretion mass before-after' over 'SVL', r^2 = 0.008, P = 0.405).
298
299 *Chemosensory tests*
300 All female lizards responded to swabs by tongue flicking. Mean latency to the first TF
301 differed significantly among conditions (repeated-measures ANOVA; Wilk's λ =
302 0.064, $F_{6,13}$ = 31.612, P < 0.001; Fig 5a). Latencies to the first TF in response to male
303 stimuli were significantly shorter than to water (Tukey post-hoc test, all P < 0.002).
304 Latency times in response to scent stimuli were similar for C- and T-males
305 (comparing secretions from one thigh: P = 0.222; from both thighs: P = 0.772; from
306 faeces: P = 0.191). However, females reacted more rapidly when offered large
307 amounts of epidermal secretions (from both thighs) than when offered smaller
308 quantities (from one thigh only) (comparing C-males: P < 0.001; comparing T-males:

309 $P < 0.001$). TF latency times did not differ with origin (one thigh, two thighs, faeces)
310 when stimuli were taken from C-males ($P \geq 0.191$). T-males faeces and secretions
311 from both thighs elicited earlier reactions than T-male secretions from one thigh ($P <$
312 0.001).
313 The mean number of TFs differed among stimulus conditions (repeated-
314 measures ANOVA, Wilk's $\lambda = 0.026$, $F_{6,13} = 81.282$, $P < 0.001$; Fig 5b). Swabs
315 impregnated with male epidermal gland secretions or faeces elicited significantly
316 more TFs than control swabs with water (all $P < 0.001$). Regardless of whether
317 secretions were obtained from a single thigh or from both, swabs with epidermal
318 secretions from T-males elicited more TFs than secretions from C-males ($P < 0.001$).
319 The amount of male secretion did not matter (quantity-effect in C-males: $P = 0.120$;
320 in T-males: $P = 0.164$). Females did not TF more when confronted with faeces from
321 T-males than with C-male faeces ($P = 0.253$). Similar numbers of TFs were
322 performed in response to C-male epidermal secretions and C-male faeces ($P = 0.120$),
323 but epidermal secretions of T-males elicited more TFs than T-male faeces ($P <$
324 0.001).
325
326 **Discussion**
327 *Testosterone and the epidermal gland system*
328 Firstly, the results of this study show that artificial T administration does not cause
329 any changes in the size of male femoral pores in *Podarcis muralis*. Pore size also did
330 not correlate with body size or secretion production. Interestingly, while the results of
331 this study does not support the idea that femoral pore size can be used as a proxy for
332 the intensity of secretion production in male wall lizards, studies on other lizard
333 species found opposite results. Femoral pore size in male green iguanas correlates

334 strongly with secretion mass, indicating that pore size probably provides a good
335 estimate of gland productivity in *Iguana iguana* (Alberts *et al*. 1992). More in line
336 with our results, Van Wyk (1990) did not find a relationship between testis volume
337 and femoral pore size in the cordylid lizard *Karusasaurus polyzonus*. Although, based
338 on histological section of the glands, the researcher did find a correlation between
339 testis volume and the depth of the epidermal glands. While we are not certain on the
340 true origin of these disparate findings, it is possible that they derive from interspecific
341 differences in gland morphology (e.g. tubular, tubulo-acinar, tubulo-alveolar), where
342 the form of the glands might determine if high secretory activity affect the expansion
343 of the pore-bearing scales. Detailed histological studies on this topic should be
344 encouraged. Nevertheless, pore size can easily be used as a tool for sex discrimination
345 in *Podarcis muralis,* for male femoral pores were almost twice the size of female
346 pores. Male pores are known to be notability larger than those of females in most
347 lizard taxa (Valdecantos *et al*. 2014; Baeckens *et al*. 2015; Mayerl *et al*. 2015). This
348 sexual difference may be due to increased plasma oestrogen levels associated with
349 female reproductive maturity, since administration of exogenous oestrogen inhibits
350 glandular activity in other lizard species (Forbes 1941; Chiu *et al*. 1975).
351 Secondly, our results show that T supplementation provokes substantial
352 changes in the secretory production of epidermal glands in male *Podarcis muralis*
353 lizards: males with artificially increased T concentrations produced on average 19.3%
354 more secretion than control males. In castrated *Ctenophorus ornatus*, daily T
355 injections incite a 23.4% increase in secretion production (Fergusson *et al*. 1985).
356 Research on seasonal T fluctuations and peak secretory activity in dominant *Iguana*
357 *iguana* males showed that during the month in which the highest plasma T
358 concentrations were observed (January), epidermal secretion production was

359 approximately 150% higher than during the period with the lowest T concentration
360 (May) (Alberts *et al*. 1992). Martins *et al*. (2006) have argued that an increased
361 secretion production during the mating season may help male *Sceloporus graciosus*
362 lizards to mark larger territories. As T levels are highest at the start of the
363 reproductive season (Manzo *et al*. 1994; Oppliger *et al*. 2004), they may stimulate
364 secretion production and, in some cases, lizard mobility (e.g. *Lacerta agilis,* Olsson *et*
365 *al*. 2000). Then, it could be assumed that as lizards move around more often, they
366 may release more glandular material over a larger area — which is made possible by
367 the high (T-induced) secretion production rate — and, therefore, expand their scent
368 marking space substantially. However, more research is necessary to confirm this.
369 In this study, large *Podarcis muralis* males produced larger amounts of
370 secretion than small males. Similarly, dominant *Iguana iguana* males (equipped with
371 large heads) produce gland secretions at a higher rate than subordinates with small
372 heads (Alberts *et al*. 1992). Because secretion production is most probably an
373 energetically costly affair (Martín and López 2015), it is very likely that only
374 individuals in a good condition can afford high secretory activity rates. Therefore,
375 large males with high T levels – which are often considered as most dominant
376 (Sinervo *et al*. 2000; Huyghe *et al*. 2007, 2009; Husak *et al*. 2009) and in best
377 condition (Tokarz 1985; Jakob *et al*. 1996), may produce high amounts of secretion,
378 and are thus able to mark more sites and create a more widespread territory than small
379 lizards. This is the case with sagebrush lizards (*Sceloporus graciosus*): territorially
380 aggressive males deposit high amounts of epidermal secretion, and this over a large
381 area (Martins *et al*. 2006).
382
383 *Testosterone and signal detectability*

Squamate chemoreception is said to work in a hierarchical fashion, with chemicals being received initially through the nares, processed by the nasal organs, and finally triggering tongue-flick mediated vomerolfaction (Halpern 1992; Cooper 1994; Schwenk 1995). As such, different latencies to the first TF or the number of TFs elicited toward stimuli indicate distinct chemosensory activities and, thus, stimuli discrimination (Cooper 1994). Our chemosensory experiments suggest that female *Podarcis muralis* can detect and distinguish between epidermal gland secretion of C- and T-males, as they react more quickly and more strongly with TF behaviour to secretion of T-males than to secretion of C-males. This result corroborates the findings by Martín *et al*. (2007*b*) in the closely related *Podarcis hispanicus*. Females may exploit T-induced differences in the chemical composition of the epidermal gland secretion or changes in the concentrations of relevant chemicals in the secretion. The ability to discriminate between differential T levels may be an important tool in lizard mate assessment, as male T concentration is commonly known to correlate strongly with various lizard performance traits, such as sprint speed (Klukowski *et al*. 1998) and bite force (Husak *et al*. 2007; Huyghe *et al*. 2009, 2010), and with overall dominant behaviour (Greenberg and Crews 1990; Pratt *et al*. 1992).

Although the amount of epidermal secretion did not affect female TF rates, females did respond more rapidly when offered double-loaded swabs — irrespectively of whether secretions were obtained from C- or T-males. These findings imply that the absolute amount of detectable airborne chemicals increases with increasing secretion quantity. As the absolute amount of airborne chemicals in the environment is determined by the amount of volatile chemicals in the secretion (Apps *et al*. 2015), we can infer that an increase in secretion quantity corresponds

409 with an increase in the absolute amount of volatiles. High amounts of epidermal gland
410 secretion may, therefore, increase signal detectability, with lizards responding more
411 rapidly by vomerolfactory behaviour. These results indicate that secretion quantity —
412 rather than T-induced changes in the physico-chemical properties of the secretion —
413 affect chemosignal transmission and detectability in *Podarcis muralis* lizards.
414 Accordingly, Escobar *et al*. (2001, 2003) found that *Liolaemus* lizards occurring at
415 high altitudes and low latitudes are equipped to produce high amounts of secretion, a
416 hypothesized compensation strategy to prevent signal loss due to the harsh signalling
417 environment. In addition to changes in secretion quantity, changes in the chemical
418 profile of the secretion may affect signal detectability too. For example, natural
419 seasonal changes in T level concentrations increase the proportion of unsaturated
420 lipids in the secretions of male *Iguana iguana*, which are postulated to increase
421 volatility and detectability of secretions (Alberts 1992). A recent study by Martín *et*
422 *al*. (2015) established interpopulational differences in secretion composition in
423 *Podarcis hispanicus*, enabling maximum signal detectability (and persistence) under
424 different climatic conditions.
425 Our results also show that although *Podarcis muralis* females showed interest
426 in male faeces, they could not discriminate between males with dissimilar T levels
427 based on scent of faeces alone. Previous research has already established the ability of
428 lacertid lizards to discriminate chemicals from faeces, and even suggested faeces as a
429 player in intraspecific lizard communication. Tongue-flick assays showed that
430 Carpetan rock lizards (*Iberolacerta cyreni*) are able to distinguish between own and
431 conspecific faeces (López *et al*. 1998), and that Iberian rock lizards (*Iberolacerta*
432 *monticola*) react differently when confronted with juvenile or adult faecal deposits
433 (Moreira *et al*. 2008). Although faecal scent elicited substantial vomerolfactory

434 behaviour in female *Podarcis muralis* in this study, the fact that females were unable
435 to detect T level differences in faeces (while the T enzyme immunoassay revealed
436 clear T level variation in the faecal pellets), suggests that faeces may not only serve as
437 a chemical signal. It has been suggested that lizards use faecal deposits to mark their
438 territory, in which individuals exploit both the chemical and visual aspect of the mark
439 to convey information. Male *Iberolacerta cyreni* typically deposit their excrements on
440 rock-tops, which is thought to facilitate visual detection by conspecifics (López *et al*.
441 1998). After first locating and approaching the signal visually, a lizard could
442 investigate the mark more closely through chemosensory channels (Duvall *et al*.
443 1987; Alberts 1989). It is generally assumed that — when the lizard moves —
444 femoral pores scrape against the substrate and small portions of glandular material are
445 transferred onto the substrate (Maderson 1986; Jared *et al*. 1999; Khannoon *et al*.
446 2013; de Villiers *et al*. 2015). Following this line of thought, it is probable that sites
447 with faecal deposits will also be labelled with epidermal secretions. Martins *et al*.
448 (2006) found a strong association between the amount of epidermal secretion and the
449 amount of faecal matter on a certain marking spot in *Sceloporus graciosus*. It is,
450 therefore, reasonable to hypothesize that lizards use faecal deposits as an initial long-
451 range visual signal providing the first cue, and carrying minimal chemical information
452 (e.g. own vs. conspecific) once encountered. The neighbouring epidermal gland
453 secretion deposits may act as chemosignal-reservoirs containing more elaborate
454 information on an individuals' condition, e.g. hormonal level, parasitic infection,
455 immune response, diet quality or fighting ability (López *et al*. 2003; Labra 2006;
456 Martín and Lopéz 2006*a,b*; Martín *et al*. 2007*a,b*, 2008; Khannoon *et al*. 2010, 2011;
457 Kopena *et al*. 2014).
458

In conclusion

Our data demonstrate that testosterone affects epidermal gland activity in male *Podarcis muralis* lizards with an increased secretory production, but without any changes in the size of the pore-bearing scales. Based on the scent of male epidermal gland secretion alone, and not on the scent of faecal deposits, female conspecifics are able to discriminate between males with different testosterone levels using vomerolfaction behaviour. Moreover, females react more quickly towards higher amounts of secretion, implying that epidermal gland secretion quantity affects lizard chemosignal detectability. Still, more research on lizard scent marking behaviour is necessary to elucidate the importance of secretion quantity in a natural setting.

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782 **Figures**

783 **Fig. 1—** An illustration of the cotton-swab technique, designed to test lizard's tongue-flick
784 behaviour towards chemical stimuli. Drawn by Detlef Tibax.

785

786 **Fig. 2—** Mean (±SE) faecal testosterone (metabolite) concentrations of *Podarcis muralis*
787 males from the experimental group, which received a testosterone treatment (T-males) and
788 males from the control group (C-males). Means are shown 'before' and 'after' the three-week
789 administration period. Steroid concentration is presented as the mass of immunoreactive
790 testosterone metabolites per dried faecal mass. FTM = faecal testosterone metabolites.
791 Asterisks indicate statistical differences.

792

793 **Fig. 3—** Relationship between epidermal gland secretion mass and snout-vent length (SVL)
794 in *Podarcis muralis* males.

795

796 **Fig. 4—** Mean (±SE) difference in epidermal secretion mass (i.e. secretion mass after three-
797 week administration period subtracted by secretion mass before administration period) for
798 control males (C) and males with experimentally increased testosterone levels (T). Asterisks
799 indicate statistical differences.

800

801 **Fig. 5— (a)** Mean latency (in seconds) to the first TF and **(b)** mean number of tongue-flicks
802 (TF) elicited in 1 minute by female conspecifics in response to different stimuli (symbolized
803 by different background colours) originating from control males (C) and males with
804 experimentally increased testosterone levels (T). The stimuli are: deionized water (W); a
805 single portion of male epidermal gland secretion (red); a double portion of male epidermal
806 gland secretion (green); faeces (blue). Error bars represent SE. The same letters above the
807 bars denotes that means are not significantly different from each other.