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Sex identification of juvenile sand lizards, *Lacerta agilis* using digital images

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Abstract. Sexing neonate animals is necessary for many evolutionary and ecological studies. Yet non-invasive sex identification of neonate reptiles is often problematic because these do not exhibit salient differences in colouration and body proportions. We examined digital images of the ventral body surface in 214 adult or subadult individuals (95 males + 119 females), 59 juveniles (29 + 30), and 156 hatchlings of the Eurasian sand lizard, *Lacerta agilis*. Two quantitative traits, the number of transverse rows of ventral scales and the width/length ratio of the anal plate, which are easily recordable from digital images and show no substantial correlation with body size, exhibit pronounced sexual dimorphism. A discriminant function derived from these two characters allows correct identification of the sex in 90% of juvenile individuals when males and females of older stages are used as reference samples. Also, we introduce a new qualitative trait, namely the presence/absence of the skin hyperaemia behind cloaca in hatchlings, and provide indirect evidence that this trait is likely to be strongly associated with sex.

Keywords: anal plate index, hatchlings, *Lacerta agilis*, lizards, sex identification, sexual dimorphism, ventral scales.

Introduction

Sexing neonate animals is a necessary prerequisite for many important fields of evolutionary and ecological studies. Data on the secondary sex ratio and the sex-specific values of phenotypic traits (particularly body size) allow us to address various issues related to sex-allocation theory (Trivers and Willard, 1973; Olsson et al., 2005; Uller et al., 2006), ontogeny of sexual size dimorphism (Badyaev, Whittingham and Hill, 2001; Le Galliard et al., 2006), and other important topics (e.g., Uller and Olsson, 2003; Braña, 2008). For lizards, which are a model group for studying the evolution of life-histories (Vitt and Pianka, 1994; Shine, 2005), non-invasive sex identification in newborns and juveniles is often problematic: unlike adults, hatchlings do not exhibit salient differences in colouration and body proportions. Harlow (1996) suggested an effective and apparently harmless method of sexing hatchling lizards: a gentle pressure on tail base elicits everting hemipene(s) in male individuals. This technique, eventually with slight modifications, has been applied in several studies (e.g., Olsson et al., 2005; Braña, 2008; Li et al., 2013). Yet for such tiny and fragile creatures as hatchlings of small-sized lizard species, manipulations of the kind are potentially stressful, because the study animal should be restrained either by cooling in a refrigerator, or with the assistance of a second person (Harlow, 1996). Therefore, searching for diagnostic traits in external morphology of neonates which would enable an identification of their sex with minimal disturbance for the animal remains an important methodological issue. Ideally, sexing should be possible from digital images which can be easily archived and examined with no pressure of time and other constraints for the researcher and no stress for the animal.

In most lacertid lizards, the number of transverse rows of ventral scales (ventralia) differs between the sexes, males having on average 2-3 rows fewer than females (e.g., Darevsky, 1967; Orlova, 1975; Roitberg, 1989). Considering that meristic scale counts, such as ventralia, do not change after birth (Bauwens and Thoen, 1982;

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Roitberg, 1989), several researchers (Bauwens and Thoen, 1982; Lecomte, Clobert and Massot, 1992; Märtens, 1996) tried to distinguish between male and female juveniles using the sex-specific distributions of this trait in adults from the same locality as reference samples. The extent of sexual differences, and respectively the discriminating power of ventralia obviously differ among species and populations. In a *Zootoca vivipara* population from Massif Central in France, more than 95% of hatchlings and juveniles can be correctly sexed based on this trait (Lecomte, Clobert and Massot, 1992; Le Galliard et al., 2006), whereas in a Belgian population of this species (Bauwens and Thoen, 1982), as well as in a *Lacerta agilis* population from eastern Germany (Märtens, 1996), less than 75% juveniles could be surely assigned to males or females. Searching for further easily recordable diagnostic traits is therefore needed.

For adult *L. agilis*, consistent sexual differences were reported for the shape of the anal plate, this scale tending to be more elongated transversally in males than in females (Darevsky, Shcherbak and Peters, 1976; Majláth, Šmajda and Kundrátet, 1997; Zavialov, Tabachishin and Shlyakhtin, 2000; Simonov, 2007, 2008). However, these reports are based on relatively small samples. Furthermore, it is unclear, if appreciable sexual differences in the shape of the anal plate also occur in juveniles.

This study was aimed to promote methods of non-invasive sex determination of juvenile lacertid lizards. We show that in Eurasian sand lizards (*Lacerta agilis*) a set of two traits, ventralia and the shape of the anal plate, which are easily recordable from digital images, correctly sexed a vast majority of juveniles. Also, we introduce a new character which describes the state of the skin behind cloaca in hatchlings and provide indirect evidence that this trait is likely to be strongly associated with sex.

Material and methods

The following samples were used for this study. Sample 1 included 214 adult and subadult individuals who were reliably sexed based on their colouration, body proportions, and the presence/absence of hemipenes. These animals come from two sites (Togliatti, 53°28′N, 49°21′E; Mordovo, 53°10′N, 49°27′E) located in the Middle Volga Region of Russia, i.e. clearly within the range of *Lacerta agilis exigua*. Sample 2 included 59 preserved juveniles of *Lacerta agilis boemica* which were collected near Makhachkala (Republic Daghestan, Russia) in early 1980s for other purposes (Roitberg, 1989). These juveniles had SVL from 31-52 (mostly <42) mm and did not show sex-specific external morphology; they were sexed via autopsy. Sample 3 included 156 unsexed hatchlings. The hatchlings have been obtained via monitoring of gravid females which were caught from the same sites as sample 1 and held in captivity for a few days or weeks until oviposition. Eggs were incubated at 25°C, and hatchling traits were recorded within 12 hours after hatching.

The following two characters were recorded in all study animals: Character 1, the number of transverse rows of ventral scales (ventralia). The scales were counted in the second longitudinal row from the collar fold to and including the first scale contacting femoral pores (fig. 1A; see also: Roitberg, 1994). This trait was recorded on the right and the left side of the body to mitigate the bias due to occasional asymmetry and other deviations from a regular pattern of scale rows. Character 2, the width/length ratio of the anal plate, quantifies the extent of elongateness of this scale in the transverse direction. The width and the length were measured as indicated in fig. 1D, G.

Hatchlings were additionally examined for the state of the skin behind cloaca (Character 3). Two states can be distinguished: two short strips (which sometimes merge to a single transverse strip) of hyperaemic skin of rose colour (apparent males, fig. 1B-D), and a lack of the above pattern (apparent females, fig. 1E-G). The three characters exhibited a high repeatability of records (>98% for all characters) and a considerable sexual dimorphism.

To record the above traits (and three body size characters) with minimal disturbance of the animal the following protocol was applied to hatchlings. An animal was gently taken (caught up) on a concave sheet of paper, placed into a Petri dish, and weighted with a digital balance. During weighing the hatchling becomes quiet, and the operator can partly open the cap and gently stretch the hatchling's body with a blunt probe or another fine tool. Snout-vent length (SVL) and tail length was recorded by fixing the ruler on the outer surface of the dish along the animal's body axis (fig. 2). This procedure approximates a usual measuring immediately on the ventral surface of the body. The routine presented here is related to that of Wapstra (2005) who used a transparent plastic bag instead of a Petri dish. After measuring SVL and tail length, several digital images of the ventral surface of the body were collected for subsequent recording the traits used in this study. Adults, subadults, and preserved juveniles were handled by hands, without placing into Petri dish. Except preserved lizards, all study animals were released in the localities of their capture.

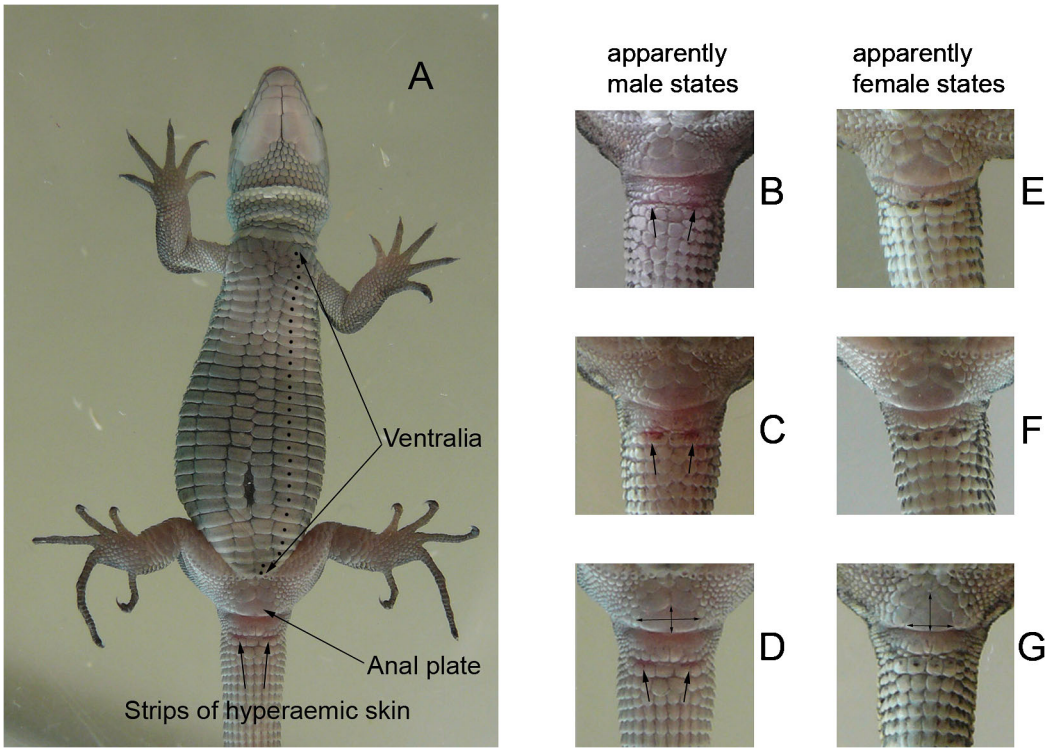

Figure 1. Ventral body surface of hatchling *Lacerta agilis exigua* and our study traits. See text for explanations. This figure is published in colour in the online version.

Because of a high correlation between the ventralia counts on the left and the right side of the body ($r = 0.75$-0.85, this study) their average value was used as a single character. An anal plate index, the Width/Length ratio of this scale (Darevsky et al., 1976; Zavialov et al., 2000; Simonov, 2007, 2008), was used to quantify the extent of transverse elongation of the anal plate. Use of a ratio could not be avoided here, because the images included no reference length so that the width and length values were comparable within an image but not between the images. For statistical analyses, we used a transformation LN(100 × anal plate width/anal plate length). Note that the logarithm of another ratio of two correlated traits, population means of male size and female size, was shown to have reasonable statistical properties and suggested as an appropriate metric for sexual size dimorphism (Smith, 1999).

Discriminant analysis was used to evaluate the extent of separation between the sexes in the morphospace of characters 1 and 2 and to measure the relative contribution of these characters to this separation. Males and females of sample 1 were used as reference groups. Scores of the discriminant function (Z) derived from this analysis were then calculated for each individual of samples 2 and 3 treated as unknown groups. Sample 2 served for validation the procedure on small-sized animals which were not represented in the reference groups. For hatchlings (sample 3), which were not sexed, we examined whether the two groups exhibiting alternative states of Trait 3 differed in their Z values in the same way as males and females of samples 1 and 2. In each sample, the proportion of total variance of Z explained by sex (samples 1 and 2), or by the presence/absence of skin hyperaemia (sample 3), and the significance of these predictors were estimated by a one-way ANOVA.

Statistical independence of the studied quantitative traits from one another and from body size (SVL) was tested using Pearson correlation coefficient.

Results

In the morphospace of two quantitative characters, ventralia and the anal plate index, both the extent and the pattern of separation between males and females, as well as between the individuals exhibiting presence vs. absence of the skin hyperaemia, are strikingly similar in the studied samples (fig. 3).

The discriminant function analysis of sample 1 showed that both characters contributed substantially (and nearly equally) to the discrimination: standardized canonical discriminant function coefficients were −0.732 for ven-

tralia and 0.756 for anal plate index. The power of this discriminant function was substantially higher than those of single characters: two characters, Wilk's Lambda 0.282, canonical correlation 0.847; ventralia, Wilk's Lambda 0.467, canonical correlation 0.730; anal plate index, Wilk's Lambda 0.452, canonical correlation 0.740. The discriminant function derived from sample 1 ($Z = -0.737 \times$ ventralia $+ 6.146 \times$ LN(100 $\times$ anal plate width/anal plate length) $- 10.631$) was calculated for each individual of this and the other study samples. In all three samples, Z scores show a pronounced differentiation of males vs. females, and hatchlings with presence vs. absence of the skin hyperaemia (fig. 4). These differences explain from 69.5-73.6% of the total variance in Z (table 1).

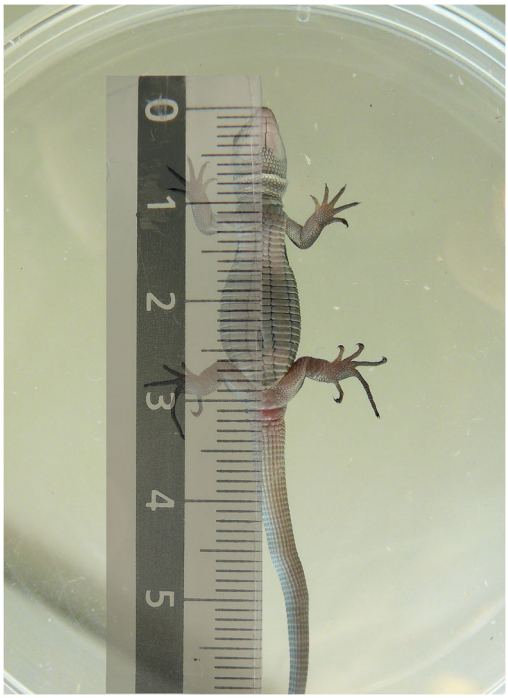

Figure 2. Hatchling *Lacerta agilis exigua* closed in a Petri dish for measuring the snout-vent length and taking pictures of the ventral surface of hatchling's body. See text for details. This figure is published in colour in the online version.

In the reference samples, percent of incorrectly classified individuals was 2.2 (2 from 90) in males and 1.7 (2 from 116) in females (see caption to fig. 4 for the definition of classification criteria). When the same criteria were applied to sample 2, the rate of misclassifications was 17.2% (5 from 29) in males and 3.3% in females. In sample 3, in which the classification based on Z scores was confronted to the presence (presumed males) or absence (presumed females) of the skin hyperaemia, suspected misclassifications amounted respectively 0.0% ($n = 70$) and 17.4% (15 from 86).

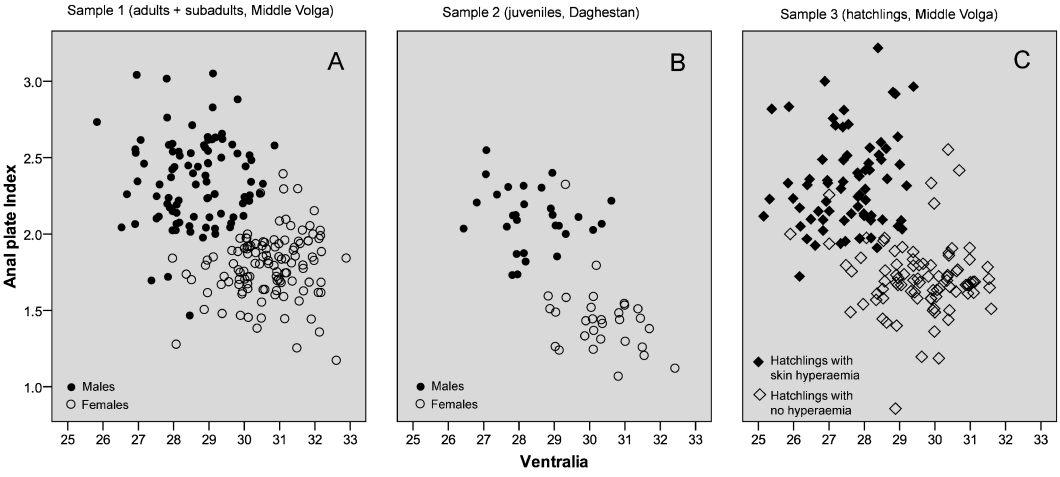

Figure 3. Variation in the number of transverse rows of ventral scales (Ventralia) and the extent of transverse elongation of the anal plate (Anal plate index) in the three study samples of *Lacerta agilis*. Data points are jittered horizontally to reduce superposing. See Methods for sample locations.

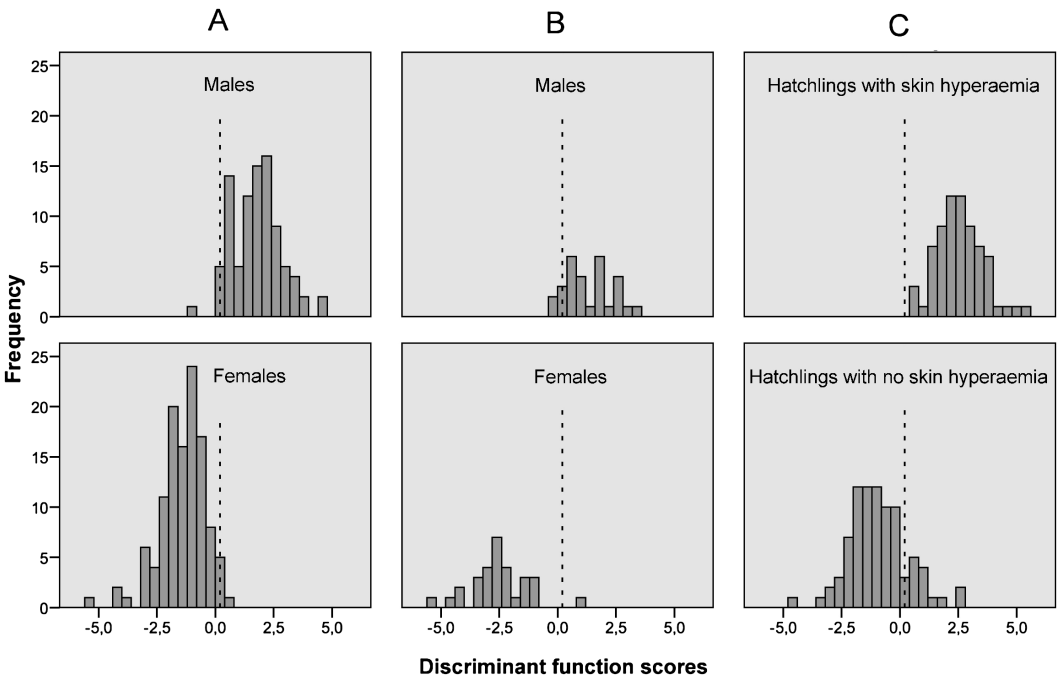

Figure 4. Distributions of the discriminant function scores for non-juvenile males and females of *Lacerta agilis exigua* (reference samples, A), juvenile males and females of *Lacerta agilis boemica* (B), and hatchlings of *L. a. exigua* exhibiting presence vs. absence of the skin hyperaemia (C). See Methods for sample locations. Vertical broken lines indicate the midpoint between centroids of the reference samples ($Z = 0.202$). For scores above this value, the probability of membership in Group 1 (Males) is greater than 0.5. For scores lower than 0.202, the probability of membership in Group 2 (Females) is greater than 0.5.

Table 1. One-way ANOVAs with Z-score as the response variable, and sex (males vs. females) or state of the skin hyperaemia (presence vs. absence) as the predictor. See text for details.

	df1	*df2*	*MS*	*F*	*P*	% variance (partial eta × 100)
Sample 1						
Corrected model	1	204	520.076	520.06	<0.001	71.8
Sex	1	204	520.076	520.06	<0.001	71.8
Sample 2						
Corrected model	1	57	219.965	159.00	<0.001	73.6
Sex	1	57	219.965	159.00	<0.001	73.6
Sample 3						
Corrected model	1	154	461.380	351.53	<0.001	69.5
State of the skin	1	154	461.380	351.53	<0.001	69.5

To check whether the two quantitative traits, ventralia and anal plate index, tend to be correlated with one another and with body size (SVL) at the level of individual variability, Pearson correlation coefficients for the respective pairs of traits were computed for subsamples of same-sex individuals within samples 1 and 2, and for subsamples of individuals with the same state of the skin behind cloaca within sample 3. Correlations between ventralia and anal plate index were also computed for the sex-combined samples for comparative purposes. All within-subsample correlations were weak and inconsistent (table 2). In contrast, the sex-combined

Table 2. Pearson correlation coefficients (r) and their significance (P) between three traits within several samples of *Lacerta agilis* (see Methods for sample details). Hyp+ and Hyp− designate respectively the presence and absence of skin hyperaemia behind cloaca in hatchlings.

Samples		n	Ventralia – Anal index		Ventralia – SVL		Anal index – SVL	
			r	P	r	P	r	P
Sample 1	males	90	0.058	0.587	−0.066	0.540	0.113	0.173
	females	116	0.130	0.164	0.038	0.687	0.072	0.441
	combined	206	**−0.490**	<0.001				
Sample 2	males	29	−0.132	0.494	−0.279	0.143	0.042	0.831
	females	30	−0.364	0.048	0.075	0.693	0.014	0.941
	combined	59	**−0.708**	<0.001				
Sample 3	Hyp+	70	0.200	0.096	0.191	0.113	0.007	0.952
	Hyp−	86	−0.057	0.601	−0.192	0.077	0.260	0.015
	combined	156	**−0.497**	<0.001				

The P values are related to single tests. The two correlations with $0.01 < P < 0.05$ become non-significant when corrected for multiple comparisons ($k = 18$). The correlations given in bold remain significant after this correction.

samples showed relatively strong and highly significant negative correlations between ventralia and anal plate index (table 2).

Discussion

Our study demonstrates that *Lacerta agilis* exhibits pronounced sexual differences in the extent of transverse elongation of the anal plate, quantified as the width/length ratio of this scale (anal plate index). This dimorphism is as strong as that in the number of transverse rows of ventral scales (ventralia). A discriminant function derived from these two characters using non-juvenile males and females from the Middle Volga area (sample 1) provides correct identification of the sex in 98% individuals of the reference samples and in nearly 90% specimens of the test sample (sample 2). This result seems promising considering the following two points. (1) Sample 2 consists of small juveniles with SVL 31-52 (mostly <42) mm vs. 50-105 (mostly >60) mm in the reference sample. (2) Sample 2 represents a geographically distant and genetically distinct population (*L. a. boemica* from the south-eastern North Caucasus; see Andres et al., 2014 and references therein for a strong genetic separation of *L. a. boemica* from the rest of the species). Note that actual overlap in Z scores of males and females of the test sample is not higher than in the reference samples (fig. 4A, B), a relatively high percent of misclassifications in males being obviously due to an overall female-bias of sample 2, as compared to sample 1, in the morphospace of ventralia and anal plate index (fig. 3A, B). It is, therefore, likely that the rate of misclassifications would be even lower if the tested juveniles belonged to the same subspecies as sample 1.

As expected from the lack of appreciable dependence of ventralia and anal plate index on body size at the level of individual variability (table 2), the extent and pattern of variation in the morphospace of the two traits in hatchlings (fig. 3C) is similar to that of adult and subadult lizards from the same geographic region (fig. 3A). A small bias to lower values of ventralia in hatchlings may reflect between-cohort variation (Lecomte, Clobert and Massot, 1992) or/and other effects, including slight inconsistencies in applying the counting protocol (e.g. scales of the most caudal row possibly tend to increase in their relative size during the early postnatal ontogeny so that this row is less frequently considered in hatchlings than in older animals). In any event, this bias is small, as compared to sexual differences, and is unlikely to substantially affect sex determination

in hatchlings and juveniles when using a discriminant function derived from older animals.

A striking similarity of differentiation in the morphospace of ventralia and anal plate index between the groups of hatchlings exhibiting presence or absence of the skin hyperaemia with the differentiation between males and females of older stages (figs 3 and 4) is noteworthy. This similarity suggests that the presence of the hyperaemia is strongly associated with male sex, and the absence with female sex. The following two patterns also argue for this hypothesis. (1) The predictor presence/absence of the skin hyperaemia explains as much of the total variation in Z scores in sample 3 as the predictor sex in samples 1 and 2 (table 1). (2) A lack of a negative correlation between ventralia and anal plate index within "homogenous" subsamples (same-sex individuals or hatchlings with the same state of skin behind cloaca – table 2) shows that the similarity of the considered differentiation (fig. 3) cannot be explained by intrinsic non-independence of the two characters at the level of individual variability. Together, the presented results provide highly suggestive (even though indirect) evidence that the state of the skin behind cloaca in hatchlings is strongly associated with sex. The hyperaemia behind cloaca is supposedly related to the hemipene ontogeny at latest prenatal stages; it was not observed in larger juveniles and non-juvenile animals. In line with this hypothesis, all hatchlings with the hyperaemia exhibit Z scores typical for males, whereas a small proportion of hatchlings in which the hyperaemia was not observed also show male-like Z scores (fig. 4C). This latter group is likely to consist of males in which the hyperaemia has not arisen or quickly disappeared.

The main direction of future research is a direct validation of the suggested procedures in hatchlings using Harlow's (1996) method, autopsy or a follow-up sexing of individuals grown up to maturity. In particular, it should be clarified whether the presence/absence of the skin hyperaemia is associated with sex so tightly that this trait would better differentiate male from female hatchlings solely than in concert with the two quantitative traits. Future research should also show how strong are the gender differences for the three traits in hatchlings (and for the two quantitative traits in older animals), in other *L. agilis* populations and in related species.

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