Does Sexual Selection Influence Ornamentation of Hemipenes in Old World Snakes?

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ABSTRACT

In the present study, we investigated and documented the morphology of the male copulatory organs (hemipenes) in fifteen wide-ranging snake species. The species represent four families (Boidae, Colubridae, Lamprophiidae, and Viperidae) and ten genera. We applied the same preparation techniques for all species, successfully everting and expanding the organs completely. The detailed description of the general morphology of the male copulatory organs was based on 31 specimens. Our data were compared with published observations and we point out some incorrectly described details in previous investigations. We provide the first description of the hemipenial morphology for three ophidian species (Elaphe sauromates, Telescopus fallax, and Malpolon insignitus). In addition to the morphological characteristics of the hemipenes presented in the research, we propose the adoption of a standardized index describing the hemipenial proportions. The immense variation in hemipenial morphology presupposes its dynamic evolution, but we suggest that many of the significant structures observed here may have escaped previous researchers due to differing methodologies. Some of the highly ornamented morphologies that we describe are consistent with a locking mechanism during copulation. However, other morphologies may relate to the variety of mating behaviors observed. As a result, we propose that sexual selection is the major driver affecting the hemipenial ornamentation in snakes. Anat Rec, 300:1680-1694, 2017. © 2017 Wiley Periodicals, Inc.

Key words: mating; copulatory organ; male; phylogeny; constraints

INTRODUCTION

The morphology of the male copulatory organ in snakes (the hemipenis) has been a subject of interest for over 120 years. Since the seminal study of Cope (1895) that describes 234 species, the general morphology of the hemipenis is considered to be species specific (see also Dowling and Savage, 1960; Keogh, 1999; Zaher, 1999). Although a limited number of studies describe intraspecific variations (e.g. Bernardo et al., 2012; Inger

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Species	Side	Date	Locality (current name)	UTM	Coll. N
Eryx jaculus	l + r	26.07.1930	Nadezhden, Harmanli	MG13	III-17–30
	r^*	20.05.1920	Nadezhden, Harmanli	MG13	III-17–38
Coronella austriaca	$l + r^*$	20.06.1926	Granitovo, Belogradchik	FP33	III-13–48
	1	23.09.2012	Divchevoto, Teteven	KH74	_
Dolichophis caspius	l + r	01.08.2003	Arkutino, Primorsko	NG58	_
	r	02.07.1927	Poruchik Minkov station	FM80	III-12–38
	r^*	20.05.1931	Strandzha mountain	_	III-12–36
Elaphe quatuorlineata	$l + r^*$	1938	Breznitsa, Sandanski	GM21	III-4–4
Elaphe sauromates	1*	05.06.2010	Dervent Heights	_	_
Platyceps collaris	$l + r^*$	30.07.1973	Lozenets, Tsarevo	NG67	_
Platyceps najadum	$l + r^*$	15.06.1938	Breznitsa, Sandanski	GM21	III-11–18
Telescopus fallax	r^*	03.09.1900	Greece, from Sofia zoo	_	III-116–1
Zamenis longissimus	\mathbf{r}^*	29.06.1931	Near Petrich	_	III-9–14
	1	14.05.2000	Tabachka, Ruse	MJ12	_
	1 + r	20.06.1918	Stargach mountain	_	III-9–6
Zamenis situla	$l + r^*$	08.2007	General Todorov	FL99	_
Natrix natrix	1 + r	19.07.1926	Chamkoria, Rila mountain	GM18	III-14–34
	r^*	20.05.1926	Harmanli	MG04	III-14–80
	1 + r	26.07.1930	Nadezhden, Harmanli	MG13	III-14–41
Natrix tessellata	r^*	02.06.2007	Ruse, near Danube river	MJ15	_
Malpolon insignitus	$l + r^*$	08.06.1925	Nadezhden, Harmanli	MG13	III-10–21
	1	26.05.1921	Harmanli	MG04	III-10–23
	r	10.05.1927	Haskovo	_	III-10–24
Vipera ammodytes	l + r	22.03.1933	Belidie khan, Sofia	FN75	_
	l + r	01.05.1926	Konstantinovo	NH67	III-1–57
	l + r	15.06.1931	Yablanitsa, Lukovit	KH66	III-1–79
	1*	10.05.1928	Velinovo, Tran	FN33	III-1–52
	r	26.07.1930	Ali Botush	GL28	III-1–49
Vipera berus	l + r	1905	Evksinograd, Varna	NH88	III-2–23
	1*	01.05.1925	Kutsina, Veliko Tarnovo	LH89	III-2–60
	l + r	15.09.1918	Aigidik, Rila mountain	GM06	III-2–31

TABLE 1. List of specimens used for hemipenial extraction

Side—"1" = left hemipenis, "r" = right hemipenis; Date—specimen's date of collection; UTM—name of the 10×10 km grid cell in the UTM grid zones 34/35T; Coll. N—NMNHS collection number; * denotes specimen used for measurements and shown on corresponding figure.

and Marx, 1962; Klaczko et al., 2014; Myers, 1974; Zaher, 1999; Zaher and Prudente, 1999), it is considered to be an exception rather than a rule, occurring mostly in highly diverged subgroups of the species. Except for a few cases (e.g. Bernardo et al., 2012; Zaher and Prudente, 1999), it does not affect the general shape and ornamentation, but more inconspicuous characteristics of the hemipenial morphology.

The huge variety of hemipenial shapes and ornamentation within snakes (even on the generic level) raises questions about the evolutionary development of this organ. The mechanisms constraining the morphology of the ophidian male copulatory organs are not understood in detail, although there are some studies dealing with that matter (Hollis, 2006; Jadin et al., 2010; Jenner and Dowling, 1985; King et al., 2009; Malhotra and Thorpe, 2004; Utiger et al., 2002), as well as a general review by Myers and McDowell (2014). The same ambiguity applies to the evolution of male genitalia in general, although it has also been a hot topic for the last 65 years (Langerhans et al., 2016). The first hypothesis on the evolution of male genitalia (Dufour, 1844) was provided even before the formulation of the evolutionary concept in "Origin of species" (Darwin, 1859). Considering the limitations in the widespread hypotheses for the mechanism of male genitalia evolution i.e. "lock-and-key" mechanism (Dufour, 1844), pleiotropy (Mayr, 1963) and sexual selection (Eberhard, 1985, 2001; Lloyd, 1979;

Waage, 1979), it remains a challenge to comment on the evolution of the hemipenial morphology in snakes.

In the present study, we investigated the hemipenial morphology of fifteen snake species from four families (Boidae, Colubridae, Lamprophiidae, and Viperidae). We provide the first morphological description of the male copulatory organ for three species—*Elaphe sauromates* (Pallas, 1814), *Telescopus fallax* (Fleischmann, 1831), and *Malpolon insignitus* (Geoffroy, Saint-Hilaire, 1827). Improved techniques for dissection and documentation of the hemipenis were proposed and a comparison with published descriptions of the hemipenial morphology at the specific and generic levels was provided. We analyze our findings in the evolutionary context and discuss potential phylogenetic and ethological factors that may impact hemipenial design.

MATERIALS AND METHODS

We investigated the hemipenial morphology of 15 species of widely distributed snakes from four families (Boidae, Colubridae, Lamprophiidae, and Viperidae). We examined the following 15 extant species, all but one occurring in Bulgaria: *Eryx jaculus* (Linnaeus, 1758) (2 ind.), *Coronella austriaca* Laurenti, 1768 (2 ind.), *Dolichophis caspius* (Gmelin, 1779) (3 ind.), *Elaphe sauromates* (1 ind.), *E. quatuorlineata* (Lacépède, 1789) (1 ind.), *Platyceps collaris* (Müller, 1878) (1 ind.), *P.*



Fig. 1. Selected examples of hemipenial characteristics in three species (*Eryx jaculus*, left; *Platyceps najadum*, middle; *Coronella austriaca*, right): 1–small spines; 2–large spines; 3–flounces; 4–calyces; 5–undivided sulcus spermaticus, and 6–divided sulcus spermaticus. On the hemipenis of *C. austriaca* the main hemipenial parts are shown – base, body and apical part.

najadum (Eichwald, 1831) (1 ind.), Telescopus fallax (1 ind.), Zamenis longissimus (Laurenti, 1768) (3 ind.), Z. situla (Linnaeus, 1785) (1 ind.), Natrix natrix (Linnaeus, 1758) (3 ind.), N. tessellata (Laurenti, 1768) (1 ind.), Malpolon insignitus (3 ind.), Vipera ammodytes (Linnaeus, 1758) (5 ind.), V. berus (Linnaeus, 1758) (3 ind.) (Table 1). We investigated all 31 available specimens at the collection of the National Museum of Natural History in Sofia. No suitable specimen of Xerotyphlops vermicularis (Merrem, 1820) was present in the collection. The species V. ursinii (Bonaparte, 1835) and V. aspis (Linnaeus, 1758) were not included, being considered extinct in Bulgaria (Stojanov et al., 2011). We used the classification proposed by Uetz and Hošek (2015) and present the species in the phylogenetic order proposed by Pyron et al. (2013). Considering the widespread concept that the general shape and ornamentation of the hemipenes are specific, only one hemipenis per species is necessary for the hemipenial description (see Cope, 1895; Dowling and Savage, 1960). Some of the organs were damaged or over-expanded during the preparation, so we used only the best hemipenes prepared for description and measurements. Both hemipenes of every specimen were extracted if available. All specimens used had been fixed and stored in alcohol. To

avoid potential artifacts from the ontogenetic shift in the morphology of the hemipenes (Jadin and King, 2012), we used only adults (identified after Stojanov et al., 2011).

The hemipenes were prepared using slight modifications of the methods described by Pesantes (1994) and additionally developed by Zaher and Prudente (2003) and Myers and Cadle (2003). After extracting the organ, it was soaked in 2% KOH solution for 30 min to 6 hr, depending on its size and duration of preservation in alcohol. During initial trials, we found that the three days soaking proposed by Pesantes (1994) was inappropriate and injurious for the organ. We decreased the duration of soaking as Zaher and Prudente (2003) suggest, but without increasing the concentration. After the soaking, the hemipenis was gently everted manually using tweezers and filled with petroleum jelly. It was colored by soaking it in a solution of Alizarin Red S (few crystals diluted in 100 ml of 50% ethanol) (Harvey and Embert, 2008; Nunes et al., 2012; Passos et al., 2013). Afterward, the hemipenis was placed in $\sim 75\%$ ethanol for permanent storing.

After coloration, we photographed the best-prepared hemipenis using a high-resolution digital camera (Nikon COOLPIX P510) by placing the object on a glass slide positioned about 20 cm above a black background and



Fig. 2. The hemipenis of *Eryx jaculus* (NMNHS III-17–38). Sulcate (left) and asulcate (right) view. Scale bar = 10 mm.

illuminating it by two opposite light sources to reduce shadows.

The terminology to describe hemipenial morphology is primarily after Dowling and Savage (1960) and Zaher (1999), with minor additions such as the division of the sulcus spermaticus and some of the categories for the apical part (i.e. rounded and pointed). We present the following data: 1) presence and level of bilobation; 2) shape (subcylindrical, attenuate, bulbous, or clavate; 3) type of capitation; 4) shape of the apical part (rounded, pointed, disked, terminal awns); 5) division of the sulcus spermaticus. We also described the presence of ornamentation of the different parts of the hemipenis (base, body, and apical part) i.e. spines, papillae, calyces, and flounces (see Fig. 1). We recorded the micro-ornamentation of flounces and calvces (smooth, scalloped, papillated or spinulated). In the case of large spines at the base, we use the term "hooks", which we found most appropriate, considering the terminology used by Cope (1895) and Keogh (1999).

In addition, we propose previously unreported terminology concerning hemipenial proportions because we hypothesize the form plays an important role in copulation and, therefore, it should be reflected in future descriptions. We calculated a "Hemipenis Proportion Index" (HPI), where the maximal width of the hemipenis is divided by its total length (measured from the base to the apex). For our morphometric measurements, we used digital calipers with precision up to 0.001 mm. A hemipenis with HPI > 0.5 was considered "stubby"; one with HPI between 0.5 and 0.25— "medium formed"; one with HPI < 0.25— "elongated".

We noticed that the organs shrunk, sometimes up to 10% in length for the large organs, after staying in 75% ethanol for ca. two years. All the measurements



Fig. 3. The hemipenis of *Coronella austriaca* (NMNHS III-13-48). Sulcate (left) and asulcate (right) view. Scale bar-10 mm.

presented in the results are made after the shrinking of the hemipenes.

RESULTS

In this section, we provide a detailed morphological description and the results of our morphometrical investigations of the hemipenes in fifteen snake species from four families. We represent the calculated "Hemipenis Proportion Index" (HPI) for every species. We provide the first morphological description of the male copulatory organ for E. sauromates, T. fallax, and M. insignitus.

Family Boidae

Eryx jaculus (Javelin sand boa). The hemipenis is simple, clavated (although on the pictures it looks subcylindrical) and noncapitated (Fig. 2). The s. spermaticus is undivided, terminating laterally. The base and the body are completely nude, without ornamentation. The apical part is disked and few flounces with scalloped edges are recognizable. The hemipenis is "medium formed" (HPI = 0.307).

Family Colubridae

Coronella austriaca (Smooth snake). The hemipenis is bilobed, subcylindrical, noncapitated (Fig. 3).



Fig. 4. The hemipenis of *Dolichophis caspius* (NMNHS III-12–36). Sulcate (left) and asulcate (right) view. Scale bar-10 mm.

The s. spermaticus is undivided, terminating laterally. Many small spines are notable on the base of the hemipenis and an increase of the spines' size is present along the body. The apical part of the hemipenis is richly ornamented with numerous small and evenly dispersed spines. The lobes are pointed. An interesting and recognizable characteristic is the nude area on the medial side of the lobes. The hemipenis is "elongated" (HPI = 0.232).

Dolichophis caspius (Caspian whipsnake). The hemipenis is simple, bulbous, and noncapitate (Fig. 4). The s. spermaticus is undivided and terminates laterally on one of the lobes. The base is nude, with no structures except a small swelling of the tissue. Numerous small spines, few moderately sized spines and calyces with spinulated edges are present on the body. The apical part, presented with one lobe only, is rounded and highly ornamented with spinulated calyces. A nude area on the top of the lobe is present. We suspected that this could be an artifact of preparation i.e. overexpansion of the lobe due to the soft tissue on the top of it, but all the extracted hemipenes showed the same characteristic even with low-pressure filling. The hemipenis is "medium formed" (HPI = 0.305).

Elaphe quatuorlineata (Four-lined snake). The hemipenis is slightly bilobed, bulbous, and noncapitate (Fig. 5A). The s. spermaticus is undivided and terminates laterally. The base of the organ is covered with many moderately sized spines and a few hooks. The body is ornamented with small spines and spinulated calyces. The apical lobes of the hemipenis are rounded and also ornamented with spinulated calyces. The hemipenis is "medium formed" (HPI = 0.370).

Elaphe sauromates (Blotched snake). The organ is very similar in shape and ornamentation to that of *E. quatuorlineata* (Fig. 5B). The hemipenis is slightly bilobed, bulbous, and noncapitate. The s. spermaticus is undivided and terminates laterally. The base of the hemipenis is ornamented with numerous moderately sized spines and few hooks. The body is covered with a lot of moderate spines, smoothly transforming into spinulated calyces. The apical part is ornamented with spinulated calyces and the lobes are rounded. A nude area is also present on the asulcal side of the apical part. The hemipenis is "medium formed" (HPI = 0.322).

Platyceps collaris (Red whip snake). The hemipenis is simple, subcylindrical, and noncapitate (Fig. 6A). The s. spermaticus is undivided and terminates in the center of the lobe. The base is covered with numerous small spines. These structures are present also along the body with a higher density. The apical lobe is rounded and ornamented with a lot of small spines and spinulated calyces. The hemipenis is "medium formed" (HPI = 0.364).

Platyceps najadum (Dahl's whip snake). The hemipenes is very similar to that of *P. collaris*, being simple, subcylindrical, and noncapitate (Fig. 6B). The s. spermaticus is undivided and terminates in the center of the lobe. On the base and the body numerous small spines are recognizable. The apical part is ornamented with spinulated calyces which turn into papillated calyces in the distal part of the lobe. Along with the value of HPI, this could be considered the main difference between the morphologies of the hemipenis of *P. najadum* and *P. collaris*. The hemipenis of *P. najadum* is "elongated" (HPI = 0.238).

Telescopus fallax (European cat snake). The hemipenis is simple, subcylindrical to bulbous, and non-capitate (Fig. 7). The s. spermaticus is undivided and terminates in the center of the lobe. All over the base and the body there are a lot of small spines, with higher density on the body. The apical part is ornamented with spinulated calyces. The apex is disked. The hemipenis is "medium formed" (HPI = 0.479).

Zamenis longissimus (Aesculapian snake). The organ is slightly bilobed, bulbous, and noncapitate (Fig. 8A). The s. spermaticus is undivided and terminates laterally on one of the lobes. Few medium sized spines and hooks are conspicuous on the base. Large spines are missing on the body, but the medium sized spines are presented with higher density and a few small spines are also recognizable. The apical part is covered with papillated calyces. The apical lobes are small and rounded. The hemipenis is "medium formed" (HPI = 0.306).

Zamenis situla (European ratsnake). The hemipenis is slightly bilobed, subcylindrical, and noncapitate (Fig. 8B). The organ is notably asymmetrical. The s. spermaticus is undivided and terminates laterally on one of the lobes. The base is ornamented with numerous small spines and two big hooks. The body is covered with many small and moderate spines and a few large spines. The apical part is classified as pointed and one of

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Fig. 5. The hemipenis of *Elaphe quatuorlineata* (NMNHS III-4-4) – sulcate (A, left) and asulcate (A, right) view and the hemipenis of *E. sauro-mates* (absence of museum number, the specimen is found on the Dervent Hights, Boliarovo, 2010) – sulcate (B, left) and asulcate (B, right) view. Scale bar-10 mm.



Fig. 6. The hemipenis of *Platyceps collaris* (absence of museum number, specimen is found near Lozenets village, 1973) —sulcate (A, left) and asulcate (A, right) view of and the hemipenis of *P. najadum* (NMNHS III-11-18) —sulcate (B, left) and asulcate (B, right) view. Scale bar—10 mm.

the lobes is significantly larger than the other one. Spinulated calyces are presented on the proximal part of the apex and smooth calyces are visible on the distal part of the lobes. The hemipenis is "medium formed" (HPI = 0.479).

Natrix natrix (Grass snake). The hemipenis is slightly bilobed, subcylindrical, and noncapitate (Fig. 9A). The s. spermaticus is undivided and terminates centrally. Numerous small spines are visible on the base of the organ and one hook as well. The same structures are

present on the body, but with higher density. The apical lobes are pointed and covered with small spines. A nude area is visible on the medial side of the lobes. The hemipenis is "medium formed" (HPI = 0.416).



Fig. 7. The hemipenis of *Telescopus fallax* (NMNHS III-6–2). Sulcate (left) and asulcate (right) view. Scale bar-10 mm.

Natrix tessellata (Dice snake). The hemipenis of *N. tessellata* is very similar to that of *N. natrix* (Fig. 9). Differences are found in the apical part of the hemipenes and no hook is visible on the base of the *N. tessellata* hemipenis. The hemipenis of *N. tessellata* (Fig. 9B) is slightly bilobed, subcylindrical, and noncapitate. The s. spermaticus is undivided and terminates centrally. A lot of small spines are visible along the whole organ and few moderate spines are present on the base of the hemipenis. The lobes are pointed. The hemipenis is "elongated" (HPI = 0.248).

Family Lamprophiidae

Malpolon insignitus (Eastern Montpellier snake). The hemipenis is simple, noncapitate, and attenuate (Fig. 10). No structures are present along the organ. The s. spermaticus is undivided and terminates centrally. The hemipenis is relatively small compared to the snake's large body length—the SVL of one of the individuals used for the extraction of the hemipenis is 1363 mm total length, and its hemipenis is only 9 mm in length. The organ is "medium formed" (HPI = 0.277).

Family Viperidae

Vipera ammodytes (Nose-horned viper). For the description of *V. ammodytes* hemipenis we used newly prepared hemipenes, but the detailed intraspecific variation of the hemipenes among the three clades *V. a. ammodytes*, *V. a. montandoni* and *V. a. meridionalis* (Andonov and Tzankov, unpublished data) is not considered essential for the general morphology presented here. The hemipenis is noncapitated, divided and



Fig. 8. The hemipenis of Zamenis longissimus (NMNHS III-9–14) –sulcate (A, left) and asulcate (A, right) view and the hemipenis of Z. situla (no museum number was available, the specimen has been found near General Todorov, 2007) –sulcate (B, left) and asulcate (B, right) view. Scale bar-10 mm.

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Fig. 9. The hemipenis of *Natrix natrix* (NMNHS III-14–80) —sulcate (A, left) and asulcate (A, right) view and the hemipenis of *N. tessellata* (no museum number was available, the specimen is found near Ruse, 2007) —sulcate (B, left) and asulcate (B, right) view. Scale bar—10 mm.



Fig. 10. The hemipenis of *Malpolon insignitus* (NMNHS III-10–21). Sulcate (left) and asulcate (right) view. Scale bar-10 mm.

subcylindrical to bulbous in shape (Fig. 11A). The s. spermaticus is divided at the distal part of the body, surrounding a barely visible intrasulcular region. The base is ornamented with numerous small spines and a few hooks. The body is covered with small, moderate, and a few large spines. Small spines and papillated calyces are

present on the apical lobes. Terminal awns are recognizable on the top of the lobes. The hemipenis is "stubby" (HPI = 0.571).

Vipera berus (Common European viper). The hemipenis of *V. berus* is bilobed and more gracile than the hemipenis of *V. ammodytes.* It is subcylindrical and noncapitate (Fig. 11B). The s. spermaticus is divided at the distal part of the body, without surrounding the conspicuous intrasulcular region. The base is weakly ornamented with only a few hooks present. Few moderate and large spines are present on the body. The lobes are covered with a lot of small and moderate spines on their proximal part and spinulated calyces on the distal part. Terminal awns are easily recognizable on the top of the lobes. The hemipenis is "medium formed" (HPI = 0.425).

DISCUSSION

In the range of the discussion we compare the morphology of the hemipenes we extracted to other known descriptions, within the species and the genus. We also noted some differences with previously published descriptions of the organs, so we comment on methodologies of hemipenial preparation. The variety of hemipenial shapes we observed provoked us to discuss the evolution of these complicated structures.

Comparison of the Hemipenial Morphology at the Intrageneric Level, with Comments on Previous Descriptions

We provide an intrageneric comparison of the hemipenial morphology except for three species where no



Fig. 11. The hemipenis of *Vipera ammodytes* (NMNHS III-1–52) --sulcate (A, left) and asulcate (A, right) view and the hemipenis of *V. berus* (NMNHS III-2–60) --sulcate (B, left) and asulcate (B, right) view. Scale bar-10 mm.

congeneric species have been described—*C. austriaca*, *D. caspius*, and *T. fallax*.

Figure 2 (op. cit.), the hemipenis presented there is more elongated (HPI = 0.470), but still in the same category ("medium formed") as in our calculation (HRI = 0.305).

Family Boidae

Eryx jaculus. We compared our data with the description of Tokar and Obst (1993) and found some major differences. On Figure 6 (op. cit.), the hemipenis looks stubby, with no other structures but smooth calyces. According to our results, the morphology of the organ is rather different. The variation in the morphological descriptions can be explained by the application of different methods of preparation. We propose that the description made by Tokar and Obst (1993) is based on not fully everted hemipenis, thus, some of the structures remained unrecognizable.

A description of the hemipenial morphology is available for only one other species of this genus - *E. johnii* (Russell, 1801) (Kluge, 1993). The hemipenes of *E. johnii* and *E. jaculus* are quite similar and only small differences are notable. The main difference is in the s. spermaticus, which is undivided in *E. jaculus* and divided in its distal section in *E. johnii*. Both species have smooth flounces on the apical part of the copulatory organs, however, in *E. johnii* flounces are visible on the body and smooth calyces are presented on the apical part along the flounces. In both species the morphology of the hemipenes is rather basal and lacks carbonated structures.

Family Colubridae

Coronella austriaca. The extracted hemipenis fully matched the description by Branch and Wade (1976).

D. caspius. The extracted hemipenes showed similarities with the description by Schätti (1986), although we noted some differences. The typical proximal swelling on the base of the hemipenis we describe is not mentioned by Schätti (1986). Based on calculations we made on

Elaphe sp.. Before we discuss this genus, we have to identify the species dissected from Dowling and Fries (1987). Their study was published prior to the split of *E. quatuorlineata* and *E. sauromates* into separate species. The specimen that Dowling and Fries (1987) describe— (HISS-75528) was sought out in the American Museum of Natural History database. Unfortunately, we could not find the exact specimen, but close numbers clearly belonging to *E. quatuorlineata* were checked. According to their description and localities of origin, we conclude that the animal dissected by Dowling and Fries (1987) belongs to *E. quatuorlineata*. Thus, here we provide the first description of the hemipenis morphology in *E. sauromates*.

We found some differences between the description made by Dowling and Fries (1987) and our data. The hemipenis we prepared is "medium formed", while the drawing included in the op. cit. represents the organ much stubbier. Based on our experience, it could be the outcome of overexpansion in one of the extractions or an inaccuracy in the representation.

Guo et al. (2012) described a third congener—the King ratsnake *E. carinata* (Günter, 1864). The species has a different hemipenial morphology compared to the closely resembling *E. sauromates* and *E. quatuorlineata*. The King ratsnake has a simple ornamented hemipenis with papillae instead of spines on the body. The form is more extended in the apical part compared to the hemipenes of *E. sauromates* and *E. quatuorlineata*, and it is clavate. Despite these differences, the ornamentation of the hemipenis is similar. A possible difference in the expansion of the hemipenes due to the application of different techniques could explain the diverged shape in *E. carinata*.

Platyceps sp.. We found considerable differences upon comparing our results on *P. collaris* with those of Rehak and Obst (1993). We believe the hemipenis they described was not fully everted. We extracted more elongated organs with easily recognizable rounder apical lobes, covered with calyces. Although we inadvertently caused slight damage at the base of the hemipenes of *P. collaris* that we studied (Fig. 6), and full expansion was not possible, the organs were still completely everted, suggesting that the description we provide is more accurate.

Concerning *P. najadum*, we identified several differences with the description of the hemipenial morphology presented by Darewskij and Ščerbak (1993). We propose that Darewskij and Ščerbak (1993) describe a non-fully everted hemipenis, resulting in incomplete data and erroneous presentation of a number of characteristics. The organ we extracted is more elongated, with rounded apical lobes instead of disked. In our specimens, the apical parts were covered with calyces and not spines.

We found hemipenial descriptions for three more congeneric species-P. bholanathi (Sharma, 1976) described by Seetharamaraju and Srinivasulu (2013); P. rhodorachis (Jan, 1865) by Schätti et al. (2014); P. ventromaculatus (Gray, 1834) by Schätti and Schmitz (2006). The hemipenes of all five described species are generally similar-the form is "simple", subcylindrical and noncapitated. The s. spermaticus is undivided and terminates in the central part of the lobe. Differences can be found in the carbonated structures in the base and on the body-P. collaris, P. najadum, and P. ventromaculatus have numerous small spines with similar density. The hemipenis of P. bholanathi has a low number of moderatelysized spines, while P. rhodorachis has both small as well as moderate spines. A visible difference is present also on the apical part. In all species except P. bholanathi calyces are found on the apical part, but in *P. bholanathi* only a few moderate spines are recognizable.

Zamenis sp.. We compared our description of Z. longissimus to the one made by Böhme (1993). We found no particular differences, except that the organs we extracted look more elongated.

We compared our data on Z. situla to that in Obst et al. (1993) and found significant differences. Thus, we consider the description of Obst et al. (1993) incomplete, being based on not fully everted hemipenis. The drawing in Obst et al. (1993) likely represents only the base and part of the hemipenial body.

The hemipenis of Z. situla is conspicuously different from that of Z. longissimus and only a few similar patterns are present. The main difference is their general shape—bulbous in Z. longissimus and subcylindrical in Z. situla. The density of the structures is also quite different—the calyces on the apical part are papillated in Z. longissimus and smooth and spinulated in Z. situla.

We found no other descriptions of congeneric species.

Natrix sp.. We compared the description made by Branch and Wade (1976) of the hemipenis in *N. natrix* to our dataset (Fig. 9A) and noted no considerable differences. A description of the *N. tessellata* hemipenis was also made by Darewskij in Gruschwitz et al. (1999). The main difference we found refers the apical parts. The

lobes are actually much bigger than these shown by Darewskij in Gruschwitz et al. (1999) and the s. spermaticus terminates centrally (not laterally), which is a significant difference as it may have some functional implications.

Family Lamprophiidae

Malpolon insignitus. To date, no description of the hemipenis of *M. insignitus* was found. The form of the copulatory organ of *M. monspessulanus* (Herman, 1804) was described by De Haan (1982, 1999). The hemipenes in both species share a similar design, but the organ is more elongated in *M. monspessulanus*. A hemipenial description of *Rhagerhis moilensis* (Reuss, 1834) was provided by Schleich et al. (1996). The species belongs to the Psammophiinae subfamily which was considered to be part of the *Malpolon* genus until Böhme and De Pury (2011) changed its taxonomical status. The general design of the hemipenis in *R. moilensis* is rather similar to that of *M. insignitus* and *M. monspessulanus*, but the form is more elongated than in both *Malpolon* species.

Family Viperidae

Vipera sp. Descriptions of the hemipenial morphology including illustrations for V. ammodytes and V. berus were presented by Domergue (1962) and Branch and Wade (1976), Milto and Zinenko (2005), respectively. The hemipenes we extracted showed no conspicuous differences. We found descriptions for two more congenerics—V. barani Böhme and Joger, 1983 (Joger et al., 1997) and V. ursinii (Gasc, 1968). All congenerics show a similar pattern, having bilobed, noncapitated hemipenes. The s. spermaticus is divided and the lobes have the unmistakable terminal awns.

Comments on the Methods for Description of the Male Copulatory Organ in Snakes

Comparing our results to previous descriptions, we conclude that the method used for everting and expanding the hemipenis significantly affects the description of its morphology. Most previous descriptions are based on non-fully everted hemipenes (still attached to the specimens and not filled with anything); thus, only some structures are recognizable. In the past, such kind of manipulations was used as the common technique for presenting the male snakes' genitalia.

In addition, to date, we have little information concerning the levels to which the hemipenis is expanded during copulation and what the exact functions of all different elements of the hemipenial surface are. Only a few studies discuss the fit of the hemipenis to the female cloaca (Edgren, 1953; Inger and Marx, 1962; Pisani, 1976; Pope, 1941; Siegel et al., 2011, 2012; Showalter, 2014) and the role of all structures of the hemipenis. Hence, we assume that every detail is significant for the successful copulation, and the design of every single element is constrained by sexual selection, so all of the structures should be described meticulously.

The modified method we used for this article provides the most comprehensive description of the construction of the male copulatory organs so far. The colorization

method we used allowed us to clearly distinguish carbonated from noncarbonated structures. Among all techniques for preparation and coloration of hemipenes (see Branch and Wade, 1976; Jadin and Parkhill, 2011; Ortenburger, 1923) during initial trials we found the one used herein being the most appropriate and easy for implementation.

However, we note that after two years in ethanol, the hemipenes have shrunk. Possibly, the alcohol dehydrated the tissue, but this is unlikely, considering that the specimens we used were preserved in alcohol for an extended time before this manipulation. We soaked a few of the organs in water for 12 hr to observe possible rehydration, but it did not occur and the organs did not expand, suggesting alcohol was not the primary cause for deformation. Other explanation might be found in the texture of the petroleum jelly and a possible initial presence of miniature air bubbles introduced within its structure during the filling. A more detailed research with a representative sample should be implemented for understanding the possible cause of the shrinking, so the technique could be improved further. Although the general shape of the hemipenes is not affected, it is an important occurrence to note, because it may impact the morphometric calculations.

General Analysis of the Morphological Design of the Male Mating Organ in Snakes

Multiple hypotheses have been proposed for the mechanism of the evolution of the male genitalia. Generally, we can classify them into three main categories (see also Arnqvist, 1997; Ah-King et al., 2014): "lock-and-key" mechanisms (Dufour, 1844), pleiotropy (Mayr, 1963), and sexual selection. The latter includes the cryptic female choice and Fisherian selection (Eberhard, 1985, 2001), sexual conflict (Lloyd, 1979) and sperm competition (Waage, 1979). Of course, all these hypotheses are nonmutually exclusive (Langerhans et al., 2016). Another modern hypothesis in the field of genetics concerns the expression of the Hox-genes regulation of the general morphology of the body, including male genitalia (Cohn, 2011; Gredler et al., 2014; Leal and Cohn, 2014). This hypothesis treats even the calcified ornamentation of male genitalia in the squamates as being correlated with the limbs' reduction. However, research on the gymnophthalmid lizards' male genitalia partially disproves this idea (Nunes et al., 2014).

Although several reviews were compiled on hypotheses of sexual evolution, the studies treat mainly invertebrates (e.g. Hosken and Stockley, 2004; Simmons, 2014) and not much is known about vertebrates (Brennan and Prum, 2014). Thus, we analyze the existing general hypotheses for male genital evolution while being aware of possible deviations in the mechanism of evolution among different groups of animals. Moreover, we cannot hastily apply a mechanism found in arthropods to vertebrates.

Our analysis of the hemipenial morphology in congeneric snake species indicates that there are common trends in the general design of the male copulatory organ. However, so far we cannot imply strong phylogenetical signals in the form of the hemipenis, because to date the information is rather fragmented. Congeneric species could have similar, but also totally different hemipenial morphology. For example, the genera Atractus and Dipsas show high intrageneric variation in the general shape and ornamentation (Cadle and Myers, 2003; De Lima and Prudente, 2009; Harvey and Embert, 2008; MacCulloch and Lathrop, 2004; Passos and Lynch, 2010; Passos et al., 2010; Prudente and Passos, 2010). Studies revealing intraspecific variation (Bernardo et al., 2012; Inger and Marx, 1962; Klaczko et al., 2014; Myers, 1974; Zaher, 1999) make the puzzle even more difficult to solve.

Furthermore, the evolutionary forces shaping the male copulatory organ in snakes could not be fully understood without detailed data on the morphology of the female cloaca (Ah-King et al., 2014). There is significant imbalance in the published papers devoted to the morphology of the male copulatory organs and those of females. Our knowledge on the construction of the female ophidian cloaca is even more limited than that for the hemipenis (e.g. Edgren, 1953; Inger and Marx, 1962; Pope, 1941; Pisani, 1976; Siegel et al., 2011, 2012; Showalter, 2014). Perfect fit of the hemipenis and the female cloaca is found by Pope (1941) in his study on Liophis poecilogynis (Wied-Neuwied, 1825). The author provides a detailed description of the position of the male and female copulatory organs after killing and dissecting two copulating specimens. Edgren (1953) described the fit between the male hemipenis and the female cloaca of Heterodon platirhinos Latreille, 1801, but he does not report such a close fit as described by Pope (1941). Still, the specimens used by Edgren (1953) have been preserved and tissue dehydration might have affected the form of the organs. Inger and Marx (1962) concluded that there is no correlation between the form of the cloaca and the hemipenis in Calamaria lumbricoidea Boie, 1827. However, they used both adults and subadult specimens, which probably affected the results, considering the ontogenetic changes in hemipenial (Jadin and King, 2012) and female cloacal development (Showalter, 2014). Siegel et al. (2011) performed phylogenetic analyses on the development of female cloaca in snakes and specifically on the structure between the urodaeum and the oviducts termed "pouch". Although there are synapomorphies found within the evolution of the pouch morphology and mismatches between the pouch and hemipenial morphology, Siegel concluded that the hypothesis of correlation between both could not be refuted and further research with broader sampling is required, especially given that there are instances where correlation is plausible (e.g. psammophiids). However, since there is a serious contradiction to the "lock-andkey" mechanism, more investigations are needed to refute or support this hypothesis.

On the base of his results, Nunes et al. (2014) suggests, that the highly ornamented hemipenial morphology of snakes is likely not correlated with limb reduction. The hypotheses of the pleiotropy and the effects of the hox-genes need verification and remain rather speculative in the meantime. So, we concentrate our discussion on the third main hypothesis concerning the evolution of the male copulation system in snakes the role of the sexual selection.

We have to stress that no detailed study on the evolution of the ophidian hemipenial morphology has been published to date. Only a few studies based on a limited number of species analyze the evolutionary aspect of the hemipenial morphology (Hollis, 2006; Jadin et al., 2010; Jenner and Dowling, 1985; King et al. 2009; Malhotra and Thorpe, 2004; Utiger et al., 2002), with an interesting discussion presented by Myers and McDowell (2014). Thus, the mechanisms driving the evolution of the hemipenial morphology are not yet identified. Even though hemipenial morphology is probably not affected by habitat preferences or diet of the species as some studies suggest (Branch, 1986; Dowling, 1967; Keogh, 1999), we are skeptical to consider the hemipenial morphology as conservative. Considering the large variety of hemipenial shapes even at the congeneric level (Andonov, 2016; Cadle and Myers, 2003; De Lima and Prudente, 2009; Harvey and Embert, 2008; MacCulloch and Lathrop, 2004; Passos and Lynch, 2010; Passos et al., 2010; Prudente and Passos, 2010; this study), we hypothesize that the hemipenial form is evolutionary plastic, being defined chiefly by the behavior and more specificallythe mating behavior. The hypothesis that sexual selection is the most important factor for the evolution of the male genitalia among animals is definitely not new (see Eberhard, 1985; Smith, 1984; Thornhill, 1984). According to Rivas and Burghardt (2005), polyandry among snake species occurs as often as polygyny and the dominant mating system in snakes should be most accurately termed "polygynandry" instead of promiscuity. The authors noted that multiple paternity is the norm in snakes. King et al. (2009) and Friesen et al. (2013) provided evidence for a positive correlation between hemipenial morphology, duration of the copulation, and the copulatory plug deposition for Thamnophis species. This suggests that the more ornamented hemipenis would lead to more efficient copulation. But if multiple paternity is the norm in snakes, hemipenial ornamentation would play a significant role only if it somehow affects the behavior of the female after copulation (for example by affecting the epithelia of the female cloaca and preventing further sexual activity). The latter would support the hypothesis of cryptic female choice.

Although the male competitive behavior is relatively difficult to observe, there is information about some of the species included in the present study. Male-male combats were recorded for *C. austriaca*, *Z. longissimus*, *M. insignitus*, *V. ammodytes* and *V. berus* (Andrén, 1986; Davis, 1936; Senter et al., 2014; Shine, 1994; Stojanov et al., 2011). Considering the small and naked hemipenis of *M. insignitus* and the relatively big and highly ornamented hemipenes of *V. ammodytes* and *V. berus*, according to our findings, there is no likely connection between male competitive combats and the morphology of the hemipenis. However, more species should be performed.

In the context of male-male competition, we have to comment on the design of the hemipenis in M. insignitus. The species possesses a surprisingly small hemipenis relative to its long total body size. Unfortunately, there is only limited data concerning the ecology and biology of M. insignitus after the species obtained its rank (Carranza et al., 2006); thus, we use information available for the behavior of M. monspessulanus (see De Haan, 1993, 2003, 2006; De Haan and Cluchier, 2006). Even though male-male combats are typical for this species, we propose that other ethological aspects (like its highly developed chemical communication) could explain the lack of a big and ornamented hemipenis.

Pheromonal communication among snakes, especially the sex pheromones, is currently not well-researched. Most studies on snake sex pheromones concentrate on the genus Thamnophis (e.g. Ford and Low, 1983; LeMaster and Mason, 2001; Mason et al., 1989; Shine and Mason, 2012), and only a few treat other species (e.g. Andrén, 1982; Greene and Mason, 1998). So far research on pheromones suggests that females are chemically attracting males, which then fight each other to win the female. We assume that in some species it could be the other way round-the males actively exude pheromones in order to attract females while guarding their territory. Either territorial behavior by males or male-male combats would allow females to make the choice before copulation. Therefore, "locking mechanisms," such as calcified structures, would be unnecessary. This still does not explain why some snakes with typical malemale combats have highly ornamented hemipenes while others do not. Rivas et al. (2007) reported another mechanism for successful copulation between snakes: in Eunectes murinus (Linnaeus, 1758) the male coils around the female during copulation, preventing the other males in the breeding ball from copulating with the female. This "coiling" behavior may impact the hemipenial ornamentation and serve as an alternative to successful copulation and such behaviors should definitely be considered.

CONCLUSION

We emphasize that the precise description of the morphology of the male copulatory organs in snakes is dependent on the application of proper methods for hemipenis extraction. Further research will reveal whether, besides phylogenetical factors, ecological and behavioral factors such as diet, habitat preferences, copulation duration, intensity of intraspecific mating competition, intensity of locomotion during the copulation impact the morphology of the hemipenis in snakes. Implementing ancestral state reconstructions and phylogenetic comparative methods are essential for the statistical support of the hypotheses, but a larger sample is required, and thus are beyond the scope of this article. We hypothesize that behavioral factors are the most significant drivers that affect the morphology of the ophidian copulation organs in both sexes.

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