Acta Herpetologica 19(1): 3-12, 2024
DOI: 10.36253/a_h-14145

Hemipenial morphology does not provide insight on mating barriers between the two main lineages of *Hierophis viridiflavus* (Lacépède, 1789)

Federico Storniolo[1,*], Thomas Dadda[1], Stefano Scali[2], Marco A. L. Zuffi[3], Marco Mangiacotti[1], Roberto Sacchi[1]

[1] *Dipartimento di Scienze della Terra e dell'Ambiente, Università di Pavia, Via Torquato Taramelli 24, 27100 Pavia, Italy*
[2] *Museo di Storia Naturale di Milano, Corso Venezia 55, 20121 Milano, Italy*
[3] *Museo di Storia Naturale dell'Università di Pisa, Via Roma 79, 56011 Calci (PI), Italy*
[*]*Corresponding author.* Email: federico.storniolo01@universitadipavia.it

Submitted on: 2023, 28th December; revised on: 2024, 9th January; accepted on: 2024, 16th January
Editor: Andrea Villa

Abstract. Copulatory organs are a key trait in reproductive compatibility and sexual isolation. The role of male genitalia in boosting mating success is well known and is often the outcome of behavioural and biological constraints, although no clear and common interpretation about their evolution appears broadly applicable. In snakes, hemipenial morphology has often been described under the perspective of sexual selection, taking into consideration both behavioural and morphological traits of both sexes. We investigated hemipenial morphology and ornamentation in the two subspecies of *Hierophis viridiflavus*, a male-male combating colubrid, and compared it to the sister species *H. gemonensis*, to assess intraspecific variation in size of genitalia and ornamentation richness. The male intromitted organ of this species is unilobed and bulbous, with rich ornamentations consisting of basal spines and apical calyculations. We detected no statistically significant difference in hemipenial size, basal spine count, and spine length between the two subspecies, suggesting that no copulatory barrier is present between the two clades. Although hemipenial morphology and anatomy do not seem suitable to assess intraspecific variability as shown in this study, they are often highly variable at the family or genus level, suggesting that the evolution of male genitalia is linked to phylogenetic relationships and that hemipenial divergence might be correlated to lineage splitting despite not being necessarily the main cause. Future studies should be aimed at investigating hemipenial morphology and anatomy across species under the perspective of comparative phylogeny and reproductive behaviours to address thoroughly the constraints of hemipenial evolution and development.

Keywords. *Hierophis viridiflavus*, mating barriers, hemipenes, anatomy, morphology.

INTRODUCTION

The anatomy and morphology of copulatory organs have been of great interest for herpetologists in the last century especially concerning snakes (Cadle, 2011; Folwell et al., 2022). As a matter of fact, hemipenes are postulated to play a major role in mating success, being supposedly species-specific (Cope, 1895; Keogh, 1999), and thus with marked implications in terms of reproductive biology and behaviour (Tokarz, 1988; King et al., 2009; Klaczko et al., 2017). Reptilian hemipenes show highly variable morphological traits, in terms of the gross shape of the organ itself (unilobed, bilobed) as well as of its external ornamentations, which can consist of rigid spines (spread across the organ or aggregated in a specific region, i.e., basal region or the apex; Fig. 1) or soft tissue folds, or otherwise can be completely absent (Zaher et al., 1999; Andonov et al., 2017).

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

www.fupress.com/ah

The extent of hemipenial morphological variability can remarkably vary among different families (Cadle, 2011; Andonov et al., 2017), but also at lower taxonomic levels (Inger and Marx, 1962; Branch, 1986; Zaher, 1999; Zuffi, 2002; Bernardo et al., 2012; Klaczko et al., 2014; Myers and McDowell, 2014). From this perspective, investigating the mechanisms that drive the evolution of specific features in copulatory organs can be of great interest to address phylogenetic relationships and species splitting over time as copulatory organs are some of the most rapidly evolving traits in squamates (Brennan and Prum, 2015; Klaczko et al., 2015, 2017; Folwell et al., 2022). Hypotheses have been proposed for the development of male genitalia, first of which the "lock-and-key", formerly postulated by Dufour (1844), states that male genitalia evolve to be complementary to those of females with noticeable species-specificity. Alternatively, the pleiotropy hypothesis for male genitalia differentiation has been partly supported, hypothesizing that they evolve through selective pleiotropic effects on other traits (Mayr, 1963; Edwards, 1993; Arnqvist and Thornhill, 1998; Hosken and Stockley, 2004). This hypothesis appears still unsuitable to be applied broadly as a common rule, because it assumes that the set of genes coding for general morphology codes also for genital morphological variation, which should not be selected against, implying tight genetic correlation between genital and general morphology (Arnqvist and Thornhill, 1998). These hypotheses have been revised broadly (Shapiro and Porter, 1989; Sota and Kubota Soto et al., 2013; Brennan and Prum, 2015) in an evolutionary perspective highlighting the role of genital morphology as a barrier against hybridisation, favouring coevolution between male and female genitalia (House et al., 2020; Greenwood et al., 2022).

Ophidian hemipenes have been studied vastly in the last decades (Zaher, 1999; Myers and McDowell, 2014) under the functional perspective as they are related to copulation duration as well: indeed, in New World natricines more complex and ornate hemipenes (as in *Thamnophis radix*) are associated to more prolonged copulations compared to the congeneric *T. sirtalis*, characterised by simple subcylindrical hemipenes with little ornamentation (King et al., 2009). From this perspective, the occurrence of abundant ornamentations on hemipenial surfaces of both apical and body part of the organ appears to be relevant in terms of how efficiently males remain attached to females (Friesen et al., 2014), significantly affecting the duration of copulation and thus male fitness. Additionally, Rivas et al. (2007) showed that male coiling during copulation in species that undergo mating balls (*Eunectes murinus* in this case) can impede other males from mating with the female and, in these cases, more conspicuously ornamented hemipenes (i.e., more abundant or large ornamentations) should favour copulation and operate synergically with behaviour. On the other hand, snakes that exhibit male-male combat behaviours are subject to sexual selection prior to copulation. Therefore, hemipenis morphology should not be selected to evolve more complex structures such as calyces, spines, and hooks. However, as shown by Andonov et al. (2017), hemipenial morphology does not always correlate with behavioural strategies, so the scenario of the evolution of male genitalia is a complex task to untangle.

The green whip snake *Hierophis viridiflavus* (Lacépède, 1789) is one of the most widespread species in Mediterranean Europe as it occurs from Northern Spain across France and throughout Italy to Northern Balkans (Sillero et al., 2014). From the phylogenetic point of view, this species has been object of debate and Mezzasalma et al. (2015), according to both molecular and morphological differences, have split the two subspecies *H. v. viridiflavus* and *H. v. carbonarius* (Western and Eastern clade respectively) and elevated them to the rank of species. Recently, Speybroeck et al. (2020) have proposed to pool them together as a unique species; however, the debate is still open. As a matter of fact, the phylogenetic relationships between the two lineages are still unclear and recent research on the genetic basis of its colour polymorphism (mtDNA and nDNA; Senczuk et al., 2021) has suggested that these two lineages might undergo asymmetrical gene flow from the Western into the Eastern clade, even though no decisive evidence has been gathered. Morphological variability in dentition, pholidosis, and hemipenes has also been used to characterise the phylogeny of the *Hierophis* genus by Schätti (1987, 1988), discriminating the members of this genus with respect to sister groups (i.e., *Spalerosophis, Eirenis, Platyceps* genera); however, interspecific variability patterns within genus are still unexplored (Schätti and Monsch, 2004; Utiger and Schätti, 2004). With this respect, nevertheless, Schätti and Vanni (1986) have investigated morphological traits of the target species, among which hemipenes too, but no difference among populations was found by the authors; however, hemipenial morphology was not the key subject for investigation and no in-depth study of morphology and ornamentations was performed.

In this scenario, the role of copulatory organs can be useful to cast light on the reproductive aspects of species/subspecies delimitations as marked differences in hemipenial morphology and ornamentation might impede interbreeding driving divergence between lineages; on the other hand, similar hemipenes would not act as a barrier to hybridization, favouring gene flow and inter-lineage mating (King, 1989; Sota and Kubota, 1998; Greenwood et al., 2022). For such reasons we investigated hemipenial morphology to address potential morphofunctional advantages of hemipenial structures, such as increased copulation efficiency and duration according to differences in size, shape, and ornamentation, in the scenario of intraspecific lineage diversification. Additionally, we compared the gross morphology of *H. viridiflavus* as a whole, to that of the sister species *H. gemonensis* to check the extent, if any, of morphological variability of hemipenes at the genus level.

MATERIALS AND METHODS

Gross morphology

For morphological description we referred to the categories in Dowling and Savage (1960) and Andonov et al. (2017). We collected the left hemipenis from both fresh and museum collection specimens, preserved in ~75% alcohol. For sample preparation from alcohol-preserved specimens, we slightly modified the method in Andonov et al. (2017) and Zuffi (2002): for hemipenis filling we preferred liquid paraffin over petroleum jelly (used by Pesantes, 1994; Myers and Cadle, 2003; Zaher and Prudente, 2003) because it is easier to use as it does not need to be kept in liquid state and, being less viscous, it is quicker and simpler to inject through syringe. We propose to use this technique in case the injection of petroleum jelly appears to be difficult. Firstly, the organs were soaked in 2% KOH after extraction for 30 minutes to 6 hours according to its size and duration of preservation. After this period, hemipenes were everted manually using tweezers, and subsequently filled with liquid paraffin. Hence, we sealed hemipenes at the base using a thin string. Fresh samples were processed using the same procedure as for alcohol-preserved specimens, except they were soaked in water rather than KOH solution for tissue softening before eversion. Lastly, all samples were stored in ~75% alcohol for permanent preservation. Each hemipenis was photographed on both sulcate and asulcate surfaces using a high-resolution reflex camera (NIKON D7100) by placing it on a black surface under two light sources on opposite sides to minimize shadows. We used the "magic wand" tool built in Photoshop CS3 (version 10.0) to eliminate any remaining shadow from the pictures to produce a clean photo of each object.

We prepared and analysed 10 left hemipenes for each currently accepted subspecies (20 in total, two road-killed and 18 alcohol preserved museum specimens; Table 1, for details). We also prepared a single left hemipenis of *H. gemonensis* for outgroup comparison.

Table 1. List of the specimens of *Hierophis viridiflavus* with the relative source of collection, clade, and morphometric measures. NHMPv Natural History Museum of Pavia; NHMPi: Natural History Museum of Pisa; NHMMi: Natural History Museum of Milan; Spines = number of basal spines; Length = hemipenial length; SVL = snout-to-vent length; Spine1-5 = length of five randomly selected basal spines.

Specimen	Source	Clade	Spines	Length (mm)	SVL (mm)	Spine 1 (mm)	Spine 2 (mm)	Spine 3 (mm)	Spine 4 (mm)	Spine 5 (mm)
LEC006 - 22	NHMPv	E	61	34.80	745	3.29	3.09	3.45	3.22	2.79
LEC008 - 22		E	51	30.87	875	3.64	3.53	2.79	3.14	2.56
CUN001 - 22		W	51	28.65	910	2.10	2.74	2.97	2.29	2.91
AGR001 - 22		E	46	25.98	796	3.73	2.65	2.83	2.12	3.40
LEC010 - 22		E	56	29.98	840	3.14	4.17	3.38	4.60	3.37
ARE001 - 22		W	63	28.17	950	1.99	2.63	3.38	2.15	1.72
ALE001 - 22		W	64	20.86	640	4.44	3.06	4.57	4.26	1.95
TOR006 - 22		W	52	20.45	897	2.24	1.83	2.70	2.10	1.74
1184	NHMPi	W	49	31.44	1053	4.00	3.75	2.34	4.32	3.04
1193		E	60	32.33	912	3.81	3.65	4.12	3.74	3.27
1213		E	60	36.62	910	3.22	2.25	2.36	2.45	3.11
1203		E	54	30.74	875	2.71	3.85	3.65	3.70	3.11
1206		E	39	25.06	880	4.67	4.10	2.92	3.11	2.66
1199		E	46	29.05	790	3.75	3.38	4.75	2.77	3.56
1191		W	52	31.04	972	3.42	5.37	3.84	3.67	4.01
GRO001 - 22	NHMMi	W	57	29.10	860	3.55	3.52	2.32	3.43	2.37
TOS001 - 22		W	51	30.85	740	2.64	3.68	4.29	2.50	2.54
TOS002 - 22		W	46	31.46	890	3.62	4.29	3.38	3.45	1.99
FIR001 - 22	field	W	56	22.96	715	3.04	2.66	3.22	2.15	2.76
GLP001 - 22		E	43	27.81	860	2.46	3.51	3.60	3.10	3.29

Quantitative analyses

To thoroughly describe any variation in hemipenial morphology and anatomy between the two main lineages of *H. viridiflavus* (Western and Eastern clades, hereinafter W and E respectively), we also recorded quantitative data concerning hemipenial size, number of basal spines, spine length, and snout-to-vent length (SVL) of each specimen. The count of the total number of spines was repeated three times to minimize counting error. From each hemipenis, we randomly extracted five spines from different parts of the basal region. Prior to performing any analysis, all predictors (hemipenial size, spine number, SVL, and clade) were tested for intercorrelation via Pearson's correlation test. No correlation was detected between spine count and hemipenial length (r = 0.13, P = 0.57), as well as between SVL and both hemipenial length (r = 0.37, P = 0.11) and spine count (r = -0.21, P = 0.38). Therefore, we performed a Linear Mixed-Effects (LME) model to test whether spine length depended on species clade when controlling for body size (SVL), hemipenial size, and number of spines. All those variables were implemented in the model as fixed effects. We also considered the two-way interactions between clade and both spine length and count to test for potential different effects in each group. Additionally, specimen entered the model as a random-intercept effect to account for inter-individual variability patterns that were unexplained by morphometry or lineage assignment. The model was performed with the *lme4* package (Bates et al., 2015); model visualisation was performed with the package *visreg* (Breheny and Burchett, 2017) and 95% confidence intervals were estimated with the package *bootpredictlme4* (Duursma, 2022). All analyses were performed on R 4.2.1 (R Core Team, 2022).

RESULTS

Gross morphology

The hemipenis of *Hierophis viridiflavus* is unilobed, bulbous, non-capitate (Fig. 1A-B). The basal region lacks ornamentation and does not show tissue swelling of any kind. The body part is characterised by numerous spines

Fig. 1. Hemipenes of *Hierophis viridiflavus* collected from two roadkilled specimens, respectively from the Western (A) and Eastern (B) clades, and hemipenis of *H. gemonensis* (C). The basal region (a) lacks ornamentations, whereas the body part (b) is completely covered in spines and the apical part (c) is markedly ornamented with calyculations (folds of tissue). Both the Western and Eastern clades show unilobed bulbous hemipenes and no difference in ornamentation and gross morphology. The sulcus spermaticus (white arrows) is unique and undivided from the base to the apex.

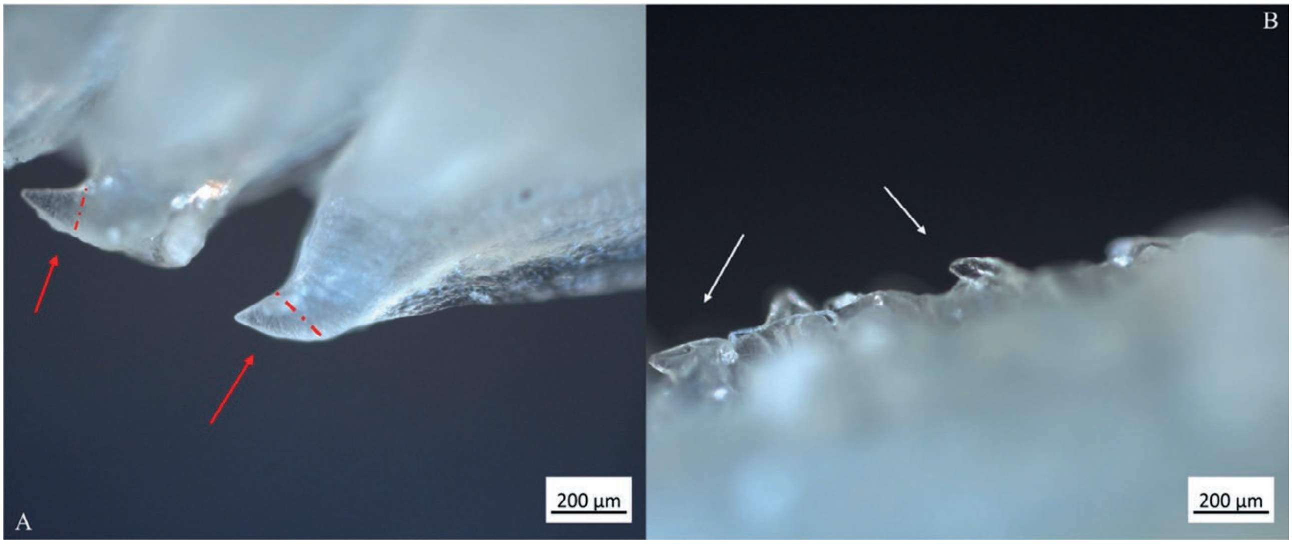

Fig. 2. High-resolution images of hemipenial spines (A, red arrows) and calyculations (B, white arrows). Picture 2A shows how spines, located across the body part of the organ, are embedded in soft tissue folds up to their terminal region (dotted red line).

(Fig. 2A), while the apical part is rounded and highly calyculate on both sulcate and asulcate surfaces (Fig. 2B). Calyculations form a reticulate pattern of more or less regular geometric shapes (hexagons, pentagons). The ridges of the calyces show small flounces of soft tissue (details in Fig. 2B). The apex is flat and ornamented with calyculations and hosts the termination of the *sulcus spermaticus*, which is oblique and undivided from the basal region to the apex (Fig. 1A). No evident morphological differences were detectable between the two clades (Fig. 1).

Similarly, the hemipenis of *H. gemonensis* is unilobed and non-capitate. However, upon inspection, its general shape is more elongate and less bulbous (Fig. 1C). The basal region lacks ornamentations and does not show any swelling. The body part is covered in large spines and the apical part is markedly calyculate on both surfaces (sulcate and asulcate); such calyculations form a reticulate pattern very much like that of the sister species. The ridges of the calyces consist of flounces of soft tissue. Also, the apex is flat and ornamented with calyculations and hosts the termination of the *sulcus spermaticus*, which in this case is less oblique and straighter than in *H. viridiflavus*, undivided from the basal region to the apex.

Quantitative analyses

Hemipenes length was on average 27.9 ± 3.3 mm when considering the whole sample of 20 whip snakes and, respectively, 27.5 ± 4.3 mm and 28.3 ± 3.0 mm for W and E clade specimens separately. The LME model run to investigate the effects of SVL, hemipenial length, spine count, and clade on the length of spines neither showed any significant effect of SVL, hemipenial length, and spine count nor any difference in spine length between the two clades. Similarly, all two-ways interactions between predictors were not significantly correlated with the response variable (Table 2). On the other hand, a marked variability of spine length at the individual level was found (LR-χ^2 = 9.760, df = 1, P = 0.0018; Fig. 3), which was not explained either by clade or morphometry, so that 18.7% of the total variance is explained by the random effect.

Table 2. Analysis of variance with Satterthwaite's method of the fixed effects and interactions implemented in the Linear Mixed-Effects model on the length of hemipenial spines in *Hierophis viridiflavus*. No significant effect was detected for any predictor nor interaction.

Fixed effect	F	df	P
SVL	0.112	1,12	0.42
clade	0.279	1,12	0.71
hemipenial length	0.075	1,12	0.30
spine count on hemipenis	0.633	1,12	0.55
SVL × clade	0.349	1,12	0.55
hemipenial length × clade	2.793	1,12	0.09
clade × spine count	0.447	1,12	0.50

DISCUSSION

Divergence in copulatory organs has been regarded as a barrier to interbreeding between closely related lin-

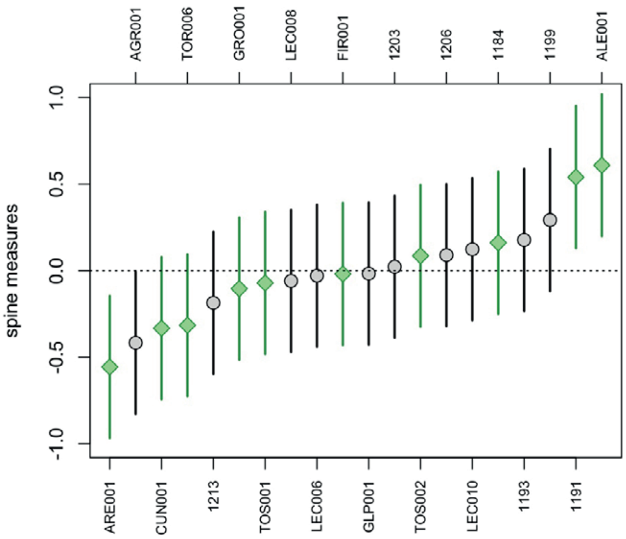

Fig. 3. Random-intercept effects on the length of hemipenial spines for each specimen, colour and symbol coded by clade (Eastern clade in grey circles; Western clade in green rhombi). Symbols correspond to each specimen's estimate ± SE retrieved from the LME model.

eages. So, we have investigated hemipenial morphology variations between the two major clades of the green whip snake *Hierophis viridiflavus*. This species is a Mediterranean colubrid that can be highly variable in phenotype (Vanni and Zuffi, 2011; Meier et al., 2023; Storniolo et al., 2023). It has been object of debate in the last decade as to whether its two major lineages should be considered as separate species according to gross morphology, karyotype, and mtDNA markers divergence (Nagy et al., 2004; Mezzasalma et al., 2015), while no investigation in reproductive barriers has ever been performed.

In our study we show that the hemipenis of *H. viridiflavus* is markedly ornamented in both subspecies, with noticeable morphological differentiation between the apical part (completely covered in tissue folds - calyculations) and the body region (covered in rigid spines). Furthermore, upon investigation, we have found that the general hemipenial morphology of our target species is markedly similar to that of *H. gemonensis*, especially in terms of ornamentations, with the body part covered in spines and the apical part in calyculations of soft tissue folds. Minor differences, however, were found, so that the organ itself is not bulbous and more elongate in the Balkan whip snake with respect to *H. viridiflavus* and the *sulcus spermaticus* is not oblique but straighter from the base to the apex.

Concerning the quantitative investigations for *H. viridiflavus*, no difference in snake size (SVL), hemipenial length, ornamentations, and length of spines was detected between the two major clades, suggesting the lack of copulatory barriers between the two lineages. On the other hand, we found a significant random effect at the individual level, indicating that spines are more variable within individual rather than between individuals or subspecies.

The evolution of male genitalia was firstly supposed to be driven by complementarity to those of females (Dufour, 1844), defined as "lock-and-key" mechanism, as a way of natural selection to minimise hybridisation (Shapiro and Porter, 1989; Brennan and Prum, 2015). With this respect, however, some key assumptions must be met, such as that, in sympatry, character displacement in sister species should occurr as the outcome of reproductive isolation due to natural selection (Eberhard, 2010; Simmons, 2014; Brennan and Prum, 2015; Ng et al., 2017), which is not always true. Alternatively, pleiotropy has been suggested to be driving genital coevolution, so that modifications in genitalia are supposed to be mainly neutral and are inherited alongside positively selected mutations on genes that code for the general morphology of the organism (Mayr, 1963; Edwards, 1993; Arnqvist and Thornhill, 1998; Hosken and Stockley, 2004). This hypothesis has not been supported broadly due to limited evidence except only few cases of insects (Arnqvist and Thornhill, 1998; Arnqvist and Danielsson, 1999), where pleiotropic effects on both general and genital morphology were found. Concerning snakes as well, the evolutionary mechanisms driving hemipenial diversification appear still to be unclear. On one hand, phylogenetic approaches can be potent to describe hemipenial morphological patterns at higher phylogenetic levels (Keogh, 1999; Zaher, 1999; Schargel and Castoe, 2003). At lower levels instead, as in Andonov et al. (2017), sexual selection and behavioural strategies in mating appear to be more suitable to explain different adaptations in hemipenial morphology. For species in which males do not actively compete with each other to mate with partners (e.g., natricines), rendering male size less crucial, hemipenial adaptations in ornamentations can significantly affect duration and efficiency of copulation (Perry-Richardson et al., 1990; Madsen and Shine, 1993; Luiselli, 1996; King et al., 2009) as a response to the ability of larger females to disengage the hemipenis from the vagina (as in some boids and colubrids; Joy and Crews, 1985; Perry-Richardson et al., 1990; Rivas et al., 2007). Differently, concerning species that undergo male-male competitive strategies such as combats, the role of hemipenial morphology is harder to define because morphological adaptations, especially in colubrids, are extremely variable even when mating strategies match. For instance, the hemipenial morphology of *Malpolon insignitus* is relatively simple to address (Andonov et al., 2017), as it lacks any form of lobation and ornamentations, which is consistent with the assumption that combating species do not require marked morphological adaptations to enhance copulation efficiency. Contrastingly, other male-male combating colubrids such as *Chironius* and *Zamenis* (Edgar and Bird, 2006; Klaczko et al., 2014), show highly ornamented hemipenes, more similar to those of natricines (Rossman and Eberle, 1977; Ota and Iwanaga, 1997; King et al., 2009), supposedly to boost duration of copulatory events. The green whip snake is commonly known for engaging prolonged sessions of male-male combats during the mating season (Capula et al., 1995, 1997); hence, according to the sexual selection hypothesis that highlights the role of ornamentations when males do not actively compete one another for the mates, hemipenial morphology should not be markedly developed (Perry-Richardson et al., 1990; King et al., 2009; Andonov et al., 2017). However, the differentiation and complexity we recorded is in contrast with this statement and suggests that hemipenial adaptations cannot be thoroughly explained under the perspective of sexual selection (Andonov et al., 2017; Klaczko et al., 2014). Alternatively, phylogenetic relationships, despite not being suitable to address hemipenial morphology at lower levels (species or subspecies), seem to be a good predictor of variability when considering closely related groups at the family level, such as *Hierophis* in the present study and its sister group *Dolichophis* from other studies (Andonov et al., 2017). Nevertheless, how and whether marginal populations interbreed along the contact zone between the two subspecies (Mezzasalma et al., 2015) is still untangled, making behavioural, reproductive and genetic studies an open and intriguing field of investigation to address the putative evolutionary history of *Hierophis viridiflavus*. With this respect, it needs to be pointed out that, in order to have a broad and accurate investigation of this matter, also female genitalia should be taken into consideration.

In conclusion, our study shows that variability in hemipenial morphology is not potent enough to discriminate populations at the subspecies level, as both major clades of *H. viridiflavus* show almost identical hemipenial features, in accordance to partial evidence collected by Schätti and Vanni (1986). Even by comparing hemipenial morphology between species, the extent of the diversification is limited: the hemipenes of *H. viridiflavus* and *H. gemonensis* are very similar to one another, which leads to potentially intriguing perspectives. If, on one hand, the similarity in copulatory organs at the subspecies level is consistent with morphological and molecular evidence (Meier et al., 2023; Storniolo et al., 2023), and suggests marked gene flow events between the two lineages, on the other hand the extent, if any, of gene flown between the two species is unknown but cannot be excluded, at least according to anatomy. With this respect, hemipenial morphology does not seem to be potentially impeding copulation. If these taxa are indeed reproductively isolated, alternative mechanisms other than genital compatibility must be at play to keep the two entities distinct, such as behavioural strategies (e.g., chemical communication as in Fornasiero et al., 2007) or selection against hybrids (Servedio et al., 2004). In accordance with past research, hemipenial features of both species are not consistent with their mating strategy, suggesting that hemipenial morphology and its evolution are more challenging then expected to investigate under the perspective of common hypotheses. Therefore, we believe that future research on hemipenial anatomy should address this matter under different approaches, such as comparative phylogeny and behavioural ecology with the aim to investigate the evolutionary and biological constraints of hemipenial evolution and development along with a thorough examination of female genitalia as well.

ACKNOWLEDGEMENTS

The authors would like to thank the Natural History Museum of the University of Pisa, the Natural History Museum of Milan, and the Natural History Museum of the University of Pavia, and their respective curators, especially Edoardo Razzetti of the NHM of the University of Pavia, for granting access to alcohol-preserved specimens and for allowing hemipenes extraction for the analyses.

We would also thank three anonymous reviewers for their interest in our research and the very useful comments that significantly improved the form of this work.

REFERENCES

Andonov, K., Natchev, N., Kornilev, Y.V., Tzankov, N. (2017): Does sexual selection influence ornamentation of hemipenes in Old World snakes? Anat. Rec. **300**: 1680-1694.

Arnqvist, G., Danielsson, I. (1999): Copulatory behavior, genital morphology, and male fertilization success in water striders. Evolution **53**: 147-156.

Arnqvist, G., Thornhill, R. (1998): Evolution of animal genitalia: patterns of phenotypic and genotypic variation and condition dependence of genital and non-genital morphology in water strider (Heteroptera: Gerridae: Insecta). Genet. Res. **71**: S0016672398003279.

Bates, D., Maechler, M., Bolker, B., Walker, S. (2015). Fitting Linear Mixed-Effects models using lme4. J. Stat. Softw. **67**: 1-48.

Bernardo, P.H., MacHado, F.A., Murphy, R.W., Zaher, H. (2012): Redescription and morphological variation of *Oxyrhopus clathratus* Duméril, Bibron and Duméril, 1854 (Serpentes: Dipsadidae: Xenodontinae). South. Am. J. Herpetol. **7**: 134-148.

Branch, W.R. (1986): Hemipenial morphology of African snakes: A taxonomic review. Part 1. Scolecophidia and Boidae. J. Herpetol. **20**: 285-299.

Breheny, P., Burchett, W. (2017): Visualization of regression models using visreg. The R Journal **9**: 56-71.

Brennan, P.L., Prum, R.O. (2015): Mechanisms and evidence of genital coevolution: the roles of natural selection, mate choice, and sexual conflict. Cold Spring Harb. Perspect. Biol. **7**: a017749.

Cadle, J.E. (2011): Hemipenial morphology in the North American snake genus *Phyllorhynchus* (Serpentes: Colubridae), with a review of and comparisons with natricid hemipenes. Zootaxa **3092**: 1-25.

Capula, M., Filippi, E., Luiselli, L. (1995): Annual mating in female colubrid snakes with irregular reproductive frequency. Herpetozoa **8**: 11-15.

Capula, M., Filippi, E., Luiselli, L., Jesus, V.T. (1997): The ecology of the Western whip snake, *Coluber viridiflavus* (lacépède, 1789), in Mediterranean Central Italy. Herpetozoa **10**: 65-79.

Cope, E.D. (1895): The classification of the Ophidia. Trans. Am. Phil. Soc. **18**: 186-219.

Dowling, H.G., Savage, J.M. (1960): A guide to the snake hemipenis: a survey of basic structure and systematic characteristics. Zoologica **45**: 17-28.

Dufour, L. (1844): Anatomie générale des Dipteres. Ann. Sci. Nat. **1**: 244-264.

Duursma, R. (2022): bootpredictlme4: predict method for lme4 with bootstrap. R package version 0.1.

Eberhard, W.G. (2010): Evolution of genitalia: theories, evidence, and new directions. Genetica **138**: 5-18.

Edgar, P., Bird, D.R. (2006): Action plan for the conservation of the Aesculapian snake (*Zamenis longissimus*) in Europe. In: Convention on the Conservation of European Wildlife and Natural Habitats. Council of Europe, Strasbourg.

Edwards, R. (1993): Entomological and mammalogical perspectives on genital differentiation. Trends. Ecol. Evol. **8**: 406-409.

Folwell, M.J., Sanders, K.L., Brennan, P.L., Crowe-Riddell, J. (2022): First evidence of hemiclitores in snakes. Proc. Royal Soc. B. **289**: 20221702.

Fornasiero, S., Dendi, F., Bresciani, E., Cecchinelli, E., Zuffi, M.A.L. (2007): The scent of the others: chemical recognition in two distinct populations of the European whip snake, *Hierophis viridiflavus*. Amphibia-Reptilia **32**: 39-47.

Friesen, C.R., Uhrig, E.J., Squire, M.K., Mason, R.T., Brennan, P.L.R. (2014): Sexual conflict over mating in red-sided garter snakes (*Thamnophis sirtalis*) as indicated by experimental manipulation of genitalia. Proc. Royal Soc. B **281**: 20132694.

Greenwood, J.F., Lara Granados, G., Secor, S.M., Todd, B.D., Showalter, I., Hedrick, B.P., Brennan, P.L.R. (2022): Divergent genital morphologies and female–male covariation in watersnakes. Integr. Comp. Biol. **62**: 569-580.

Hosken, D.J., Stockley, P. (2004): Sexual selection and genital evolution. Trends Ecol. Evol. **19**: 87-93.

House, C.M., Lewis, Z., Sharma, M.D., Hodgson, D.J., Hunt, J., Wedell, N., Hosken, D.J. (2021): Sexual selection on the genital lobes of male *Drosophila simulans*. Evolution **75**: 501-514.

Inger, R.F., Marx, H. (1962): Variation of hemipenis and cloaca in the colubrid snake *Calamaria lumbricoidea*. Syst. Zool. **11**: 32-38.

Joy, J.E., Crews, D. (1985): Social dynamics of group courtship behavior in male red-sided garter snakes (*Thamnophis sirtalis parietalis*). J. Compar. Psychol. **99**: 145-149.

Keogh, J.S. (1999): Evolutionary implications of hemipenial morphology in the terrestrial Australian elapid snakes. Zool. J. Linn. Soc. **125**: 239-278.

King, R.B. (1989): Sexual dimorphism in snake tail length: sexual selection, natural selection, or morphological constraint? Biol. J. Linn. Soc. **38**: 133-154.

King, R.B., Jadin, R.C., Grue, M., Walley, H.D. (2009): Behavioural correlates with hemipenis morphology in New World natricine snakes. Biol. J. Linn. Soc. **98**: 110-120.

Klaczko, J., Gilman, C.A., Irschick, D.J. (2017): Hemipenis shape and hindlimb size are highly correlated in *Anolis* lizards. Biol. J. Linn. Soc. **122**: 627-634.

Klaczko, J., Ingram, T., Losos, J. (2015): Genitals evolve faster than other traits in *Anolis* lizards. J. Zool. **295**: 44-48.

Klaczko, J., Montingelli, G.G., Zaher, H. (2014): A combined morphological and molecular phylogeny of the genus *Chironius* Fitzinger, 1826 (Serpentes: Colubridae). Zool. J. Linn. Soc. **171**: 656-667.

Lacépède, B.G. (1789): Histoire naturelle des quadrupèdes ovipares et des serpens. Hôtel de Thou, Paris.

Luiselli, L. (1996): Individual success in mating balls of the grass snake, *Natrix natrix*: size is important. J. Zool. **239**: 731-740.

Madsen, T., Shine, R. (1993): Male mating success and body size in european grass snakes. Copeia **1993**: 561-564.

Mayr, E. (1963): Animal species and evolution. Harvard University Press, Cambridge.

Meier, N., Lucek, K., Zuffi, M.A.L., Storniolo, F., Mezzasalma, M., Geniez, P., Dubey, S., Sacchi, R., Scali, S., Ursenbacher, S. (2023): Extensive gene flow suggests lack of reproductive barriers between the two subspecies of the green whip snake, *Hierophis viridiflavus* (Squamata: Colubridae). Biol. J. Linn. Soc. **141:** 133-147.

Mezzasalma, M., Dall'Asta, A., Loy, A., Cheylan, M., Lymberakis, P., Zuffi, M.A.L., Tomović, L., Odierna, G., Guarino, F.M. (2015): A sisters' story: Comparative phylogeography and taxonomy of *Hierophis viridiflavus* and *H. gemonensis* (Serpentes, Colubridae). Zool. Scr. **44**: 495-508.

Myers, C.W., Cadle, J.E. (2003): On the snake hemipenis, with notes on *Psomophis* and techniques of eversion: a response to Dowling. Herpetol. Rev. **34**: 295.

Myers, C.W., McDowell, S.B. (2014): New taxa and cryptic species of neotropical snakes (Xenodontinae), with commentary on hemipenes as generic and specific characters. Bull. Am. Mus. Nat. Hist. **385**: 1-112.

Nagy, Z. T., Lawson, R., Joger, U., Wink, M. (2004): Molecular systematics of racers, whipsnakes and relatives (Reptilia: Colubridae) using mitochondrial and nuclear markers. J. Zool. Syst. Evol. Res. **42**: 223-233.

Ng J., Geneva A.J., Noll, S., Glor, R.E. (2017): Signals and speciation: *Anolis* dewlap color as a reproductive barrier. J. Herpetol. **51**: 437-447.

Ota, H., Iwanaga, S. (1997): A systematic review of the snakes allied to *Amphiesma pryeri* (Boulenger) (Squamata: Colubridae) in the Ryukyu Archipelago. Japan. Zool J. Linn. Soc. **121**: 339-360.

Perry-Richardson, J.J., Schofield, C.W., Ford, N.B. (1990): Courtship of the garter snake, *Thamnophis marcianus*, with a description of a female behavior for coitus interruption. J. Herpetol. **24**: 76-78.

Pesantes, O.S. (1994): A method for preparing the hemipenis of preserved snakes. J. Herpetol. **28**: 93-95.

R Core Team (2022): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rivas, J.A., Muñoz, M. de C., Burghardt, G.M., Thorbjarnarson, J.B. (2007): Sexual size dimorphism and the mating system of the green anaconda (*Eunectes murinus*). In: Biology of the boas and pythons, pp. 312-325. Henderson, R.W., Powell, R., Eds, Eagle Mountain, Utah: Eagle Mountain Publishing, LC.

Rossman, D.A., Eberle, W.G. (1977): Partition of the genus *Natrix*, with preliminary observations on evolutionary trends in natricine snakes. Herpetologica **1977**: 34-43.

Schargel, W.E., Castoe, T.A. (2003): The hemipenes of some snakes of the semifossorial genus *Atractus*, with comments on variation in the genus. J. Herpetol. **37**: 718-721.

Schätti, B. (1987): The phylogenetic significance of morphological characters in the Holoartic racers of the genus *Coluber* Linnaeus, 1758 (Reptilia, Serpentes). Amphibia-Reptilia **8**: 401-418.

Schätti, B. (1988): Systematik und Evolution der Schlangengattung *Hierophis* Fitzinger, 1843 (Reptilia, Serpentes). Inaugural-Dissertation zur Erlangung der philosophischen Doctorwürde, Zürich.

Schatti, B., Monsch, P. (2004): Systematics and phylogenetic relationships of whip snakes (*Hierophis* Fitzinger) and *Zamenis andreana* Werner, 1917 (Reptilia: Squamata: Colubrinae). Rev. Suisse Zool. **111**: 239-256.

Schätti, B., Vanni, S. (1986). Intraspecific variation in *Coluber viridiflavus* Lacépède, 1789, and validity of its subspecies (Reptilia, Serpentes, Colubridae). Rev. Suisse Zool. **93**: 219-232.

Senczuk, G., Gramolini, L., Avella, I., Mori, E., Menchetti, M., Aloise, G., Castiglia, R. (2021): No association between candidate genes for color determination and color phenotype in *Hierophis viridiflavus,* and characterization of a contact zone. J. Zool. Syst. Evol. Res. **59**: 748-759.

Servedio, M.R. (2004): The evolution of premating isolation: local adaptation and natural and sexual selection against hybrids. Evolution **58**: 913-924.

Shapiro, A.M., Porter, A.H. (1989): The lock-and-key hypothesis: evolutionary and biosystematic interpretation of insect genitalia. Ann. Rev. Entomol. **34**: 231-245.

Sillero, N., Campos, J., Bonardi, A., Corti, C., Creemers, R., Crochet, P.A., Crnobrnja Isailović, J., Denoël, M., Ficetola, G.F., Gonçalves, J., Kuzmin, S., Lymberakis, P., Pous, P. de, Rodríguez, A., Sindaco, R., Speybroeck, J., Toxopeus, B., Vieites, D.R., Vences, M. (2014): Updated distribution and biogeography of amphibians and reptiles of Europe. Amphibia-Reptilia **35**: 1-31.

Simmons, L.W. (2014): Sexual selection and genital evolution. Austral Entomol. **53**: 1-17.

Speybroeck, J., Beukema, W., Dufresnes, C., Fritz, U., Jablonski, D., Lymberakis, P., Martinez-Solano, I., Razzetti, E., Vamberger, M., Vences, M., Voros, J., Crochet, P.A. (2020): Species list of the European herpetofauna-2020 update by the Taxonomic Committee of the Societas Europaea Herpetologica. Amphibia-Reptilia **41**: 139-189.

Sota, T., Kubota, K. (1998): Genital lock-and-key as a selective agent against hybridization. Evolution **52**: 1507-1513.

Soto, I.M., Carreira, V.P., Soto, E.M., Márquez, F., Lipko, P., Hasson, E. (2013): Rapid divergent evolution of male genitalia among populations of *Drosophila buzzatii*. Evol. Biol. **40**: 395-407.

Storniolo, F., Mangiacotti, M., Zuffi, M.A.L., Scali, S., Sacchi, R. (2023). Large scale phenotypic characterisation of *Hierophis viridiflavus* (Squamata: Serpentes): climatic and environmental drivers suggest the role of evolutionary processes in a polymorphic species. Evol. Ecol. **37**: 419-434.

Tokarz, R.R. (1988): Copulatory behaviour of the lizard *Anolis sagrei*: alternation of hemipenis use. Anim. Behav. **36**: 1518-1524.

Utiger, UAK, Schätti, B. (2004): Morphology and phylogenetic relationships of the Cyprus racer, *Hierophis cypriensis*, and the systematic status of *Coluber gemonensis gyarosensis* Mertens (Reptilia: Squamata: Colubrinae). Rev. Suisse Zool. **111**: 225-238.

Vanni, S., Zuffi M.A.L. (2011): *Hierophis viridiflavus* (Lacépède, 1789). In: Corti C., Capula M., Luiselli, L., Razzetti E., Sindaco R. (Eds), Fauna d'Italia, Reptilia. pp. 509-516. Calderini, Bologna.

Zaher, H. (1999): Hemipenial morphology of the South American xenodontine snakes, with a proposal for a monophyletic Xenodontinae and a reappraisal of colubroid hemipenes. Bull. Am. Mus. Nat. Hist. **240**: 1-168.

Zaher, H., Prudente, A.L.C. (2003): Hemipenes of *Siphlophis* (Serpentes, Xenodontinae) and techniques of hemipenial preparation in snakes: a response to Dowling. Herpetol. Rev. **34**: 302-306.

Zuffi, M.A.L. (2002): A critique of the systematic position of the asp viper subspecies *Vipera aspis aspis* (Linnaeus, 1758), *Vipera aspis atra* Meisner, 1820, *Vipera aspis francisciredi* Laurenti, 1768, *Vipera aspis hugyi* Schinz, 1833 and *Vipera aspis zinnikeri* Kramer, 1958. Amphibia-Reptilia **23**: 191-213.