

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

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

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15
16 **Abstract.** Copulatory organs are a key trait in reproductive compatibility and sexual isolation. The
17 role of male genitalia in boosting mating success is well known and is often the outcome of
18 behavioural and biological constraints, although no clear and common interpretation about their
19 evolution appears broadly applicable. In snakes, hemipenial morphology has often been described
20 under the perspective of sexual selection, taking into consideration both behavioural and
21 morphological traits of both sexes. We investigated hemipenial morphology and ornamentation in the
22 two subspecies of *Hierophis viridiflavus*, a male-male combating colubrid, and compared it to the
23 sister species *H. gemonensis*, to assess intraspecific variation in size of genitalia and ornamentation
24 richness. The male intromitted organ of this species is unilobed and bulbous, with rich ornamentations
25 consisting of basal spines and apical calyculations. We detected no statistically significant difference
26 in hemipenial size, basal spine count, and spine length between the two subspecies, suggesting that

27 no copulatory barrier is present between the two clades. Although hemipenial morphology and
28 anatomy do not seem suitable to assess intraspecific variability as shown in this study, they are often
29 highly variable at the family or genus level, suggesting that the evolution of male genitalia is linked
30 to phylogenetic relationships and that hemipenial divergence might be correlated to lineage splitting
31 despite not being necessarily the main cause. Future studies should be aimed at investigating
32 hemipenial morphology and anatomy across species under the perspective of comparative phylogeny
33 and reproductive behaviours to address thoroughly the constraints of hemipenial evolution and
34 development.

35

36 **Running title:** Hemipenial morphology of *Hierophis viridiflavus*.

37

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

39

40

INTRODUCTION

41 The anatomy and morphology of copulatory organs have been of great interest for
42 herpetologists in the last century especially concerning snakes (Cadle, 2011; Folwell et al., 2022). As
43 a matter of fact, hemipenes are postulated to play a major role in mating success, being supposedly
44 species-specific (Cope, 1895; Keogh, 1999), and thus with marked implications in terms of
45 reproductive biology and behaviour (Tokarz, 1988; King et al., 2009; Klaczko et al., 2017). Reptilian
46 hemipenes show highly variable morphological traits, in terms of the gross shape of the organ itself
47 (unilobed, bilobed) as well as of its external ornamentations, which can consist of rigid spines (spread
48 across the organ or aggregated in a specific region, i.e. basal region or the apex; Fig. 1) or soft tissue
49 folds, or otherwise can be completely absent (Zaher et al., 1999; Andonov et al., 2017). The extent of
50 hemipenial morphological variability can remarkably vary among different families (Cadle, 2011;
51 Andonov et al., 2017), but also at lower taxonomic levels (Inger and Marx, 1962; Branch, 1986;
52 Zaher, 1999; Zuffi, 2002; Bernardo et al., 2012; Klaczko et al., 2014; Myers and McDowell, 2014).

53 From this perspective, investigating the mechanisms that drive the evolution of specific features in
54 copulatory organs can be of great interest to address phylogenetic relationships and species splitting
55 over time as copulatory organs are some of the most rapidly evolving traits in squamates (Brennan
56 and Prum, 2015; Klaczko et al., 2015, 2017; Folwell et al., 2022). Hypotheses have been proposed
57 for the development of male genitalia, first of which the “lock-and-key”, formerly postulated by
58 Dufour (1844), states that male genitalia evolve to be complementary to those of females with
59 noticeable species-specificity. Alternatively, the pleiotropy hypothesis for male genitalia
60 differentiation has been partly supported, hypothesizing that they evolve through selective pleiotropic
61 effects on other traits (Mayr, 1963; Edwards, 1993; Arnqvist and Thornhill, 1998; Hosken and
62 Stockley, 2004). This hypothesis appears still unsuitable to be applied broadly as a common rule,
63 because it assumes that the set of genes coding for general morphology codes also for genital
64 morphological variation, which should not be selected against, implying tight genetic correlation
65 between genital and general morphology (Arnqvist and Thornhill, 1998). These hypotheses have been
66 revised broadly (Shapiro and Porter, 1989; Sota and Kubota Soto et al., 2013; Brennan and Prum,
67 2015) in an evolutionary perspective highlighting the role of genital morphology as a barrier against
68 hybridisation, favouring coevolution between male and female genitalia (House et al., 2020;
69 Greenwood et al., 2022).

70 Ophidian hemipenes have been studied vastly in the last decades (Zaher, 1999; Myers and
71 McDowell, 2014) under the functional perspective as they are related to copulation duration as well:
72 indeed, in New World natricines more complex and ornate hemipenes (as in *Thamnophis radix*) are
73 associated to more prolonged copulations compared to the congeneric *T. sirtalis*, characterised by
74 simple subcylindrical hemipenes with little ornamentation (King et al., 2009). From this perspective,
75 the occurrence of abundant ornamentations on hemipenial surfaces of both apical and body part of
76 the organ appears to be relevant in terms of how efficiently males remain attached to females (Friesen
77 et al., 2014), significantly affecting the duration of copulation and thus male fitness. Additionally,
78 Rivas et al. (2007) showed that male coiling during copulation in species that undergo mating balls

79 (*Eunectes murinus* in this case) can impede other males from mating with the female and, in these
80 cases, more conspicuously ornamented hemipenes (i.e. more abundant or large ornamentations)
81 should favour copulation and operate synergically with behaviour. On the other hand, snakes that
82 exhibit male-male combat behaviours are subject to sexual selection prior to copulation. Therefore,
83 hemipenis morphology should not be selected to evolve more complex structures such as calyces,
84 spines, and hooks. However, as shown by Andonov et al. (2017), hemipenial morphology does not
85 always correlate with behavioural strategies, so the scenario of the evolution of male genitalia is a
86 complex task to untangle.

87 The green whip snake *Hierophis viridiflavus* (Lacépède, 1789) is one of the most widespread
88 species in Mediterranean Europe as it occurs from Northern Spain across France and throughout Italy
89 to Northern Balkans (Sillero et al., 2014). From the phylogenetic point of view, this species has been
90 object of debate and Mezzasalma et al. (2015), according to both molecular and morphological
91 differences, have split the two subspecies *H. v. viridiflavus* and *H. v. carbonarius* (Western and
92 Eastern clade respectively) and elevated them to the rank of species. Recently, Speybroeck et al.
93 (2020) have proposed to pool them together as a unique species; however, the debate is still open. As
94 a matter of fact, the phylogenetic relationships between the two lineages are still unclear and recent
95 research on the genetic basis of its colour polymorphism (mtDNA and nDNA; Senczuk et al., 2021)
96 has suggested that these two lineages might undergo asymmetrical gene flow from the Western into
97 the Eastern clade, even though no decisive evidence has been gathered. Morphological variability in
98 dentition, pholidosis, and hemipenes has also been used to characterise the phylogeny of the
99 *Hierophis* genus by Schätti (1987, 1988), discriminating the members of this genus with respect to
100 sister groups (i.e. *Spalerosophis*, *Eirenis*, *Platyiceps* genera); however, interspecific variability
101 patterns within genus are still unexplored (Schätti and Monsch, 2004; Utiger and Schätti, 2004). With
102 this respect, nevertheless, Schätti and Vanni (1986) have investigated morphological traits of the
103 target species, among which hemipenes too, but no difference among populations was found by the

104 authors; however, hemipenial morphology was not the key subject for investigation and no in-depth
105 study of morphology and ornamentations was performed.

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

116

117 MATERIALS AND METHODS

118 *Gross morphology*

119 For morphological description we referred to the categories in Dowling and Savage (1960) and
120 Andonov et al. (2017). We collected the left hemipenis from both fresh and museum collection
121 specimens, preserved in ~75% alcohol. For sample preparation from alcohol-preserved specimens,
122 we slightly modified the method in Andonov et al. (2017) and Zuffi (2002): for hemipenis filling we
123 preferred liquid paraffin over petroleum jelly (used by Pesantes, 1994; Myers and Cadle, 2003; Zaher
124 and Prudente, 2003) because it is easier to use as it does not need to be kept in liquid state and, being
125 less viscous, it is quicker and simpler to inject through syringe. We propose to use this technique in
126 case the injection of petroleum jelly appears to be difficult. Firstly, the organs were soaked in 2%
127 KOH after extraction for 30 minutes to 6 hours according to its size and duration of preservation.
128 After this period, hemipenes were everted manually using tweezers, and subsequently filled with
129 liquid paraffin. Hence, we sealed hemipenes at the base using a thin string. Fresh samples were

130 processed using the same procedure as for alcohol-preserved specimens, except they were soaked in
131 water rather than KOH solution for tissue softening before eversion. Lastly, all samples were stored
132 in ~75% alcohol for permanent preservation. Each hemipenis was photographed on both sulcate and
133 asulcate surfaces using a high-resolution reflex camera (NIKON D7100) by placing it on a black
134 surface under two light sources on opposite sides to minimize shadows. We used the “magic wand”
135 tool built in Photoshop CS3 (version 10.0) to eliminate any remaining shadow from the pictures to
136 produce a clean photo of each object.

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

140

141 *Quantitative analyses*

142 To thoroughly describe any variation in hemipenial morphology and anatomy between the two main
143 lineages of *H. viridiflavus* (Western and Eastern clades, hereinafter W and E respectively), we also
144 recorded quantitative data concerning hemipenial size, number of basal spines, spine length, and
145 snout-to-vent length (SVL) of each specimen. The count of the total number of spines was repeated
146 three times to minimize counting error. From each hemipenis, we randomly extracted five spines
147 from different parts of the basal region. Prior to performing any analysis, all predictors (hemipenial
148 size, spine number, SVL, and clade) were tested for intercorrelation via Pearson's correlation test. No
149 correlation was detected between spine count and hemipenial length ($r = 0.13$, $P = 0.57$), as well as
150 between SVL and both hemipenial length ($r = 0.37$, $P = 0.11$) and spine count ($r = -0.21$, $P = 0.38$).

151 Therefore, we performed a Linear Mixed-Effects (LME) model to test whether spine length depended
152 on species clade when controlling for body size (SVL), hemipenial size, and number of spines. All
153 those variables were implemented in the model as fixed effects. We also considered the two-way
154 interactions between clade and both spine length and count to test for potential different effects in
155 each group. Additionally, specimen entered the model as a random-intercept effect to account for

156 inter-individual variability patters that were unexplained by morphometry or lineage assignment. The
157 model was performed with the *lme4* package (Bates et al., 2015); model visualisation was performed
158 with the package *visreg* (Breheny and Burchett, 2017) and 95% confidence intervals were estimated
159 with the package *bootpredictlme4* (Duursma, 2022). All analyses were performed on R 4.2.1 (R Core
160 Team, 2022).

161

162

RESULTS

163 *Gross morphology*

164 The hemipenis of *Hierophis viridiflavus* is unilobed, bulbous, non-capitate (Fig. 1 A-B). The basal
165 region lacks ornamentation and does not show tissue swelling of any kind. The body part is
166 characterised by numerous spines (Fig. 2a), while the apical part is rounded and highly calyculate on
167 both sulcate and asulcate surfaces (Fig. 2b). Calyculations form a reticulate pattern of more or less
168 regular geometric shapes (hexagons, pentagons). The ridges of the calyces show small flounces of
169 soft tissue (details in Fig. 2b). The apex is flat and ornamented with calyculations and hosts the
170 termination of the *sulcus spermaticus*, which is oblique and undivided from the basal region to the
171 apex (Fig. 1a). No evident morphological differences were detectable between the two clades (Fig.
172 1).

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

181

182 *Quantitative analyses*

183 Hemipenes length was on average 27.9 ± 3.3 mm when considering the whole sample of 20 whip
184 snakes and, respectively, 27.5 ± 4.3 mm and 28.3 ± 3.0 mm for W and E clade specimens separately.
185 The LME model run to investigate the effects of SVL, hemipenial length, spine count, and clade on
186 the length of spines neither showed any significant effect of SVL, hemipenial length, and spine count
187 nor any difference in spine length between the two clades. Similarly, all two-ways interactions
188 between predictors were not significantly correlated with the response variable (Table 2). On the other
189 hand, a marked variability of spine length at the individual level was found ($LR-\chi^2 = 9.760$, $df = 1$, P
190 $= 0.0018$; Fig. 3), which was not explained either by clade or morphometry, so that 18.7% of the total
191 variance is explained by the random effect.

192

193 DISCUSSION

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

202 In our study we show that the hemipenis of *H. viridiflavus* is markedly ornamented in both
203 subspecies, with noticeable morphological differentiation between the apical part (completely
204 covered in tissue folds - calyculations) and the body region (covered in rigid spines). Furthermore,
205 upon investigation, we have found that the general hemipenial morphology of our target species is
206 markedly similar to that of *H. gemonensis*, especially in terms of ornamentations, with the body part
207 covered in spines and the apical part in calyculations of soft tissue folds. Minor differences, however,

208 were found, so that the organ itself is not bulbous and more elongate in the Balkan whip snake with
209 respect to *H. viridiflavus* and the *sulcus spermaticus* is not oblique but straighter from the base to the
210 apex.

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

216 The evolution of male genitalia was firstly supposed to be driven by complementarity to those
217 of females (Dufour, 1844), defined as “lock-and-key” mechanism, as a way of natural selection to
218 minimise hybridisation (Shapiro and Porter, 1989; Brennan and Prum, 2015). With this respect,
219 however, some key assumptions must be met, such as that, in sympatry, character displacement in
220 sister species should occur as the outcome of reproductive isolation due to natural selection
221 (Eberhard, 2010; Simmons, 2014; Brennan and Prum, 2015; Ng et al., 2017), which is not always
222 true. Alternatively, pleiotropy has been suggested to be driving genital coevolution, so that
223 modifications in genitalia are supposed to be mainly neutral and are inherited alongside positively
224 selected mutations on genes that code for the general morphology of the organism (Mayr, 1963;
225 Edwards, 1993; Arnqvist and Thornhill, 1998; Hosken and Stockley, 2004). This hypothesis has not
226 been supported broadly due to limited evidence except only few cases of insects (Arnqvist and
227 Thornhill, 1998; Arnqvist and Danielsson, 1999), where pleiotropic effects on both general and
228 genital morphology were found. Concerning snakes as well, the evolutionary mechanisms driving
229 hemipenial diversification appear still to be unclear. On one hand, phylogenetic approaches can be
230 potent to describe hemipenial morphological patterns at higher phylogenetic levels (Keogh, 1999;
231 Zaher, 1999; Schargel and Castoe, 2003). At lower levels instead, as in Andonov et al. (2017), sexual
232 selection and behavioural strategies in mating appear to be more suitable to explain different
233 adaptations in hemipenial morphology. For species in which males do not actively compete with each

234 other to mate with partners (e.g. natricines), rendering male size less crucial, hemipenial adaptations
235 in ornamentations can significantly affect duration and efficiency of copulation (Perry-Richardson
236 et al., 1990; Madsen and Shine, 1993; Luiselli, 1996; King et al., 2009) as a response to the ability
237 of larger females to disengage the hemipenis from the vagina (as in some boids and colubrids; Joy
238 and Crews, 1985; Perry-Richardson et al., 1990; Rivas et al., 2007). Differently, concerning species
239 that undergo male-male competitive strategies such as combats, the role of hemipenial morphology
240 is harder to define because morphological adaptations, especially in colubrids, are extremely variable
241 even when mating strategies match. For instance, the hemipenial morphology of *Malpolon insignitus*
242 is relatively simple to address (Andonov et al., 2017), as it lacks any form of lobation and
243 ornamentations, which is consistent with the assumption that combating species do not require
244 marked morphological adaptations to enhance copulation efficiency. Contrastingly, other male-male
245 combating colubrids such as *Chironius* and *Zamenis* (Edgar and Bird, 2006; Klaczko et al., 2014),
246 show highly ornamented hemipenes, more similar to that of natricines (Rossman and Eberle, 1977;
247 Ota and Iwanaga, 1997; King et al., 2009), supposedly to boost duration of copulatory events. The
248 green whip snake is commonly known for engaging prolonged sessions of male-male combats during
249 the mating season (Capula et al., 1995, 1997); hence, according to the sexual selection hypothesis
250 that highlights the role of ornamentations when males do not actively compete one another for the
251 mates, hemipenial morphology should not be markedly developed (Perry-Richardson et al., 1990;
252 King et al., 2009; Andonov et al., 2017). However, the differentiation and complexity we recorded is
253 in contrast with this statement and suggests that hemipenial adaptations cannot be thoroughly
254 explained under the perspective of sexual selection (Andonov et al., 2017; Klaczko et al., 2014).
255 Alternatively, phylogenetic relationships, despite not being suitable to address hemipenial
256 morphology at lower levels (species or subspecies), seem to be a good predictor of variability when
257 considering closely related groups at the family level, such as *Hierophis* in the present study and its
258 sister group *Dolichophis* from other studies (Andonov et al., 2017). Nevertheless, how and whether
259 marginal populations interbreed along the contact zone between the two subspecies (Mezzasalma et

260 al., 2015) is still untangled, making behavioural, reproductive and genetic studies an open and
261 intriguing field of investigation to address the putative evolutionary history of *Hierophis viridiflavus*.
262 With this respect, it needs to be pointed out that, in order to have a broad and accurate investigation
263 of this matter, also female genitalia should be taken into consideration.

264 In conclusion, our study shows that variability in hemipenial morphology is not potent enough
265 to discriminate populations at the subspecies level, as both major clades of *H. viridiflavus* show
266 almost identical hemipenial features, in accordance to partial evidence collected by Schätti and Vanni
267 (1986). Even by comparing hemipenial morphology between species, the extent of the diversification
268 is limited: the hemipenes of *H. viridiflavus* and *H. gemonensis* are very similar to one another, which
269 leads to potentially intriguing perspectives. If, on one hand, the similarity in copulatory organs at the
270 subspecies level is consistent with morphological and molecular evidence (Meier et al., 2023;
271 Storniolo et al., 2023), and suggests marked gene flow events between the two lineages, on the other
272 hand the extent, if any, of gene flow between the two species is unknown but cannot be excluded,
273 at least according to anatomy. With this respect, hemipenial morphology does not seem to be
274 potentially impeding copulation. If these taxa are indeed reproductively isolated, alternative
275 mechanisms other than genital compatibility must be at play to keep the two entities distinct, such as
276 behavioural strategies (e.g. chemical communication as in Fornasiero et al., 2007) or selection against
277 hybrids (Servedio et al., 2004). In accordance with past research, hemipenial features of both species
278 are not consistent with their mating strategy, suggesting that hemipenial morphology and its evolution
279 are more challenging than expected to investigate under the perspective of common hypotheses.
280 Therefore, we believe that future research on hemipenial anatomy should address this matter under
281 different approaches, such as comparative phylogeny and behavioural ecology with the aim to
282 investigate the evolutionary and biological constraints of hemipenial evolution and development
283 along with a thorough examination of female genitalia as well.

284

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465 **Table 1.** List of the specimens of *Hierophis viridiflavus* with the relative source of collection, cladem and morphometric measures. NHMPv Natural
 466 History Museum of Pavia; NHMPi: Natural History Museum of Pisa; MHMMi Natural History Museum of Milan.

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	MHMPv	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	MHMPi	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

468 **Table 2.** Table of the analysis of variance with Satterthwaite's method of the fixed effects and
469 interactions implemented in the Linear Mixed-Effects model on the length of hemipenial spines in
470 *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	0.094
Clade × spine count	0.447	1,12	0.50

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CAPTIONS TO FIGURES

473

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

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

483 **Figure 3.** Random-intercept effects on the length of hemipenial spines per each specimen, colour and
484 symbol coded by clade (Eastern clade in grey circles; Western clade in green rhombi). Symbols
485 correspond to each specimen's estimate \pm SE retrieved from the LME model.

486

487 Fig. 1

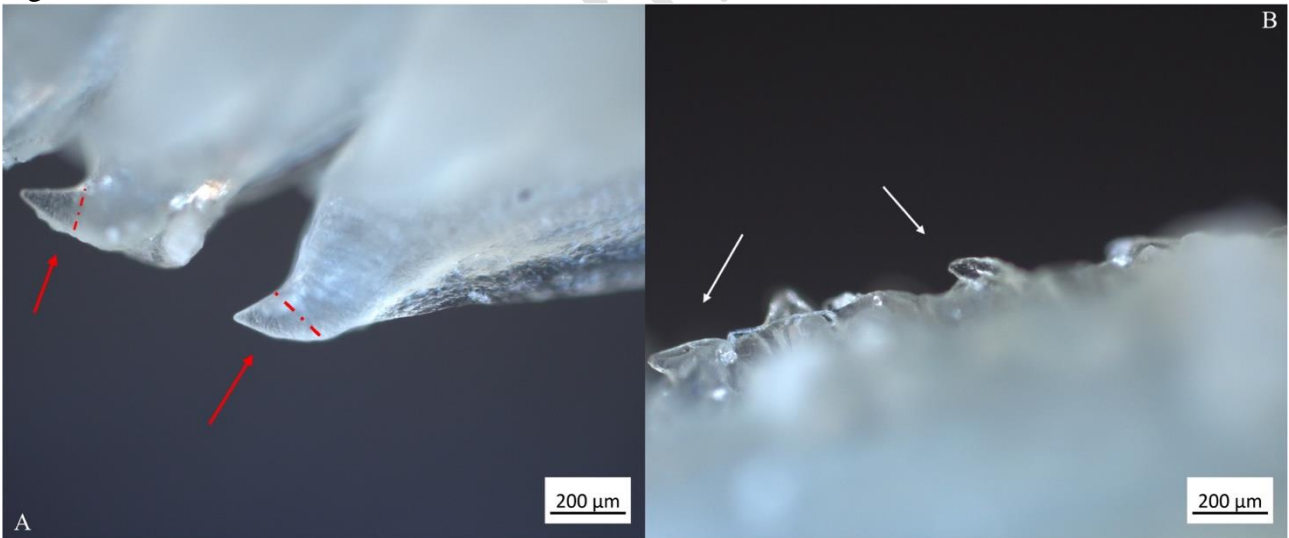


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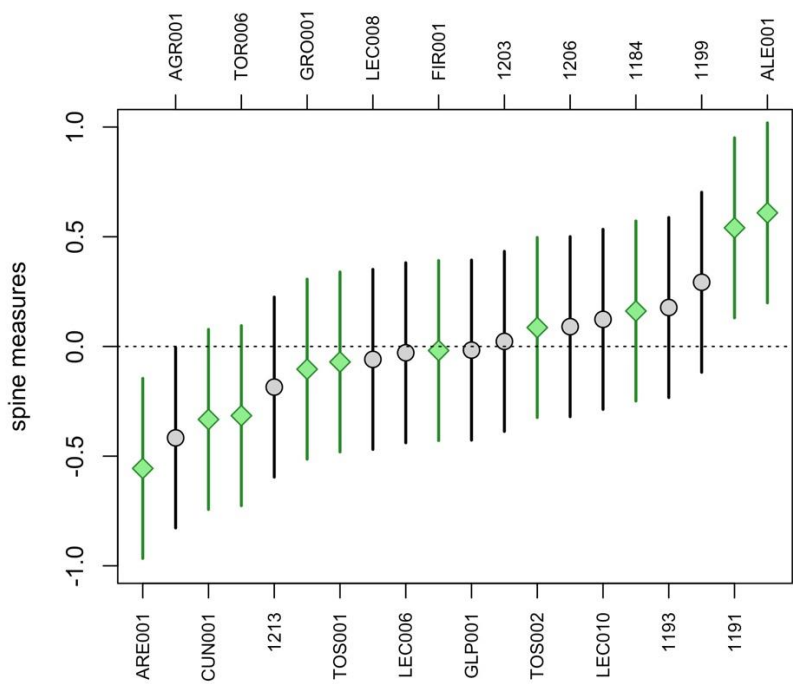
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Fig. 2



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