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4 **Evolution of sperm morphology in anurans: insights into the**
5 **roles of mating system and spawning location**

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23

24 **Abstract**

25 **Background:** The degree of postcopulatory sexual selection, comprising variable
26 degrees of sperm competition and cryptic female choice, is an important evolutionary
27 force to influence sperm form and function. Here we investigated the effects of
28 mating system and spawning location on the evolution of sperm morphology in 67
29 species of Chinese anurans. We also examined how relative testes mass as an
30 indicator of the level of sperm competition affected variation in sperm morphology
31 across a subset of 29 species.

32 **Results:** We found a significant association of mating system and spawning location
33 with sperm morphology. However, when removing the effects of body mass or
34 absolute testes mass for species for which such data were available, this effect became
35 non-significant. Consistent with predictions from sperm competition theory, we found
36 a positive correlation between sperm morphology and relative testes mass after taking
37 phylogeny into account.

38 **Conclusions:** Our findings suggest that sexual selection in Chinese anurans favors
39 longer sperm when the level of sperm competition is high. Pre-copulatory male-male
40 competition and spawning location, on the other hand, do not affect the evolution of
41 sperm morphology after taking body mass and absolute testes mass into account.

42

43 **Keywords**

44 Anurans, Mating system, spawning location, sperm morphology, sperm competition,

45 testes mass

46

47 **Background**

48 Spermatozoa exhibit a striking degree of variation in size and shape within and across
49 species [1-3]. In addition to phylogeny and the mode of fertilization [4-5],
50 postcopulatory sexual selection, comprising variation in the level of sperm
51 competition [6] and cryptic female choice [7], is thought to be one of the main
52 selective forces responsible for variation in sperm morphology [8-13]. However, the
53 detailed evolutionary causes and consequences of the remarkable diversity of
54 spermatozoa are still poorly understood.

55 Since the main biological role of sperm is to fertilize eggs, such variation is
56 intimately associated with sperm function. Theoretical approaches to understand
57 variation in sperm morphology are often based on assumptions concerning the
58 relationship between sperm size and parameters such as sperm swimming speed and
59 longevity [14-15]. These models predict that an increased risk of sperm competition
60 among species can favor longer sperm with higher swimming speed. Empirical
61 studies on a broad range of species including internal and external fertilizers have
62 however resulted in empirical evidence which both supports [13, 16-21] and rejects
63 [22-25] associations between sperm size and swimming speed. It has further been
64 suggested that the ratio between head length and tail length is a good predictor for the
65 swimming speed of sperm [7, 26-27]. Simpson et al. [27] reveal that sperm with a
66 relatively long flagellum swam faster in external fertilizers, in which females have no
67 opportunity to affect sperm motility. By contrast, sperm with a relatively short
68 flagellum swam faster in internally fertilizing species where females can significantly
69 affect sperm motility.

70 Sperm competition occurs when sperm from different males attempt to fertilize the
71 same set of ova [6]. Males in polygamous species should suffer a higher risk of sperm

72 competition than their monogamous counterparts. As a response, many studies
73 showed that polygamous males have larger relative testes size [14, 16, 28-32] and
74 longer sperm [9, 11, 16, 33-37]. However, the association between sperm competition
75 and sperm size could not be confirmed across all groups [38-41], and it has been
76 suggested that that evolution of sperm morphology might also be influenced by the
77 need to overcome hydrodynamic drag associated with the complexity of the sperm
78 head [42]. A growing body of evidence suggests that species which experience greater
79 levels of sperm competition have faster swimming sperm than species where sperm
80 competition is relaxed or absent [16, 20, 22, but see also 13].

81 There is every reason to expect that the spawning environment should further affect
82 sperm morphology [11, 43-44]. For example, Byrne et al. [11] revealed for anurans
83 that species with terrestrial spawning have a larger sperm head and a longer sperm tail
84 than aquatically spawning species. However, more subtle effects of spawning
85 environments on the evolution of sperm morphology across a broad range of anurans
86 still await further exploration.

87 Here, we investigated the effects of mating system and spawning location on the
88 evolution of sperm morphology in Chinese anurans. We also examined the amount of
89 covariation between relative testes mass (as an indicator of level of sperm competition)
90 and sperm morphology. The aim of this study was to investigate patterns and possible
91 causes of variation in sperm morphology in anurans, and to test the hypothesis that
92 polygamous species have longer sperm than their monogamous counterparts. To this
93 end, we analyzed a dataset on sperm morphology comprising 67 anurans from the
94 Hengduan Mountains, China. We further analysed a data set on 29 species which also
95 covered information on absolute testes mass and body mass, to test whether relative
96 testes size is correlated with of the level of sperm competition and sperm morphology.

97 **Methods**

98 The reported experiments comply with the current laws of China concerning animal
99 experimentation, and permission to collect anurans was received from the Ethical
100 Committee for Animal Experiments in China. All experiments involving the sacrifice
101 of these live animals were approved by the Animal Ethics Committee at China West
102 Normal University. All fieldworks performed were complied with the Convention on
103 Biological Diversity and the Convention on the Trade in Endangered Species of Wild
104 Fauna and Flora local ethical regulations and agreements.

105 For our analyses we combined our primary data with data derived from the
106 literature; taken together, we compiled a data set on sperm morphology for 67 species,
107 and a further data set which also incorporates body mass and absolute testes mass for
108 29 species (Additional Table S1). To obtain the primary data, we collected 5-6 males
109 of each species by hand at night using a flashlight during the breeding season at the
110 Hengdian Mountains, China, from 2009 to 2013. Individuals were kept singly in
111 wire-netting rectangular containers (20×10×15 cm; L×W×H) placed in a tank
112 (90×40×40 cm; L×W×H) with a depth of 10 cm of fresh water at room temperature.
113 We weighed body mass to the nearest 0.1 mg with an electronic balance and killed
114 animals by double-pithing. We dissected all individuals and removed both testes.
115 After weighing the testes to the nearest 0.1 mg, we immediately crushed them and
116 released sperm into reverse-filtered tap water. We pipetted 50 μ l of the suspension
117 onto microscope slides and air-dried the slides before staining them with acid carmine
118 for 40 seconds. We then captured images of mature sperm using a Motic BA300
119 digital camera attached to a Moticam2006 light microscope at a 400x magnification.
120 Abnormal spermatozoa (broken tail, damaged or missing acrosome) were not
121 considered in the analysis. Sperm morphology (total length, head length and tail

122 length) was measured using a line chain tool in the Motic Images Advanced 3.2
123 software. Measurements comprised 20 sperms from each male. All measurements
124 (testes and sperm) were taken without knowledge of the species identification to
125 prevent observer bias. The repeatability [45] was high when we compared three
126 measurements on 20 sperm ($R = 0.94$). To further enhance the reliability of sperm size
127 data, we measured the same 20 spermatozoa three times, using average values in the
128 analysis. For the three species *Rhacophorus chenfui*, *R. dugritei* and *R. omeimontis*,
129 the length of sperm heads was calculated as $L = \pi DN$ (L: length of sperm head, D =
130 diameter of helix, N = number of turns in the helix) [46]. We used the ratio between
131 flagellum and head length as a possible predictor of sperm swimming speed [26].

132 Following Byrne and Roberts [47], we used mating system as an imperfect
133 surrogate for the intensity of sperm competition on a two-point scale: 1 =
134 simultaneous polyandry, where multiple males clasp a female and sperm from males
135 to simultaneously compete to fertilize eggs over the course of a breeding season; 2 =
136 monandry, where a female mates with one male over the course of a breeding season
137 and deposits a single clutch. In *Chaparana quadrana* we observed that multiple males
138 participated in fertilising the eggs deposited by a single female without amplexus
139 (similar to *C. taihangnicus*, 48), and we regarded this species as simultaneously
140 polyandrous. Following Li et al. [49], we classified spawning location on a four-point
141 scale: 1 - arboreal: spawning occurs mostly occur on trees, eggs in foam nests; 2 -
142 terrestrial: spawning occurs on the ground, eggs laid in foam nests in holes or on the
143 ground near ponds; 3 - lentic aquatic – eggs in ponds; 4 - lotic aquatic –eggs in
144 running water.

145 Comparative analyses of interspecific data may require phylogenetic control, as
146 closely related species share parts of their evolutionary history. To control for

147 phylogeny we employed comparative analyses by independent contrasts [50]. We
148 used an established phylogeny [51-52] to reconstruct phylogenetic trees for the 67 and
149 29 anurans species, respectively (Additional files, Figure S1 and Figure S2). Because
150 information on branch lengths was not available, they were first arbitrarily set to 1
151 based on the suggestions of Pagel [53]. Felsenstein [54] provided the details of the
152 general procedure for estimating the character values in the ancestors. With 67 and 29
153 species at the tips of the reconstructed trees, respectively, 66 (67-1) and 28 (29-1)
154 pairs of contrasts could be computed for pairs of nodes sharing an immediate common
155 ancestor, and then re-scaled and analysed as suggested by Garland et al. [55].

156 Allometric effects were controlled for by correcting for body mass. All data were
157 log-transformed in all analyses. None of the distributions of log-transformed variables
158 (such as body mass, testes mass or sperm morphology) were significantly different
159 from normal, and we used parametric tests throughout.

160 In the two datasets used in the GLMs, there was only a small number of
161 polygamous, arboreal and terrestrial species compared to larger numbers of
162 monandrous species and the two aquatic categories. This made it difficult to compare
163 the effects of mating system and spawning location *per se* with an analysis which also
164 takes the effects of absolute testes mass and body mass into account. Hence, we
165 combined arboreal and terrestrial species. Both are characterized by foam nests, which
166 as a hypothesis enable fertilization in a rather protected environment. As a result, the
167 categories of spawning locations as terrestrial, lentic and lotic were used in all
168 analysis.

169 To test for the effect of mating system and spawning locations on sperm
170 morphology among 67 species, we first used a multivariate GLM to test for the effects
171 of mating system and spawning locations on independent contrasts in sperm

172 morphology, using independent contrasts in sperm morphology as dependent
173 variables and mating system and spawning locations as fixed factors. For the 29
174 species for which the required data were available, we conducted a multivariate GLM
175 on independent contrasts in relative testis mass as dependent variables and mating
176 system and spawning location as fixed factors and independent contrasts in body mass
177 as covariate to test relative testes size differences. In order to control for collinearity
178 between mating system and relative testes mass, we also ran a separate analysis with
179 relative testes mass, spawning location and body mass; obtaining the same results for
180 both analyses would lend support to an association between them. We also ran GLMs
181 with sperm morphology as dependent variable, mating system/spawning location,
182 species types and their interaction as fixed factors to test the difference in relationship
183 between sperm morphology and mating system/spawning location between 67 and 29
184 species. Finally, we used phylogenetically controlled multiple regression models (i.e.,
185 including body mass as a covariate) to test for correlations between independent
186 contrasts in relative testes mass and sperm morphology. All tests were conducted by
187 using Type III sums of squares.

188

189 **Results**

190 We used GLMs to determine if sperm morphology is influenced by the mating system
191 and spawning location in 67 anurans. Mating system and spawning location
192 significantly affected independent contrasts in total sperm length, head length and tail
193 length, but not the ratio between sperm head length and tail length (Table 1).
194 Polyandrous species had longer sperm than monogamous species (Figure 1).
195 Furthermore, post-hoc tests revealed that there was no difference in tail length of
196 sperm between species with terrestrial and lotic oviposition ($P = 0.058$). However,

197 species with terrestrial oviposition had longer sperm than those with aquatic
198 oviposition (all $P < 0.045$). Species with lotic oviposition had longer sperm than those
199 with lentic oviposition (Figure 2; both $P < 0.008$).

200 Species with a polygamous mating system had significantly larger relative testes
201 mass than monogamous species when correcting body mass (phylogenetically
202 controlled GLMs, $F_{1,27} = 9.936$, $P < 0.001$). Relative testes mass also significantly
203 differed among spawning locations (phylogenetically controlled GLMs, $F_{2,27} = 3.661$,
204 $P = 0.041$).

205 The mating system and spawning location significantly affected sperm morphology
206 across the 29 species for which data on testes mass were available (phylogenetically
207 controlled GLMs: mating system, $F_{1,27} > 4.343$, $P < 0.038$; spawning location, $F_{2,27} >$
208 3.953 , $P < 0.029$). Neither mating system nor spawning location affected the ratio
209 between head length and tail length (mating system: $F_{1,27} = 1.201$, $P = 0.283$;
210 spawning location: $F_{2,27} = 0.950$, $P = 0.400$). However, when this analysis is based on
211 mating system and spawning location as two predictors, the independent contrasts in
212 sperm morphology did not differ between polygamous and monogamous mating
213 systems and spawning locations (Table 2). We also found that independent contrasts
214 in sperm morphology were unrelated to spawning location ($F_{2,27} < 2.817$, $P > 0.085$)
215 and relative testes mass ($F_{1,27} < 2.115$, $P > 0.159$) when mating system was replaced
216 by relative testes mass in the model. The converging results for both analyses
217 demonstrate the lack of collinearity between mating system and relative testes mass.

218 We found significant differences in mating systems between spawning locations
219 when considering 67 species ($F_{2,66} = 35.303$, $P < 0.001$) as well as when focusing on
220 the 29 species for which data on testes mass were available ($F_{2,28} = 13.152$, $P < 0.001$).
221 The GLMs also revealed non-significant differences in the relationship between

222 sperm morphology and mating system as well as spawning location between both
223 datasets (Table 3), suggesting that body mass and absolute testes mass rather than
224 species number affect variation in sperm morphology.

225 We further examined the correlation between relative testes mass and sperm
226 morphology using phylogenetically controlled multiple regression models including
227 body mass as a covariate. We found that independent contrasts in relative testes mass
228 were further positively correlated with independent contrasts in sperm morphology
229 (total length, $t = 3.229$, $P = 0.004$; head length, $t = 2.895$, $P = 0.008$; tail length, $t =$
230 3.682 , $P = 0.005$; ratio of head to tail, $t = 2.465$, $P = 0.012$; Figure 3).

231

232 **Discussion**

233 Mating system and spawning location significantly affect sperm morphology among
234 67 anurans species. Polyandrous species have longer sperm than monogamous species.
235 Species with arboreal spawning locations have longer sperm than species with aquatic
236 spawning, and species with both terrestrial and lotic spawning locations have longer
237 sperms than those with lentic spawning sites. These patterns remain unchanged when
238 analyzing 29 species for which data on body mass and testes mass are available.
239 However, after correcting for the effects of body mass or absolute testes mass, the
240 effects of mating system and spawning location on sperm morphology disappear.
241 Moreover, we find that relative testes mass (as a proxy for the risk of sperm
242 competition) is positively correlated with sperm total length, head length and tail
243 length. This finding is consistent with the prediction that sperm competition should
244 favor longer sperm. A positive correlation between relative testes mass and the ratio
245 between sperm head size and tail size also suggests that sperm competition might
246 promote faster sperm in anurans.

247 We find that sperm length varies by a factor of 8.1 across 67 Chinese anurans
248 species. *Rhacophorus dennysi* have the longest sperm (235 μm), whereas
249 *Hoplobatrachus tigrina* have the shortest sperm (29 μm). An earlier study on
250 myobatrachid frogs has suggested that variation in sperm head and tail length is not
251 associated with variation in body size after controlling for phylogeny [11]. However,
252 we observe a consistent positive correlation between sperm length and relative testes
253 mass. For Australian frogs, a number of selective pressures result in the observed
254 variation in sperm morphology [11]. Our results provide evidence that particularly the
255 mating system may account for the evolution in sperm morphology across Chinese
256 anurans.

257 Across vertebrates, there is ample empirical evidence which supports predictions
258 from sperm competition theory about sperm morphology at the interspecific level
259 [8-12, 37, 43, 56-59]. The majority of studies suggest that selection favors longer
260 sperm when the intensity and risk of sperm competition is high. However, in a few
261 cases, either negative relationships or no influence of sperm competition on sperm
262 length are observed [39-40, 42]. In our study, we provide clear evidence that more
263 intense sperm competition results in longer sperm. Selection on relative testis size
264 does not necessarily result in variation in sperm morphology. Sperm competition
265 might result in selection for longer sperm if these have a competitive advantage and in
266 selection for larger testes either because the production of longer sperm requires
267 larger testes or because larger testes can produce more sperm (or both combined) [9,
268 11-12, 43]. The relationship between sperm morphology and relative testes mass was
269 also supported by our finding that polygamous species have longer sperm than
270 monogamous species.

271 The mating system is often used as an indicator of the intensity of sexual selection
272 [60]. Our results show that sperm morphology is affected by mating system across 67
273 species, suggesting that more intense sexual selection results in longer sperm, in line
274 with previous evidence from e.g. fishes [43]. Variation in sperm morphology could
275 also be attributed to the intensity of sperm competition as measured by relative testis
276 size. Furthermore, our study confirms previous studies for anurans which show that
277 the mating system affects relative testes size [30, 61-63].

278 The ratio between head length and tail length is frequently used as an indicator of
279 sperm speed [17, 26-27]. In our study across 67 species, the mating system does not
280 affect this ratio, suggesting that the intensity of male-male competition does not
281 increase putative sperm swimming speed. However, a positive correlation between
282 relative testes size and the ratio between head and tail length across the second data
283 set (29 species) reveals that sperm competition can promote sperm with short tails and
284 long head, implying that sperm swimming speed is slower when the risk of sperm
285 competition is high. In line with results from other studies [2, 64-66] this suggests that
286 there is a possible interaction between sperm longevity and swimming speed. Based
287 on theory, an increase of sperm competition should favor increasing or decreasing
288 sperm length when sperm longevity is negatively or positively correlated with sperm
289 length, respectively [15]. It is important to consider that differences in sperm
290 morphology, velocity and longevity between anurans and fish might arise from
291 different targets of selection. Assuming a trade-off between sperm velocity and
292 viability, selection can act on sperm to be fast (and hence short-lived due to energy
293 depletion) or to live longer (and thus being slower as a consequence). An important
294 difference between fish and anurans, for example, is that anuran sperm often have to
295 penetrate layers of jelly and so can stay motile for over an hour, although swimming

296 slowly, to work their way through. In most externally fertilizing fish, however, sperm
297 tend to be released close to the eggs and so swim a short distance through water,
298 where speed might be more important than longevity. This may then also be reflected
299 in differences in sperm morphology and selection on it. In addition, there are likely to
300 be differences in the risk of sperm loss or dilution, so that any trade-off between
301 sperm size and number might also vary between these taxa and differentially constrain
302 the evolution of sperm morphology. As a consequence, it depends on the
303 interpretation of the theoretical predictions as to how to interpret Stockley et al.'s
304 results. Contrary to this prediction, however, sperm length in fish is significant
305 negatively correlated with sperm competition despite a negative correlation between
306 sperm longevity and sperm length [39]. Unfortunately we currently lack data on the
307 relationship between sperm length and longevity for anurans.

308 Amphibians deposit their eggs on a range of aquatic and terrestrial substrates [49,
309 67-68], and previous studies have shown that sperm morphology can depend on
310 spawning location [11, 69]. Byrne et al. [11] reveal that spawning location
311 significantly affects the length of sperm heads, but not the length of sperm tails. In our
312 study, terrestrial species have longer sperm than aquatic species, and the shortest
313 sperm is found in species with lentic aquatic ovipositing. In line with a previous study
314 on fish which showed that buccal-fertilizing cichlids having shorter sperm than
315 substrate fertilizers [43], this suggests that the locomotor ability of sperm needs to be
316 higher in lotic sites than in lentic sites. However, when correcting for the effects of
317 body mass and absolute testes mass, these relationships disappear, and our data
318 therefore do not provide clear evidence for a link between water turbulence affecting
319 sperm total length across 29 species.

320 In conclusion, our study shows that mating system and spawning location
321 significantly influence the evolution of sperm morphology, but that the effect is
322 largely due to differential body mass and absolute testes mass. We find that the
323 influence of the mating system and spawning location on the evolution of sperm
324 morphology remains unchanged when accounting for phylogeny. We also find a
325 relationship between sperm morphology and relative testes size, suggesting that the
326 risk of sperm competition has a strong influence on sperm morphology.

327

328 **Availability of supporting data**

329 The sperm morphology data set supporting the results of this article is available in the
330 Dryad Digital Repository, with identifier doi: DOI: 10.5061/dryad.8cj79
331 (<http://doi.org/DOI:doi:10.5061/dryad.8cj79>).

332

333 **Additional files**

334 Additional file 1: Table S1. Species, mean body mass, absolute testes mass, sperm
335 morphology, mating system, oviposition locations and references of published papers.
336 For spawning location we placed species into one of four nominal categories, 1 -
337 arboreal; 2 -terrestrial; 3 - lentic aquatic. 4 - lotic aquatic; Mating system as an
338 imperfect surrogate of the intensity of sexual selection was quantified on a two-point
339 scale: 1 - simultaneous polyandry where sperm from multiple males compete to
340 fertilize eggs of a female over the course of a breeding season; 2 monandry where a
341 females mates with one male over the course of a breeding season by depositing a
342 single clutch (following Byrne et al. 2003).

343

344 Additional file 2: Figure S1 The phylogenetic tree of the 67 anurans species used in
345 the comparative analysis following Jiang et al. (2003) and Pyron and Wiens (2011).

346

347 Additional file 3: Figure S2 The phylogenetic tree of the 29 anurans species used in
348 the comparative analysis following Jiang et al. (2003) and Pyron and Wiens (2011).

349

350 **Competing interests**

351 The authors have declared that no competing interests exist.

352

353 **Authors' contributions**

354 YZ and SLL carried out the analyses and drafted the manuscript. WBL and YZ
355 designed the study. WBL and RJ wrote the paper. All the authors read and approved
356 the final manuscript.

357

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531 **Figure Legends**

532 Fig. 1 Differences in sperm morphology between polyandrous and monogamous
533 mating system across 67 anurans species. 1 – sperm total length; 2 – sperm head
534 length; 3 – sperm tail length.

535

536 Fig. 2 Differences in sperm morphology among spawning sites across 67 anurans
537 species. 1 – sperm total length; 2 – sperm head length; 3 – sperm tail length.

538

539 Fig. 3 Correlations between sperm morphology and relative testes mass among 29
540 anurans species. Relative testes mass was derived from residuals of observed testes
541 mass minus predicted testes mass on the basis of the allometric regression.

542

Table 1 The influences of mating system and spawning locations on variation in independent contrasts in sperm morphology across 67 anurans species using GLM^a.

Source	Sums of squares	d.f.	Mean square	<i>F</i>	<i>P</i>
Sperm total length					
Mating system	0.034	1	0.034	4.864	0.031
Spawning locations	0.059	2	0.029	4.421	0.016
Sperm head length					
Mating system	0.086	1	0.086	9.016	0.004
Spawning locations	0.120	2	0.060	6.514	0.003
Sperm tail length					
Mating system	0.057	1	0.057	5.401	0.023
Spawning locations	0.085	2	0.043	4.132	0.021
Ratio of head to tail					
Mating system	0.441	1	0.441	0.158	0.694
Spawning locations	56.556	2	28.278	0.667	0.517

^a For testing evolutionary associations, the regression was forced through the origin.

1 Table 2 The influences of mating system and spawning locations on variation in
 2 independent contrasts in sperm morphology across 29 anurans species when
 3 correcting the body mass using GLM ^a.

Source	Sums of squares	d.f.	Mean square	<i>F</i>	<i>P</i>
Sperm total length					
Mating system	0.001	1	0.001	0.007	0.932
Spawning locations	0.031	2	0.016	2.266	0.127
Body mass	0.122	1	0.122	17.655	<0.001
Sperm head length					
Mating system	0.006	1	0.006	0.500	0.487
Spawning locations	0.019	2	0.009	0.785	0.468
Body mass	0.158	1	0.158	13.365	0.001
Sperm tail length					
Mating system	0.002	1	0.002	0.199	0.660
Spawning locations	0.059	2	0.030	2.849	0.079
Body mass	0.206	1	0.206	19.880	<0.001
Ratio of head to tail					
Mating system	0.060	1	0.060	0.027	0.870
Spawning locations	0.451	2	0.225	0.103	0.903
Body mass	0.375	1	0.375	0.171	0.684

4 ^a For testing evolutionary associations, the regression was forced through the origin.

5
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 7
 8

9 Table 3 Differences in relationship between sperm morphology and mating
 10 system/spawning location between 67 and 29 species using GLM ^a.

Source	d.f.	Mean square	<i>F</i>	<i>P</i>
Sperm total length				
Mating system	1	0.011	1.322	0.253
Spawning locations	2	0.017	2.212	0.126
Species types	1	0.006	0.663	0.418
Mating system*species types	1	0.015	1.751	0.189
Spawning locations*species types	2	0.014	1.664	0.230
Sperm head length				
Mating system	1	0.037	3.194	0.077
Spawning locations	2	0.023	2.037	0.137
Species types	1	0.032	2.799	0.098
Mating system*species types	1	0.030	2.578	0.112
Spawning locations*species types	2	0.013	1.163	0.317
Sperm tail length				
Mating system	1	0.011	0.796	0.375
Spawning locations	2	0.043	2.187	0.059
Types of species	1	0.004	0.264	0.608
Mating system*species types	1	0.037	2.700	0.104
Spawning locations*species types	2	0.044	3.155	0.054

11 ^a For testing evolutionary associations, the regression was forced through the origin.

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