Hanna J. McLennan, Stefan Lüpold, Pete Smissen, Kevin C. Rowe and William G. Breed
Greater sperm complexity in the Australasian old endemic rodents (Tribe: Hydromyini) is associated with increased levels of inter-male sperm competition
Reproduction, Fertility and Development, 2016; OnlinePubl:1-10

Originally Published at: http://dx.doi.org/10.1071/RD15425

1 **Title:**

2 Greater sperm complexity in the Australasian old endemic rodents (Tribe:
3 Hydromyini) associates with increased levels of intermale sperm competition

4 **Authors:** Hanna J. McLennan[1], Stefan Lüpold[2], Pete Smissen[3], Kevin C. Rowe[3], William G. Breed[1]

5 [1]Discipline of Anatomy and Pathology and [1]Robinson Research Institute, School of Medicine, Faculty
6 of Health Sciences, The University of Adelaide, North Terrace, Adelaide, SA 5005, Australia

7 [2]Institute of Evolutionary Biology and Environmental Sciences, University of Zurich, 8057 Zurich,
8 Switzerland

9 [3] _____

10 **Keywords:** Hydromyine rodents, Muridae, spermatozoa, sperm competition

11 **Correspondence:** William G. Breed, Discipline of Anatomy and Pathology, School of Medicine,
12 Faculty of Health Sciences, The University of Adelaide, North Terrace, Adelaide, SA 5005, Australia

13 **E-mail:** bill.breed@adelaide.edu.au

14

Abstract

Word Count (max 300): 210

The male gamete, the spermatozoon, exhibits considerable interspecies morphological variation across mammals, especially among murid rodents. In Australasia most murids in the Tribe Hydromyini have a spermatozoon with a highly complex head that has, in addition to an apical hook characteristic of most murids, two further projections that extend from its upper concave surface, the ventral processes. Here we performed a phylogenetically controlled comparison of sperm morphology across 44 species of hydromyine rodents to test the hypothesis that the length and angle of both the ventral processes and apical hook, as well as the dimensions of the sperm tail, increase with relative testes mass as a proxy for differences in levels of intermale sperm competition. Although both sperm head protrusions exhibited considerable variation in their length and angle across species, only the angles increased significantly in relation to relative testes mass. Significant positive relationships were also evident between relative testes mass and lengths of the sperm midpiece and flagellum. These results suggest that in the sperm head of hydromyine rodents, the angle of the ventral processes, as well as that of the apical hook, together with the sperm tail length, are likely to be under sexual selection. The possible functional significance of these findings is discussed.

Word Count (max 5000): 3,601

Introduction

The spermatozoon is the most morphologically variable type of cell known to occur in vertebrates (Cohen 1977; Pitnick *et al.* 2009). The reason(s) for this are not clear although differences in both the mode of fertilisation and phylogeny have been suggested (Franzén 1970; Jamieson 1987). It is, however, becoming increasingly evident that sexual selection also plays a major role in determining the species specific form of the spermatozoon (for reviews see Pitnick *et al.* 2009; Simmons and Fitzpatrick 2012) with mounting evidence suggesting that both sperm size and shape co-vary with differences in levels of intermale sperm competition (see Snook 2005; Pitnick *et al.* 2009; Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014 for reviews). Within a species, sperm morphology also appears to respond to experimentally manipulated levels of sperm competition (e.g. LaMunyon and Ward 2002; Palopoli *et al.* 2015), and has been found to contribute to fertilisation success under competitive conditions (e.g. LaMunyon and Ward 1998; Miller and Pitnick 2002; Oppliger *et al.* 2003; García-González and Simmons 2007; Firman and Simmons 2008; Lüpold *et al.* 2012; Bennison *et al.* 2015).

A spermatozoon consists of a head, with a nucleus housing a haploid set of chromosomes and an enzyme-filled acrosome, and a tail for motility with the energy-generating mitochondria being present in its midpiece. Across species, the midpiece and total flagellum length tend to positively associate with sperm swimming speed (Gomendio & Roldan, 2008; Fitzpatrick *et al.*, 2009; Lüpold *et al.*, 2009; Gómez Montoto et al., 2011; Tourmente, Gomendio & Roldan, 2011) which is particularly evident when the size and shape of the sperm head is also taken into account (Higdon, 1979; Humphries et al. 2008).

Compared to most mammalian taxa, the morphology of the spermatozoon in murid rodents is highly diverse across species in both its size and the shape of its head (Breed, 2004, 2005; Gomendio,

Tourmente & Roldan, 2011; Tourmente *et al.*, 2011). Within the subfamily Murinae most, but not all, species have a sperm head with an apical hook whereas most rodents of the Australasian old endemic tribe Hydromyini have an even more complex sperm form with two further extensions, the ventral processes, protruding from its upper concave surface. Suggested functions of the apical hook include facilitating temporary binding of the spermatozoon to the oviduct epithelium (Smith & Yanagimachi, 1990; Firman & Simmons, 2009; Gómez Montoto et al., 2011) and/or in aiding in the formation of sperm aggregates or "trains" to enhance motility under high levels of intermale sperm competition (Moore *et al.*, 2002; Immler *et al.*, 2007; Fisher & Hoekstra, 2010) whereas the function of the sperm ventral processes may be to facilitate sperm binding to, and penetration of, the coat that surrounds the egg, the zona pellucida (Breed and Leigh, 1991; Drew et al. 2014).

In the current study, we tested the hypothesis that the length and angle of the sperm head ventral processes, that are characteristic morphological feature of the spermatozoon of most of the hydromyine rodents, are sexually selected traits and increase as the level of sperm competition is enhanced. We examined morphological data of sperm obtained from 44 species of hydromyine rodents in a phylogenetically controlled framework using relative testes mass as a proxy for sperm competition (Soulsbury 2010).

Materials and Methods

The taxonomy of the Australasian Old Endemic rodents used in the current study follows that of Musser and Carleton (2005) and Lecompte *et al.* (2008). Thus, within the Tribe Hydromyini six divisions are recognised: Hydromys, Xeromys, Uromys , Pogonomys, Lorentizmys, and Pseudomys.

Specimens and Sample Preparation

Sperm samples were obtained from 44 species that included representatives from all of the 6 hydromyine divisions (for a full list of species and source of the material see Supplementary Table 1).

Sperm were obtained from the cauda epididymides that had been fixed in 10% buffered formalin. The testis weight was determined and, when only one testis was available, its weight was doubled to provide an approximate combined testes mass for the individual. Slides of sperm smears were prepared by teasing apart the cauda epididymides with forceps under a dissecting microscope and extruding sperm from the ducts. Body mass data came from either museum or laboratory records of the relevant individuals or from the literature (e.g. Breed & Taylor, 2000; see Supplementary Table 1 for details).

Sperm Parameters

To indicate qualitative differences in sperm head morphology across species, and in particular the interspecific variation in the length and orientation of the apical hook and ventral processes, scanning electron microscopy of the sperm was carried out as previously described (see Breed, 1983, 1984; Breed & Leigh, 2010).

For quantification of trait variation, light microscopical images of morphologically intact sperm were captured with a Nikon digital camera (Olympus SC100) attached to a Nomarski light microscope (Olypus BH2), and 10 sperm per individual were measured using the image analysis program NIS-Elements BR, calibrated to 0.09 μm/pixel. Sperm were selected at random and photographically archived. Sperm head length was measured from the base of the sperm head to the base of the apical hook, and head width across the widest part of the head perpendicular to head length. The ventral process and hook length was determined by drawing a line at the base of the ventral processes, when present, and the apical hook. The lengths of the apical hook and ventral processes were measured from the base to the tip by tracing the centre line using a segmented line tool. Where two ventral processes were discernible and differences in length were evident, the longer of the two processes was recorded. The angle of the apical hook and ventral processes was measured as the reflective angle between the tangent of a line drawn through their rostral tip along the concave surface and the main longitudinal axis of the sperm head (see Immler *et al.*, 2007).

Midpiece length was measured from the connecting piece to either the cytoplasmic droplet and/or to a discernible narrowing of tail width. The lengths of the principal and end pieces were combined and measured from the posterior end of the midpiece to the tip of the tail. The total flagellum length was the sum of the midpiece, principal piece and end piece lengths. Sperm with discernible breaks were precluded from the analysis.

Statistical Analysis

Statistical analyses were performed using the statistical package R v.3.1.1 (R Core Team, 2014). Non-normal data distributions were logarithmically transformed and relative testes mass (RTM) was used as a proxy for sperm competition (e.g. Soulsbury, 2010) by including both combined testes mass and body mass as predictor variables in all analyses of sperm traits against the level of sperm competition.

Phylogenetic general linear models (PGLM) were used to account for statistical non-independence of the data due to shared common ancestry (Pagel, 1999; Freckleton, Harvey & Pagel, 2002), based on a molecular phylogeny of the Australasian old endemic rodents (P. Smissen & K. Rowe, unpublished; see Supplementary Fig. 1). The phylogenetic scaling parameter λ, estimated by the PGLM, was used to determine the level of phylogenetic dependence of the relationships. In brief, values of λ close to 0 indicate that the association between the traits under examination is largely independent of phylogeny, whereas λ values close to 1 suggest strong phylogenetic dependence. Likelihood ratio tests were used to compare the maximum likelihood estimates of λ of a given PGLM to models where λ was set to 0 or 1, respectively, and the corresponding *P*-values are shown as superscripts following λ (first superscript for λ=0, second for λ=1). We report the strength of all relationships (i.e., effect sizes) as the partial correlation coefficients, *r*, with 95% non-central confidence intervals (95% CI), calculated from the *t*-statistic of the PGLM (Nakagawa & Cuthill, 2007).

130 **Results**

131

132 The species investigated exhibited marked differences in relative testes mass (RTM) (for details see
133 Supplementary Table 1). The smallest RTM occurred in the three species of *Notomy*s (*N. alexis, N.*
134 *fuscus and N. mitchelli*) which were between 0.14% and 0.16% of body mass, whereas the largest
135 RTM occurred in *Pogonomys* species, *Mastocomys,* and several species of *Pseudomys* where it
136 ranged from 2.7% to 5.5% of body mass**.** Considerable interspecific variation in RTM was observed
137 within the one genus *Pseudomys* where it ranged from 0.4% in *P. novaehollandiae* to 3.4% in *P.*
138 *fumeus* hence spanning most of the interspecific variation for RTM throughout the hydromyine
139 species investigated (see also Breed & Taylor, 2000).

140 Scanning electron microscopy showed that in five out of six divisions of hydromyine rodents most
141 species have a sperm head with two ventral processes (Figs. 1 and 2). However, their length and
142 angle, as well as that of the apical hook, varied across species (e.g. compare Fig. 1 a-d, g-i with Fig.
143 2a-e, g-i) even though they were generally fairly consistent within a species. Within the Pseudomys
144 division, *P. novaehollandiae* (Fig. 1e) was the only species with no ventral processes but a single
145 apical hook, whereas *P. shortridgei* had neither an apical hook nor ventral processes (see Fig. 1j).
146 Furthermore, the presence of ventral processes was highly variable within *N. alexis*, but, when
147 present, the apical hook and these processes were always very short (see Fig. 1j). The Pogonomys
148 division also had interspecies variation in the presence of ventral processes with some exhibiting
149 clear processes (Fig. 2 g to i) whereas others lacked them (Table S1).

150 Based on quantitative light microscopical measurements, the length of the sperm head ranged from
151 around 4.3 µm in *Hydromys chrysogaster* to a mean of 9.2 µm in *Abeomelomys sevia***,** and its
152 maximum width ranged from 1.6 µm in *Mallomys rothschildi* to around 4 µm in *Notomys fuscus* (Fig.
153 2i) and *Anisomys imitator* (see Suppl. Table 2). When the apical hook on the sperm head was

154 present, its length ranged from 2 µm, or less, in sperm of *Notomys alexis* (Fig. 1j) to 14 µm in *A.*
155 *sevia*, with most sperm having an apical hook length of between 4 and 9 µm . The angle also varied
156 across species and ranged from 218° in *N. alexis* (Fig. 1j) to exceeding 340° in two species of
157 *Paramelomys* (e.g. Fig. 2c) (see Suppl. Table 2 for details).

158 Ventral processes were present on the sperm head of 36 out of all 44 species (Figs. 1, 2). However, in
159 *N. alexis*, when present, they measured no more than 1.0 µm. By contrast the ventral processes
160 exceeded 6 µm in several species including those of *Pseudomys desertor* (Fig. 1a)*, P. australis, P.*
161 *gracilicaudatus, P. higginsi, Leporillus conditor,* and *Mastocomys fuscus* (see Suppl. Table 2). The
162 angle of these processes varied from 204° in *N. alexis* to 360° in *Paramelomys levipes*, although the
163 majority fell between300° and 330° (see Suppl. Table 2).

164 Finally, midpiece lengths ranged from about 20 µm in *H. chrysogaster* to 55 µm in *Chiruromys vates*,
165 whereas the principal and end piece lengths varied between about 70 µm in *N. fuscus* and 126 µm in
166 *Abeomelomys sevia*.

167 Phylogenetically controlled general linear models revealed several statistically significant
168 relationships between sperm traits and relative testes mass (RTM) (see Table 1 for details). For
169 example, the ratio of head length to width, a measure of how streamlined the sperm head is,
170 covaried positively with RTM (N = 44, partial r = 0.58, p < 0.001) (Fig. 3a). The length of both the
171 apical hook and ventral processes also tended to increase with RTM. Both these associations were,
172 however, largely driven by *N. alexis* and, after removing this species from the analysis, neither *P*-
173 values were statistically significant (partial $r \leq 0.18$, $P \geq 0.25$). The angles of both the apical hook and
174 ventral processes were also positively correlated with RTM (partial $r \geq 0.63$, $P < 0.001$) with these
175 effects remaining statistically significant after removing the three influential data points (apical hook
176 angle: N = 44, partial r = 0.71, P < 0.001; ventral process angle: N = 36, partial r = 0.63, P < 0.001) (see
177 Fig. 3 b, c). In addition, midpiece length tended to increase with RTM, albeit not significantly so
178 (partial r = 0.27, P = 0.07), whereas the lengths of the combined principal and end pieces and the

179 total flagellum length were positively associated with RTM (partial $r \geq 0.50$, $P < 0.001$) (Fig. 3d).
180 None of the ratios between sperm components were significantly correlated with RTM (partial $|r| \leq$
181 0.25, $P \geq 0.09$).

182

183 **Discussion**

184 Most of the old endemic hydromyine rodents of Australasia have a highly complex sperm head in
185 which two cytoskeletal processes, the ventral processes, extend from its upper concave surface.
186 There are differences in length and orientation of these processes across the species and here we
187 tested the hypothesis that their length and orientation have evolved as a result of sexual selection.

188 Most murid rodents have elongated sperm heads with an apical hook into which the nucleus,
189 acrosome with an elongated region of cytoskeleton, the perforatorium into which part of the
190 nucleus, acrosome and cytoskeleton extend (Fawcett, 1975; Oko & Clermont, 1988; Breed, 2004),
191 and it has previously been found that the length and angle of this apical hook increases in length
192 with increase of RTM (Immler et al. 2007, Sandera et al 2013). Our current study using 44 species in
193 the murid tribe, Hydromyini, shows that, in addition to the apical hook, most species have a sperm
194 head that has, in addition to an apical hook, two ventral processes which, unlike the apical hook, are
195 largely composed of cytoskeletal material (Flaherty and Breed 1983, 1987; Breed et al., 2000). The
196 present results show that these ventral processes have a more reflective angle in species with a high
197 RTM. A finding that suggests that their angle, like that of the apical hook, is a sexually selected trait
198 that has evolved under high levels of intermale sperm competition.

199 Across the species of hydromyine rodents there are, nevertheless, marked differences in overall
200 sperm head size and shape, as well as in the length of the apical hook and ventral processes. For
201 instance in *Notomys fuscus*, unlike most hydromyines, the sperm head is around half as wide as it is
202 long with the apical hook being relatively much shorter than that of most other species with the

ventral processes also being either very short or nonexistent. These divergent features were even more evident in the sperm of a closely related species, that of *N. alexis,* which has a highly variable sperm head shape (e.g. see Suttle et al., 1988; Bauer and Breed, 2006) with the ventral processes often being absent and the curvature of the apical hook being considerably less than that of the other species. These two species of *Notomys* have the smallest relative testes mass of all species investigated and, at least in *N. alexis*, the efficiency of production of sperm per gram testis is comparatively low (Peirce and Breed, 2001; Bauer and Breed, 2008). These features suggest that intermale sperm competition in these species is weak or even lacking, and this has resulted in highly variable sperm morphology that occurs within and between males in these species similar to the situation in the greater bandicoot rat *Bandicota indica* (Thitipramote et al 2011) and naked mole rat *Heterochephalus glaber* (Van der Horst et al 2011) in which it has been suggested that due to minimal sperm competition "degenerative" sperm traits and high levels polymorphism has evolved (Van der Horst and Maree 2014).

Previous work on the functional and evolutionary significance of the apical hook of murine sperm has suggested that in the wood mouse, *Apodemus sylvaticus*, the hook may facilitate the formation of highly progressive motile groups of sperm, or "sperm trains", with sperm attaching to each other by way of their apical hook (Moore *et al.*, 2002). This finding was subsequently observed in sperm of the laboratory rat, *Rattus norvegicus* (Immler *et al.*, 2007) as well as in a species of deer mouse, *Peromyscus maniculatus* (Fisher & Hoekstra, 2010). Within the hydromyine rodents, the only published study addressing this question is that of Firman *et al.* (2013) using sperm from the Sandy Inland mouse, *Pseudomys hermannsburgensis*, in which no sperm grouping was observed in spite of a well-developed apical hook being present. However, more recent observations on sperm with similar morphology, those of *P. australis*, have indicated that sperm do indeed aggregate upon release from the epididymis into culture medium, but this was not found to occur in sperm of *N. alexis*, which lack lack a long hook and ventral processes (Kathrine Ferres and Bill Breed, unpublished observations). Based on the comparison between these two species it may be that the ventral

229 processes facilitate sperm aggregation although further evidence of the ventral processes being
230 involved in sperm behaviour is clearly needed.

231 Apart from supporting sperm aggregation, the sperm head ventral processes may also facilitate
232 sperm binding to the egg coat. For example, studies on sperm–egg interactions *in vitro* show that in
233 *P. australis* the ventral processes enhance the area of sperm binding to the egg coat (Drew *et al.*,
234 2014), and *in vivo* observations indicate that these processes enlarge the size of the penetration slit
235 in the zona (Breed & Leigh, 1991). The significance of variation in the angle of the ventral processes
236 in relation to zona pellucida binding and penetration is unknown but it may be that sperm with more
237 reflective ventral processes form tighter initial attachment to the egg coat. Even though the function
238 of these processes remains unclear at the present time, the present study clearly shows that their
239 length and orientation are generally similar to that of the apical hook; a feature that suggests that
240 these structures have coevolved.

241 In addition to sperm head variation, a significant positive relationship between relative testes mass
242 and the sperm flagellum length was also evident, a finding that is consistent with a broader range of
243 rodents (Gomendio *et al.*, 2011). The prevailing explanation for a positive association with relative
244 testes size is that sperm with a relatively long flagellum and/or larger midpiece are favoured by
245 sexual selection because they have a competitive advantage by achieving greater swimming velocity
246 than sperm with relatively shorter tails (Katz, Drobnis & Overstreet, 1989; Cardullo & Baltz, 1991;
247 Gómez Montoto *et al.*, 2011). Despite relatively little evidence within species (reviewed in
248 Humphries *et al.*, 2008; Simmons & Fitzpatrick, 2012), such a link between sperm morphology and
249 sperm velocity is supported by comparative studies using various vertebrate taxa, including cichlid
250 fishes (Fitzpatrick *et al.*, 2009), passerine birds (Lüpold *et al.*, 2009, but see Kleven *et al.*, 2009), and
251 mammals (Gomendio & Roldan, 1991; Tourmente *et al.*, 2011; Gómez Montoto *et al.*, 2011). If such
252 a link between sperm form and function also holds for hydromyine sperm, our comparative data

would suggest that selection on sperm tail length through sperm competition may be mediated by its effects on the speed of sperm swimming.

In conclusion, our results suggest that the complex sperm morphology of hydromyine rodents is, at least in part, a result of postcopulatory sexual selection. These findings extend previous reports of Immler *et al.*, (2007) and Šandera *et al.* (2013) on the apical hook of murine sperm and show that the additional ventral processes on the sperm head of the Australasian old endemic rodents may also have evolved under sexual selection. Similarly, sperm tail dimensions co-vary positively with relative testes mass, which might be the result of sperm competition selecting for faster sperm, mediated by relatively longer tails. Further studies are now required to gain more in-depth insight into the adaptive significance of the ventral processes that are such a characteristic feature of the sperm head of most of the hydromyine species of Australasia.

Acknowledgements

We should especially like to thank the relevant curators of the state museums for enabling us to gain access to specimens from which we obtained the reproductive tracts for extracting sperm from the epididymides and in particular Sandy Ingleby in Sydney as well as to the field workers who obtained the material. Visits to the state museums to obtain this material were made possible by grants from The ARC and The University of Adelaide to the corresponding author. Furthermore, we also thank the several third-year undergraduate students who carried out preliminary observations on some of the species used in this study. We also thank Dr Jono Tuke of the School of Mathematics, University of Adelaide for assistance with the statistics in an early part of this study, Tavik Morgenstern for preparing the scanning electron microscope figures and Chris Leigh and Kate McLennan for microscope assistance.

277 **References**

278 Bauer, M. & Breed, W. G. (2006). Variation of sperm head shape and tail length in a species of
279 Australian hydromyine rodent: the spinifex hopping mouse, *Notomys alexis*. *Reprod. Fert.*
280 *Dev.* **18,** 797-805.
281 Bauer, M. and Breed, W. G. (2008). Testis mass of the spinifex hopping mouse and its impact on
282 fertility potential. *J. Zool.* **274**, 349 - 356.
283 Baverstock, P. R., Watts, C. H. S., Adams, M. & Cole, S. R. (1981). Genetical relationships among
284 Australian rodents (Muridae). *Aust. J. Zool.* **29,** 289-303.
285 Baverstock, P. R., Watts, C. H. S. & Hogarth, J. T. (1977). Chromosome evolution in Australian
286 rodents. 1. Pseudomyinae, Hydromyinae and Uromys/Melomys group. *Chromosoma.* **61,** 95-
287 125.
288 Bennison, C., Hemmings, N., Slate, J. & Birkhead, T. (2015). Long sperm fertilize more eggs in a bird.
289 *Proc. R. Soc. Lond. B. Biol. Sci.* **282,** 20141897.
290 Birkhead, T. R., Immler, S., Pellatt, E. J. & Freckleton, R. P. (2006). Unusual sperm morphology in the
291 Eurasian bullfinch (*Pyrrhula pyrrhula*). *Auk* **123**, 383-392.
292 Breed, W. G. (1983). Variation in sperm morphology in the Australian rodent genus, *Pseudomys*
293 (Muridae). *Cell Tissue Res.* **229,** 611-625.
294 Breed, W. G. (1984). Sperm head structure in the Hydromyinae (Rodentia: Muridae): A further
295 evolutionary development of the subacrosomal space in mammals. *Gamete Res.* **10,** 31-44.
296 Breed, W. G. (2004). The spermatozoon of Eurasian murine rodents: Its morphological diversity and
297 evolution. *J. Morphol.* **261,** 52-69.
298 Breed, W. G. (2005). Evolution of the spermatozoon in muroid rodents. *J. Morphol.* **265,** 271-290.
299 Breed, W. G. and Adams, M. (1992). Breeding systems of Spinifex hopping mice (*Notomys alexis*) and
300 plains rats (*Pseudomys australis*): a test for multiple paternity within the laboratory. *Aust. J.*
301 *Zool.* **40**, 13 - 20.

Breed, W. G., Idriss, D. & Oko, R. J. (2000). Protein composition of the ventral processes on the sperm head of Australian hydromyine rodents. *Biol. Reprod.* **63,** 629-634.

Breed, W. G. & Leigh, C. M. (1991). The distribution of filamentous-actin in and around spermatids and in spermatozoa of Australian conilurine rodents. *Mol. Reprod. Dev.* **30,** 369-384.

Breed, W. G. & Leigh, C. M. (2010). The spermatozoon of the Old Endemic Australo-Papuan and Philippine rodents - its morphological diversity and evolution. *Acta. Zool.* **91,** 279-294.

Breed, W. G. & Taylor, J. (2000). Body mass, testes mass, and sperm size in murine rodents. *J. Mammal.* **81,** 758-768.

Cardullo, R. A. & Baltz, J. M. (1991). Metabolic regulation in mammalian sperm: Mitochondrial volume determines sperm length and flagellar beat frequency. *Cell. Motil. Cytoskel.* **19,** 180-188.

Cohen, J. (1977). *Reproduction*. London, UK: Butterworths.

Drew, S., Leigh, C. & Breed, W. G. (2014). Spermatozoa of the old endemic rodents of Australia - the possible functional significance of their ventral processes. *Reprod. Fert. Develop.* **26**, 1183-1187.

Fawcett, D. W. (1975). The mammalian spermatozoon. *Dev. Biol.* **44,** 394-436.

Firman, R. C., Bentley, B., Bowman, F., Marchant, F. G. S., Parthenay, J., Sawyer, J., Stewart, T. & O'Shea, J. E. (2013). No evidence of sperm conjugate formation in an Australian mouse bearing sperm with three hooks. *Ecol. Evol.* **3,** 1856-1863.

Firman, R. C. & Simmons, L. W. (2008). Polyandry, sperm competition, and reproductive success in mice. *Behav. Ecol.* **19,** 695-702.

Firman, R. C. & Simmons, L. W. (2009). Sperm competition and the evolution of the sperm hook in house mice. *J. Evol. Biol.* **22,** 2505-2511.

Fisher, H. S. & Hoekstra, H. E. (2010). Competition drives cooperation among closely related sperm of deer mice. *Nature.* **463,** 801-803.

Fitzpatrick, J. L. & Lüpold, S. (2014). Sexual selection and the evolution of sperm quality. *Mol. Hum. Reprod.* **20,** 1180-1189.

Fitzpatrick, J. L., Montgomerie, R., Desjardins, J. K., Stiver, K. A., Kolm, N. & Balshine, S. (2009). Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proc. Natl. Acad. Sci. USA.* **106,** 1128-1132.

Flaherty, S. P. & Breed, W. G. (1983). The sperm head of the plains mouse, *Pseudomys australis*: Ultrastructure and effects of chemical treatments. *Gamete Res.* **8,** 231-244.

Flaherty, S. P. & Breed, W. G. (1987). Formation of the ventral hooks on the sperm head of the plains mouse, *Pseudomys australis*. *Gamete Res.* **17,** 115-129.

Flannery, T. F. (1995). *Mammals of New Guinea,* 2nd edn. New South Wales: Reed Books.

Franzén, Å., (1970). Phylogenetic aspects of the morphology of spermatozoa and spermiogenesis. In: *Comparative Spermatology*: 29-45. B. Baccetti (Ed.). New York: Academic Press.

Freckleton, R. P., Harvey, P. H. & Pagel, M. (2002). Phylogenetic analysis and comparative data: A test and review of evidence. *Am. Nat.* **160,** 712-726.

García-González, F. & Simmons, L. W. (2007). Shorter sperm confer higher competitive fertilization success. *Evolution.* **61,** 816-824.

Gomendio, M. & Roldan, E. R. S. (1991). Sperm competition influences sperm size in mammals. *Proc. R. Soc. Lond. B. Biol. Sci.* **243,** 181-185.

Gomendio, M. & Roldan, E. R. S. (2008). Implications of diversity in sperm size and function for sperm competition and fertility. *Int. J. Dev. Biol.* **52,** 439-447.

Gomendio, M., Tourmente, M. & Roldan, E. R. S. (2011). Why mammalian lineages respond differently to sexual selection: metabolic rate constrains the evolution of sperm size. *Proc. R. Soc. Lond. B. Biol. Sci.* **278,** 3135-3141.

Gómez Montoto, L., Sánchez, M. V. , Tourmente, M., Martin-Coello, J., Luque-Larena, J. J. , Gomendio, M, Roldan, E. R. S. (2011). Sperm competition differentially affects swimming

velocity and size of spermatozoa from closely related muroid rodents: head first. *Reproduction*, **142**, 819-830.

Higdon, J. J. L. (1979). A hydrodynamic analysis of flagellar propulsion. *J. Fluid. Mech.* **90,** 685-671.

Humphries, S., Evans, J. P. & Simmons, L. W. (2008). Sperm competition: linking form to function. *BMC. Evol. Biol.* **8,** DOI: 10.1186/1471-2148-8-319.

Immler, S., Moore, H. D. M., Breed, W. G. & Birkhead, T. R. (2007). By hook or by crook? Morphometry, competition and cooperation in rodent sperm. *PLoS. ONE.* **2,** DOI: 10.1371/journal.pone.0000170.

Jamieson, B. G. M. (1987). *The Ultrastructure and Phylogeny of Insect Spermatozoa*. Cambridge: Cambridge University Press.

Katz, D. F., Drobnis, E. Z. & Overstreet, J. W. (1989). Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. *Gamete Res.* **22,** 443-469.

Kleven, O., Fossøy, F., Laskemoen, T., Robertson, R. J., Rudolfsen, G. & Lifjeld, J. T. (2009). Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution.* **63,** 2466-2473.

LaMunyon, C. W. & Ward, S. (1998). Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. *Proc. R. Soc. Lond. B. Biol. Sci.* **265,** 1997-2002.

LaMunyon, C. W. & Ward, S. (2002). Evolution of larger sperm in response to experimentally increased sperm competition in *Caenorhabditis elegans*. *Proc. R. Soc. Lond. B. Biol. Sci.* **269,** 1125-1128.

Lecompte, E., Aplin, K., Denys, C., Catzeflis, F., Chades, M. & Chevret, P. (2008). Phylogeny and biogeography of African Murinae based on mitochondrial and nuclear gene sequences, with a new tribal classification of the subfamily. *BMC Evol Biol*. **8,** 199.

Lifjeld, J. T., Hoenen, A., Johannessen, L. E., Laskemoen, T., Lopes, R. J., Rodrigues, P., Rowe, M. (2013). The Azores bullfinch (*Pyrrhula murina*) has the same unusual and size-variable sperm morphology as the Eurasian bullfinch (*Pyrrhula pyrrhula*). *Biol. J. Linn. Soc.* **108**, 677-687.

Lüpold, S., Calhim, S., Immler, S. & Birkhead, T. R. (2009). Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. Lond. B. Biol. Sci.* **276,** 1175-1181.

Lüpold, S., Manier, M. K., Berben, K. S., Smith, K. J., Daley, B. D., Buckley, S. H., Belote, J. M. & Pitnick, S. (2012). How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. *Curr. Biol.* **22,** 1667-1672.

Malo, A. F., Gomendio, M., Garde, J., Lang-Lenton, B., Soler, A. J. & Roldan, E. R. S. (2006). Sperm design and sperm function. *Biol. Lett.* **2,** 246-249.

Miller, G. T. & Pitnick, S. (2002). Sperm-female coevolution in *Drosophila*. *Science.* **298,** 1230-1233.

Moore, H., Dvoráková, K., Jenkins, N. & Breed, W. (2002). Exceptional sperm cooperation in the wood mouse. *Nature.* **418,** 174-177.

Musser, G. G. & Carleton, M. D., (2005). Superfamily Muroidea. In: *Mammal Species of the World : A Taxonomic and Geographic Reference*: 894-1531. D. E. Wilson & D. M. Reeder (Eds.). Baltimore: Johns Hopkins University Press.

Nakagawa, S. & Cuthill, I. C. (2007). Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev.* **82,** 591-605.

Oko, R. & Clermont, Y. (1988). Isolation, structure and protein composition of the perforatorium of rat spermatozoa. *Biol. Reprod.* **39,** 673-687.

Oppliger, A., Naciri-Graven, Y., Ribi, G. & Hosken, D. J. (2003). Sperm length influences fertilization success during sperm competition in the snail *Viviparus ater*. *Mol. Ecol.* **12,** 485-492.

Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature.* **401,** 877-884.

Palopoli, M. F., Peden, C., Woo, C., Akiha, K., Ary, M., Cruze, L., Anderson, J. L. & Phillips, P. C. (2015). Natural and experimental evolution of sexual conflict within *Caenorhabditis nematodes*. *BMC. Evol. Biol.* **15,** 93.

Peirce, E. J. and Breed, W. G. (2001). A comparative study of sperm production in two species of Australain arid zone rodents (*Pseudomy australis*, *Notomys alexis*) with marked differnces in testis size. *Reproduction* **121**, 239-247.

Pitnick, S., Hosken, D. J. & Birkhead, T. R., (2009). Sperm morphological diversity. In: *Sperm Biology: An Evolutionary Perspective*: 69-149. T. R. Birkhead, D. J. Hosken & S. Pitnick (Eds.). London: Academic Press.

R Core Team. (2014). *R: A language and environment for statistical computing*, v.3.1.1. Vienna, Austria: R Foundation for Statistical Computing.

Roldan, E. R. S., Gomendio, M. & Vitullo, A. D. (1992). The evolution of eutherian spermatozoa and underlying selective forces: female selection and sperm competition. *Biol. Rev.* **67,** 551-593.

Sánchez, M. V., Bastir, M. & Roldan, E. R. S. (2013). Geometric morphometrics of rodent sperm head shape. *PLoS. ONE.* **8,** e80607.

Šandera, M., Albrecht, T. & Stopka, P. (2013). Variation in apical hook length reflects the intensity of sperm competition in murine rodents. *PLoS. ONE.* **8,** e68427.

Simmons, L. W. & Fitzpatrick, J. L. (2012). Sperm wars and the evolution of male fertility. *Reproduction.* **144,** 519-534.

Smith, T. T. & Yanagimachi, R. (1990). The viability of hamster spermatozoa stored in the isthmus of the oviduct: the importance of sperm-epithelium contact for sperm survival. *Biol. Reprod.* **42,** 450-457.

Snook, R. R. (2005). Sperm in competition: not playing by the numbers. *Trends Ecol. Evol.* **20,** 46-53.

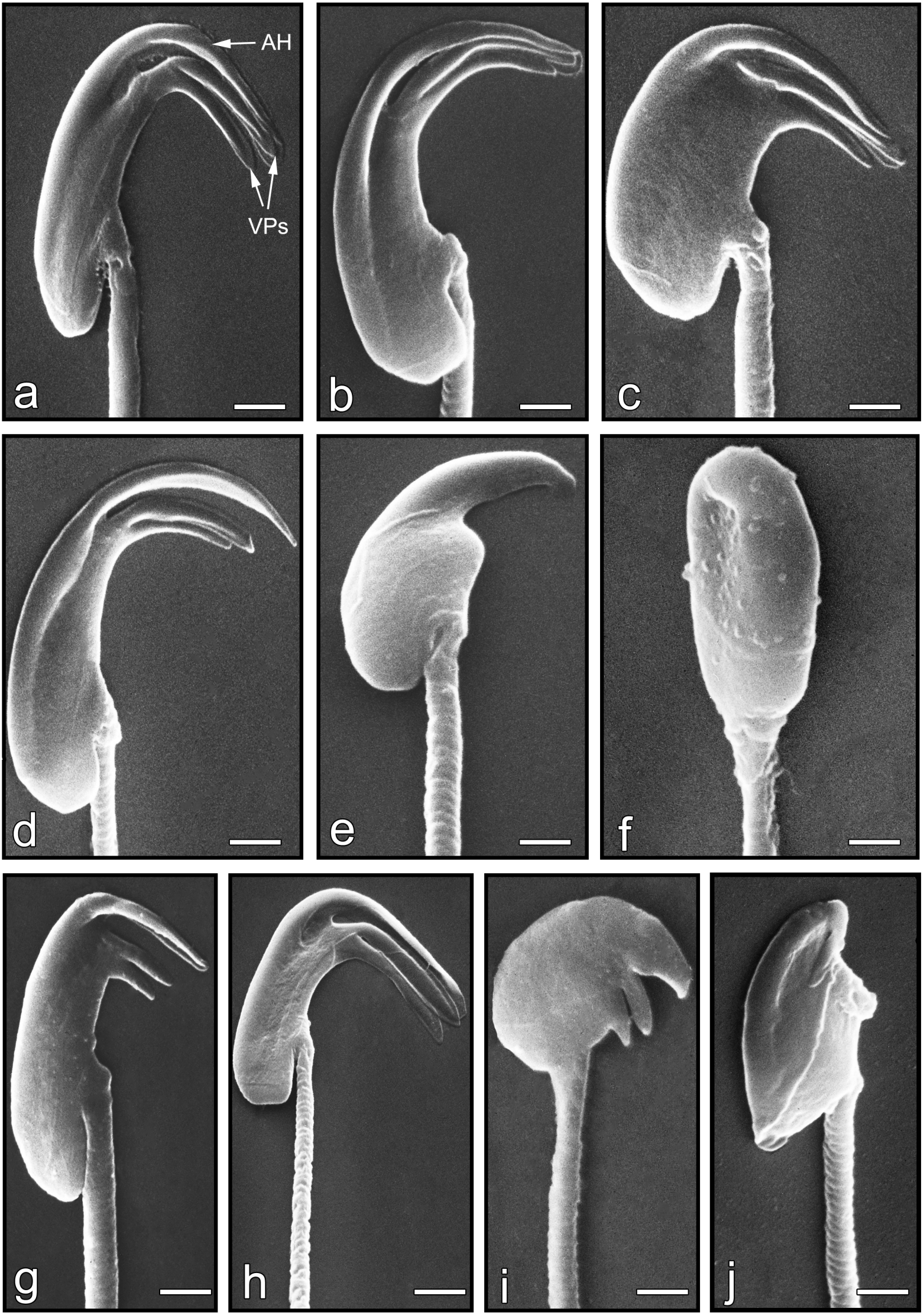
Soulsbury, C. D. (2010). Genetic patterns of paternity and testes size in mammals. *PLoS. ONE.* **5,** e9581.

Suttle, J. M., Moore, H. D. M., Peirce, E. J. & Breed, W. G. (1988). Quantitative studies on the variation in sperm head morphology of the hopping mouse, *Notomys alexis*. *J. Exp. Zool.* **247,** 166-171.

Thitipramote, N., Suwanjarat, J., Leigh, C., and Breed, W. G. (2011). Variation in sperm morphology of a murine rodent from south - east Asia: the Greater Bandicoot rat, *Bandicota indica.* Acta Zool. **92**, 201-205.

429 Tourmente, M., Gomendio, M. & Roldan, E. R. (2011). Sperm competition and the evolution of
430 sperm design in mammals. *BMC. Evol. Biol.* **11,** 12.
431 Van der Horst, G., Maree, L., Kotze, S. H. and O'Raiain, M. J. (2011). Sperm structure and motility in
432 the eusocial mole-rat *Heterocephalus glaber*: A case of degenerative orthogenesis in the
433 absence of sperm competition. BMC Evol. Biol. 11: 351.
434 Van der Horst, G. and Maree, L. (2014). Sperm form and function in absence of sperm competition.
435 *Mol. Reprod. Dev.* **81**, 204-216.
436 Van Dyck, S., Gynther, I. & Baker, A. (2013). *Field Companion to The Mammals of Australia*. London:
437 New Holland Publishers.
438 Yanagimachi, R., (1988). Mammalian Fertilization. In: *The Physiology of Reproduction*: 135-185. E.
439 Knobil & J. D. Neill (Eds.). New York: Raven Press.

440


AH
VPs
a
b
c
d
e
f
g
h
i
j

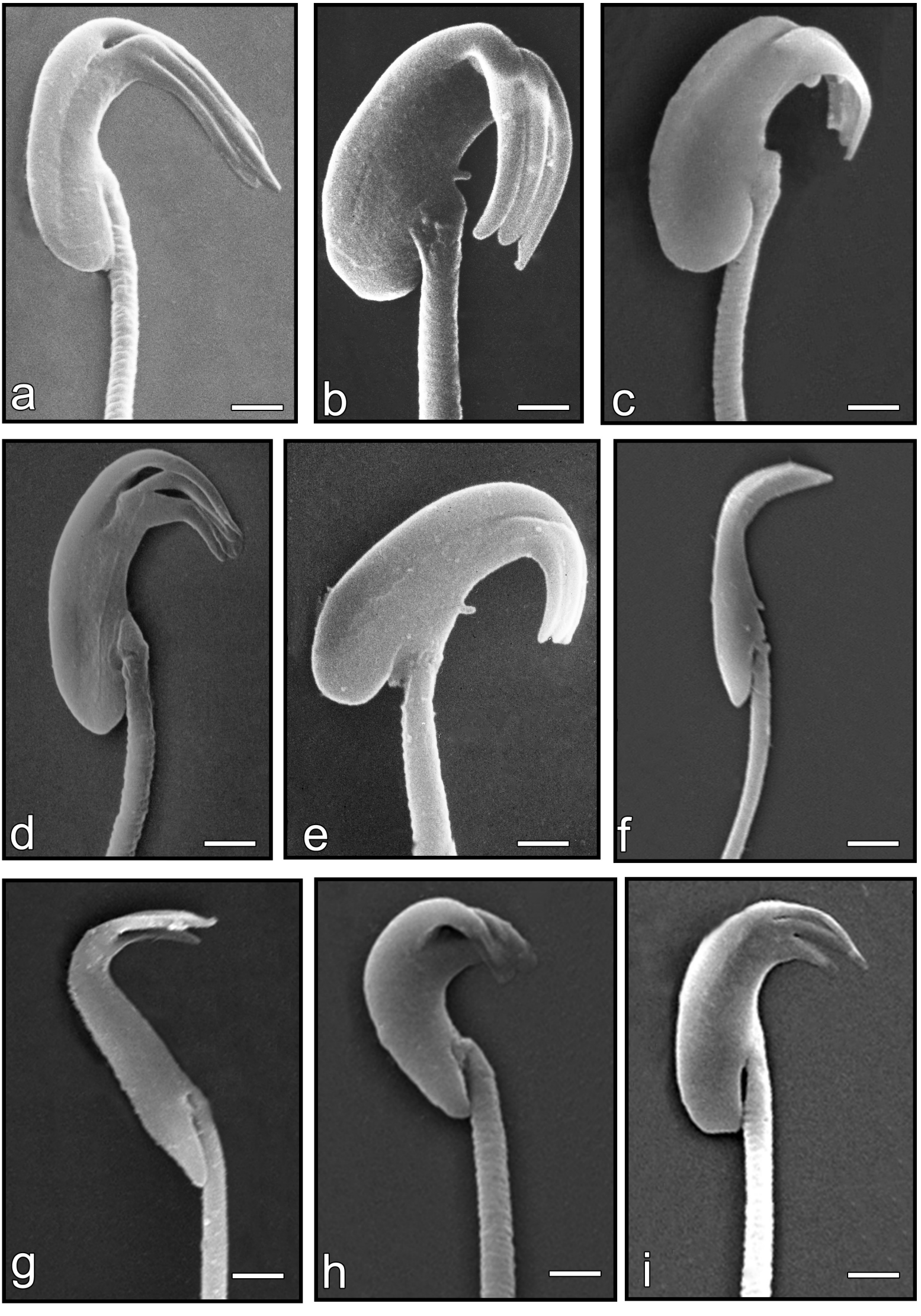

a
b
c
d
e
f
g
h
i

Table 1: Phylogenetically controlled associations between sperm morphological traits and testes mass corrected for body mass; all variables are log-transformed. Effect sizes~~(?)~~ are shown as the partial correlation coefficients *r* along with their lower (LCL) and upper (UCL) 95% confidence limits.

Commented [SL1]: Yes, this is correct. This is a statistical term referring to a statistic (in this case partial r) that expresses the strength (and direction) of an effect (or relationship).

Sperm Trait	Predictors	*df*	partial *r*	(LCL, UCL)	*t*	*P*	λ[a]
Head Length: Width Ratio	Testes Mass	42	0.41	(0.13, 0.61)	2.89	**0.006**	$0.76^{<0.001,\ <0.001}$
	Body Mass	42	-0.27	(-0.51, 0.03)	-1.81	0.08	
Apical Hook Length	Testes Mass	41	0.29	(-0.01, 0.52)	1.94	0.06[b]	$0.38^{0.12,\ <0.001}$
	Body Mass	41	-0.08	(-0.36, 0.22)	-0.51	0.61	
Apical Hook Angle	Testes Mass	41	0.72	(0.55, 0.82)	6.68	**<0.001**	$<0.001^{1.0,\ <0.001}$
	Body Mass	41	-0.50	(-0.67, -0.23)	-3.66	**<0.001**	
Ventral Process Length	Testes Mass	33	0.36	(0.03, 0.59)	2.22	**0.03**[b]	$0.24^{0.31,\ <0.001}$
	Body Mass	33	-0.16	(-0.45, 0.18)	-0.93	0.36	
Ventral Process Angle	Testes Mass	33	0.63	(0.39, 0.77)	4.72	**<0.001**	$<0.001^{1.0,\ <0.001}$
	Body Mass	33	-0.32	(-0.57, 0.01)	-1.95	0.06	
Midpiece Length	Testes Mass	42	0.08	(0.22, 0.36)	1.84	0.61	$1.00^{<0.001,\ 1.0}$
	Body Mass	42	-0.40	(-0.47, 0.08)	-1.46	0.15	
Principal and End Piece Length (PEL)	Testes Mass	42	0.34	(0.05, 0.56)	2.35	**0.02**	$0.82^{0.001,\ 0.05}$
	Body Mass	42	-0.22	(-0.47, 0.08)	-1.49	0.14	
Total Flagellum Length (TFL)	Testes Mass	42	0.34	(0.05, 0.56)	2.33	**0.02**	$0.91^{<0.001,\ 0.22}$
	Body Mass	42	-0.27	(-0.51, 0.03)	-1.81	0.08	
PEL:TFL Ratio	Testes Mass	42	0.23	(-0.07, 0.47)	1.52	0.14	$0.94^{<0.001,\ 0.39}$
	Body Mass	42	-0.04	(-0.32, 0.26)	-0.24	0.81	
Midpiece:TFL Ratio	Testes Mass	42	-0.24	(-0.49, 0.06)	-1.62	0.11	$0.90^{<0.001,\ 0.22}$
	Body Mass	42	-0.04	(-0.25, 0.33)	-0.29	0.77	
Flagellum:Head Ratio	Testes Mass	42	0.24	(-0.06, 0.49)	1.63	0.11	$<0.001^{1.0,\ 0.05}$
	Body Mass	42	-0.30	(-0.53, 0.00)	-2.04	**0.05**	

[a] Superscripts following the phylogenetic scaling parameter λ ~~estimates~~ denote significance levels of likelihood ratio tests (first superscript: against λ = 0; second superscript: against λ = 1).

[b] These positive trends are not statistically significant after removal of a single influential data point (*Notomys alexis*; both partial $r \leq 0.18$, $P \geq 0.25$).

Statistically significant *P*-values (at α = 0.05) are highlighted in bold.