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Unraveling the Sperm Bauplan: Relations Between Sperm Head Morphology and Sperm Function in Rodents[1]

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Summary sentence: Sperm competition drives changes in sperm head phenotype, with modifications in shape, curvature and length of the hook, which in turn influences sperm swimming velocity.
Key words: sperm design, sperm head morphology, hook angle, hook dimensions, sperm velocity, rodents

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ABSTRACT

Rodents have spermatozoa with features not seen in other species. Sperm heads in many rodent species bear one or more apical extensions, known as "hook(s)". The process by which hooks have evolved, together with their adaptive significance, are still controversial issues. In order to improve our understanding of the biological meaning of these sperm head adaptations, we analyzed hook curvature angles, hook length and overall hook shape in muroid rodents by using geometric morphometrics. We also searched for relationships between hook design and measures of inter-male competition to assess if postcopulatory sexual selection is an important selective force driving changes in this sperm structure. Finally, we sought possible links between aspects of sperm hook design and sperm velocity as a measure of sperm performance. Results showed that one hook curvature angle is under strong selective pressure. Similarly, hook length appears to be strongly selected by sexual selection with this selective force also exhibiting a stabilizing role reducing inter-male variation in this trait. The adaptive significance of changes in hook structure was underlied by the finding that there is a strong and significant covariation between hook dimensions and shape and between hook design and sperm swimming velocity. Overall, this study strongly suggests that postcopulatory sexual selection has an important effect on the design of the sperm head that, in turn, is important for enhancing sperm velocity, a function crucial to reaching the vicinity of the female gamete, and winning fertilizations under competitive situations.

INTRODUCTION

In eutherian mammals most species have spermatozoa with heads exhibiting an oval or paddle-like morphology. An important departure from this basic head design is found in rodents

[1, 2]. This group exhibits a wide range of sperm head morphologies, from simple paddle-shaped cells, similar to those of other eutherians, to sperm heads with a falciform shape and with different types of appendages such as single or multiple apical hooks or basal extensions [3-5]. The presence of an apical rostral hook, which contains part of the acrosomal granule and may or may not contain an extension of the cell nucleus, has only been described in myomorph rodents. Although various roles for the hook have been proposed, its function remains unclear. It has been hypothesized that hooks facilitate sperm forward progression by attaching to the walls of the female reproductive tract, particularly those of the oviduct [6, 7], that they are important for sperm adherence to the oviductal epithelium in the lower isthmus and thus promote survival and facilitate physiological changes required for fertilization (i.e., capacitation) [8], or that they could be involved in penetration of ovum vestments [9].

Changes in sperm phenotype may be driven by various selective forces. Current evidence suggests that postcopulatory sexual selection could be an important selective force behind differences in sperm form and function that, ultimately, are important for sperm fertility. In polyandrous species, sperm competition is a form of postcopulatory sexual selection that occurs when spermatozoa from one male compete with those of other males to arrive first at the site of fertilization and interact with female gametes [10-12]. Sperm competition is able to influence several reproductive traits at the physiological, cellular and molecular levels leading to improvements in male fertility [13, 14]. Thus, in a competitive context, traits which play a role in fertilization success are enhanced, namely testes mass relative to body mass [15], sperm numbers, sperm quality (i.e., normal sperm morphology, proportion of spermatozoa with acrosome integrity and proportion of motile sperm) [16, 14, 17], sperm velocity [18, 19], energy production [20] and the proportion of saturated fatty-acids (the most resistant to lipid peroxidation) in the sperm membrane in relation to the proportion of polyunsaturated fatty acids [21].

Sperm competition can also promote changes in sperm morphology. For example, in eutherian mammals, dimensions of different sperm components, and head elongation, tend to increase with sperm competition [22]. In marsupials, sperm competition favors an increase in flagellum dimensions and total sperm length [23]. On the other hand, relaxation of sperm competition appears to result in considerable variation in sperm morphology [24]. Furthermore, comparative studies have shown that increases in sperm competition promote the production of spermatozoa that are less variable in morphology between males [25-28] and within males [29-31]. Sperm competition may play a role in stabilizing sperm morphology because this feature seems to be critical for swimming speed and general sperm performance [32, 33].

From a theoretical point of view, a link between sperm head morphology and velocity is widely accepted. However, empirical evidence is scarce. An intraspecific study in mammals revealed that sperm cells with elongated heads are able to swim faster [32], which received support from subsequent studies using simulations and velocity measurements [34]. In contrast, a study in birds found a negative effect of head size on sperm velocity [33]. On the other hand, theoretical models support that sperm velocity is a balance between thrust and drag, and thus it depends mainly on the relationship between flagellum and head dimensions [35]. In any case, to correctly address this issue it is necessary to consider not only head dimensions but also sperm head design (i.e., its shape), particularly in species with complex heads morphs such as rodents.

It has been proposed that the apical hook is also a consequence of changes in the sperm head promoted by sperm competition and that it has an adaptive value because, in some species, spermatozoa may rely on these structures to establish associations to other sperm cells (in so-called "trains") and, thus, enhance swimming velocity [36-38]. This proposal is controversial

because no advantage was found in species with sperm heads bearing hooks but with individual sperm swimming faster than sperm aggregates [39-41]. Furthermore, formation of these sperm trains seems to be an exceptional behavior with only very few species exhibiting this phenomenon [42]. Other attempts to disentangle the biological function of sperm hooks have also yielded contradictory results. A positive association between hook length, hook curvature and sperm competition risk was reported for some species of murine rodents [37] leading to the suggestion that changes in the sperm head hook are adaptive responses to sperm competition. Another study in three species of murine rodents confirmed these results and also established a relationship between hook curvature and sperm lifespan [43]. On the other hand, no evidence of changes in hook shape was found in house mouse selection lines bred under different conditions of sperm competition [39, 40].

In this study we aimed to explore possible relationships between sperm head evolution, head design and sperm function focusing on the characterization of sperm head hooks by using a geometrics morphometrics tool-kit. To this end, we examined a range of murid, cricetid and arvicolid rodents in an attempt to encompass a greater variance in hook designs than what has been achieved in previous studies. We hypothesized that hook curvature and length covary with sperm competition levels and, furthermore, that differences in hook phenotype have an impact on sperm performance (i.e., sperm swimming velocity).

MATERIAL AND METHODS

Ethics Statement

This study was approved by the Ethics Committee of the Spanish Research Council (CSIC). All animal handling was done following Spanish Animal Protection Regulation RD53/2013, which conforms to European Union Regulation 2010/63.

Sperm Collection

We analyzed spermatozoa from 22 muroid rodents. Males from *Apodemus sylvaticus, Arvicola sapidus, Arvicola terrestris Microtus arvalis, Chionomys nivalis, and Myodes glareolus* were trapped in the field, with all required permissions, during the breeding season (April-June). *Lemniscomys barbarus, Mus pahari, Mus famulus, Mus macedonicus, Mus spicilegus, Mus domesticus, Mus bactrianus, Mus castaneus, Mus musculus, Mus spretus, Mus caroli, Mus cookii* came from wild-derived populations kept in captivity for 10-30 generations and maintained as outbred colonies. *Mesocricetus auratus, Phodopus campbelli, Phodopus roborovskii,* and *Phodopus sungorus* were obtained from commercial suppliers. Animals were kept in our animal facilities, in individual cages, at 23°C, with a 14 h light/10 h darkness photoperiod. Food and water were supplied *ad libitum.* A range of 4 - 5 individuals per species was sampled.

Animals were killed by cervical dislocation and weighed. Testes were dissected out and weighed immediately. Both caudae epididymides were placed in 1-3 ml of modified Tyrode medium with Hepes buffer [44] prewarmed to 37°C. Incisions were made in the epididymis to allow sperm cells to swim into the medium. Sperm suspensions were smeared onto slides, fixed with glutaraldehyde 2.5% in a phosphate buffer and stained with Giemsa [45]. Sperm cells were photographed at 1000x magnification under bright field with a digital camera (Digital Sight DS-5M, Nikon, Tokyo, Japan) attached to microscope with Pan-Fluor optics (Eclipse E-600, Nikon) and software for capture of microscopy images (NIS-Elements F v.2.20, Nikon).

Sperm Hook Analyses Using Geometric Morphometrics

Images from 25 sperm cells per male were digitized for geometric morphometrics analyses as described previously (Fig. 1A) [46] using TPS dig 2 v.2.16 (James Rohlf, Department of Ecology and Evolution, Stony Brook University, New York, USA) to obtain landmark coordinates.

Four different hook curvature angles were measured (Fig. 1B-D). Alpha 1 was defined by landmarks 9, 21 and 20, and corresponds to the inner angle on the basal side of the hook. Alpha 2 was defined by landmarks 3, 8 and 13. This angle is similar (but not identical) to that measured in two previous studies [37, 43]. The main axis of the head was used both in the present and previous studies to define one side of the angle, but the other side of the angle, which was defined previously as the tangent laid through the most apical tip of the "ventral curve" (= basal curve) [37, 43] was defined in the present study by the line passing through the tip of the hook (landmark 13) which is a clear landmark in geometric morphometrics analyses. Alpha 3 was defined by landmarks 3, 8 and 17 and corresponds to the angle between the sperm head main axis and the apical aspect of the hook; semilandmark 17 lies halfway between the tip of the hook and the apical tip of the sperm head. Alpha 4 is the outer angle of the hook curvature between landmarks 14, 8 and 17. Hook length was measured as the straight distance between landmarks 8 (apical tip of the head) and 13 (tip of the hook) (Fig. 1A). Hook curvature angles and hook length were measured using the software Morpheus et al. (Dennis Slice, Wake Forest University, Winston-Salem, North Carolina, USA). The intra-male coefficient of variation (CV) of hook curvature and length was calculated as follows: CV = (standard deviation * 100)/mean. Hook shape was analyzed from the information captured by the landmark configurations using geometric morphometric tools as described in a previous study [46].

Velocity Data

Data on sperm velocity were collected as described in earlier studies [19, 20]. Briefly, an aliquot of the original sperm suspension was placed in a 37ºC pre-warmed microscopy chamber of 20 μm of depth (Leja, Nieuw-Vennep, The Netherlands). Sperm cells were filmed under phase contrast (4x objective with pseudo-negative phase) using a video camera (Basler AG, Ahrensburg, Germany) connected to a microscope (Eclipse 50i, Nikon). Three sperm velocity parameters were assessed by averaging the individual values for 5 males of the same species for each parameter: curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s) and average path velocity (VAP, μm/s), employing a computer-aided sperm analyser (Sperm Class Analyzer, Microptic SL, Barcelona, Spain). The software was set with maximum pixel size 250 μm, minimum pixel size 3 μm, connectivity 20, contrast 600, and brightness 60. A minimum of 200 sperm trajectories were recorded in at least 6 random fields per sample. Each field was recorded for 1 second at 25 frames per second. The VAP values were determined using 15 points per trajectory. All the video captures were compared to their overlaying analyzed tracks and rectified if required [20]. No data of sperm velocity were available for *Arvicola sapidus*, *Arvicola terrestris*, *Mus famulus*, *Mus bactrianus* and *Mus cookii*.

Because velocity measures are highly correlated, we sought to obtain a new variable to integrate all this information, as done in previous studies [20]. A principal component analysis (PCA) was conducted using the mean species values for the three velocity parameters: VCL, VSL and VAP. The PCA extracted two principal components that summarized multivariate velocity variation across all species. The new variables obtained from the PCA were called overall sperm velocity (OSV). The first principal component (OSV-PC1) explained 75% of the total variance,

while the second principal component (OSV-PC2) explained 24% of the total variance. The species values for each of the three sperm velocity parameters (VCL, VSL, and VAP) were significantly correlated with PC1; VCL was the only parameter significantly correlated with PC2 (not shown).

Statistical and Phylogenetic Analyses

The increase of testes mass in relation to body mass is a widespread evolutionary response to sperm competition across taxa [17, 47]. Moreover, the increment of testes mass relative to body mass has been reported as a reliable indicator of investment in sperm production [48] and is also positively correlated with multiple genetic paternity [49]. Hence, in this study we used testes mass relative to body mass (from here onwards referred to as relative testis mass) as an indicator of the level of sperm competition across species (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org).

To ensure that the differences observed are the product of independent selective evolution rather than to phylogenetic association, relationships between hook features and relative testes mass were examined using multiple regression analyses (generalized least squares) in a phylogenetic framework (PGLS). This approach takes into account the phylogenetic structure of the sample and quantifies the phylogenetic signal estimating a parameter lambda (λ) from tree branch lengths, once data have been fitted to an evolutionary Brownian motion model. When λ values are close to 1 a strong phylogenetic association exists between variables. In contrast, when λ values are close to 0 suggests that variables have evolved independently from phylogeny. Angles alpha1, alpha 2, alpha 3, alpha 4, hook length, and coefficients of variation (CVs) of hook curvature and length were taken as dependent variables, while body mass and testes mass were used as predictor variables. The phylogenetic tree was reconstructed using data gathered from published sources [50] (Supplemental Fig. S1).

Partial Least Squares analysis (PLS) [51] was used to assess the strength of covariation between hook shape and hook length. This ordination method consists in a singular value decomposition of the matrix of covariances in two sets of variables. As a result, we obtained pairs of vectors that maximize the covariance between both sets of variables and are also mutually uncorrelated across sets.

Statistical analyses were conducted with Caper v.0.5 [52] (R Foundation for Statistical Computing) and MorphoJ v.1.06d [53]. When required, data were log-transformed to attain normal distributions.

RESULTS

Angles of Hook Curvature

Four different hook curvature angles were measured (Fig. 1). Mean values for hook curvature angles, and standard errors of the mean (± SEM), taking into account all the species examined, were as follows: alpha 1, 87.75° (± 6.82); alpha 2, 60.45° (± 4.79); alpha 3, 83.79° (± 3.00) and alpha 4, 138.95° (± 3.57). Angles showed considerable differences between species: alpha 1 ranged from 32° to 125.84°; alpha 2 from 21.66° to 87.06°; alpha 3 from 60.35° to 103.74° and alpha 4 from 102.95° to 158.38°. Mean values of the different angles for each species are summarized in Supplemental Table S2.

Of the four different angles of hook curvature, alpha 3 (landmarks 3, 8 and 17) (Fig. 1D) had a significant and negative relationship with relative testes mass ($P = 0.02$), (Table 1, Fig. 2), indicating that higher inferred levels of sperm competition associate with a higher hook

curvature. A marginally significant and negative relationship was found between alpha 4 and relative testes mass ($P = 0.06$) (Table 1). No significant relationships were found between relative testes mass and the angles alpha 1 and alpha 2 (Table 1). Regarding the coefficient of variation of the different hook angles (Supplemental Table S3), none of the CVs showed a significant correlation with relative testis mass (results not shown).

Hook Length

Hook length was quantified as the straight distance between the apical tip of the head and the tip of the hook (landmarks 8 and 13, respectively) (Fig. 1A). The mean value for hook length was 3.77 μm (± 0.347) when all species were taken into account. Differences in hook length between species were substantial, ranging from 2.5 μm to 7.83 μm. Average values of hook length for each species are given in Supplemental Table S1. Multiple regression analysis revealed that hook length was positively related to relative testis mass ($P = 0.033$) (Table 2, Fig. 3A) indicating that higher inferred levels of sperm competition associate with longer hooks. On the other hand, the coefficient of variation of hook length was negatively related to relative testis mass ($P = 0.0002$) (Table 2, Fig. 3B) suggesting that increases in inferred levels of sperm competition are accompanied by a decrease in variation in sperm hook length. We also found a significant negative correlation between hook angle alpha 3 and hook length ($P = 0.04$), (Table 3, Fig. 4) thus showing that the longer the hook, the higher the hook curvature.

Relationships Between Hook Shape and Hook Length

Hook shape was analyzed using geometric morphometric tools (Fig. 1). Partial least squares analysis revealed the existence of a covariation pattern between hook shape and hook length. The RV coefficient, which measures the strength of the association between variables, was 0.474 ($P = 0.0005$). The correlation between PLS 1 axes yielded a value of 0.788 ($P = 0.001$). Variations in shape, as associated to differences in hook length, showed that in sperm cells with relatively longer hooks, these are wider, prominent and more curved. In contrast, in spermatozoa with relatively shorter hooks, such hooks have a more sharpened shape (Fig. 5).

Relationships Between Hook Curvature and Hook Length with Sperm Swimming Velocity

The regression analysis revealed a clear trend for a negative relationship between angle alpha 3 and sperm curvilinear velocity (VCL) ($P = 0.07$), (Table 3, Fig. 6A), indicating that a higher hook curvature associates with a higher sperm curvilinear velocity. A positive relationship was found between hook length and VCL ($P < 0.0001$) (Table 3, Fig. 6B), which means that sperm with longer hooks achieve higher curvilinear velocity and hence swim faster. Regression analysis showed a significant negative association between angle alpha 3 and factor 1 of overall sperm velocity calculated in a principal component analysis (OSV-PC1; see Materials and Methods) ($P = 0.005$) (Table 3), while factor 2 of overall sperm velocity (OSV-PC2) was positively related to hook length ($P < 0.0001$) (Table 3).

DISCUSSION

Our results show evidence of a strong relationship of postcopulatory sexual selection with hook curvature and hook length. Furthermore, the design of the hook was clearly associated with sperm performance because both hook curvature and hook length were correlated with sperm swimming velocity. These results thus indicate that the design of the sperm head is under the

influence of selective forces, which may have a major impact on the chances males have in their ability to fertilize under competitive contexts.

The hook is a characteristic structure of the sperm cells of muroid rodents [54], the origin and biological meaning of which largely remains a matter of debate. Two theories have been proposed in order to provide an explanation for the evolution of the hook in muroid rodents. On the one hand, it is possible that sperm cells of extant rodents have evolved from an ancestral sperm head form that was oval and had no appendages. In Myomorpha, modifications of this simple sperm head form, and the appearance of hooks, could have taken place repeatedly in various lineages, leading to a different sperm head design in each lineage [1]. On the other hand, a falciform head with a hook could have been the ancestral sperm form of Myomorpha, before different lineages separated. Thus, the simple, oval sperm heads observed in some species today may have evolved independently in different lineages [2]. Recent studies suggest that elongation of the sperm head and the appearance of a hook could have taken place more recently, within muroid rodents, in the ancestral form of Eumuroida, leading to the presence of the hook only in the typical muroids [55].

Regarding its function, one possibility is that the hook might serve to adhere the sperm head to the epithelium of the oviduct and, thus, ensure sperm survival [6, 7]. Alternatively, the hook might have evolved as a response to sperm competition and its main function could be the formation of sperm associations (the so-called trains) that, in certain species, swim faster than individual sperm cells [36-38]. This may have happened in some exceptional cases in which the development of sperm associations may have taken advantage of the presence of the hook to further enhance sperm performance [42].

Variation in hook curvature across species has been examined in muroid rodents [37, 43] but, in both studies, only one angle was assessed. In the present study we measured several angles of hook curvature to explore possible biological meanings of this structure and its relationship with sperm movement. We anticipated that measurements of various angles that are potentially relevant for sperm kinetics, and assessments of their association with sperm competition, could uncover aspects of sperm design that are important for fertilization in a competitive context. One important feature of our study is that angles were defined by landmarks associated to relevant anatomical structures of the sperm head [46]. Using an approach based on geometric morphometrics thus allowed us to develop a more objective assessment of hook shape and curvature.

The angle alpha 1 is the innermost bend of the hook, that is, the basal angle of the hook. It has been argued that when the hook is projected forward, with respect to the main axis of the head, an area is exposed through which the sperm may be able to adhere and form trains [36]. Therefore, if train formation were a widespread feature in nature, it could be expected that this angle should be associated with postcopulatory sexual selection. Contrary to this assumption, our results show that this angle is unrelated to the level of sperm competition.

A similar angle to the one defined in this study as alpha 2 has been analyzed in previous work [37, 43]. In such early work, the outer angle formed between the head main axis and the tangent between the apical end and the hook tip associated positively with relative testes mass and, hence, the level of sperm competition. In the present study, in which a similar angle was defined by anatomical landmarks, no significant relationship was found between the angle and sperm competition level.

Sperm heads of myomorph rodents are asymmetric and this is observed even in species without a hook. Head asymmetry is important for swimming behavior in mouse sperm [56],

stabilizing swimming trajectories when sperm cells swim near surfaces. Asymmetry is generally determined by two features: a lateral insertion of the flagellum in relation to the head main axis and a more rounded morphology in the dorsal side of the head. The acquisition of the hook would confer a greater degree of asymmetry and the sperm cell needs to deal with it in order to optimize swimming behavior. Given these facts, we hypothesized that certain hook angles are key determinants of sperm movement and, thus, be under strong selective pressure. The angle alpha 4 measures whether the hook projects forward in relation to the dorsal side of the head. Through variations in this angle, evolutionary forces could shape morphological rearrangements in the most apical area of the sperm head, making it more rounded or less rounded depending on the hook curvature and, hence, such remodeling could have an effect on sperm performance. However, results gathered in this study did not reveal any association between alpha 4 and sperm competition level.

The angle alpha 3 did show a clear relationship with sperm competition levels. This angle measures the inner curvature between the sperm head main axis (landmarks 3 and 8) and the most rostral point of the hook (landmarks 8 and 17). The head main axis defines the plane of symmetry in the sperm head between the dorsal and ventral areas. Thus, variation in hook curvature in relation to this axis would increase or reduce head asymmetry. Thus alpha 3 could be informative with regards to sperm head symmetry and its potential effect on sperm hydrodynamics. The angle alpha 3 was negatively associated with the level of sperm competition. The negative relationship indicates that, at higher level of sperm competition, the angle alpha 3 is lower and, in consequence, the hook is more retracted. Our results thus suggest that this angle of hook curvature, as defined by anatomical landmarks, is informative with regards to sperm head features selected by sperm competition. Interestingly, and in contrast to our findings, two studies focusing on intraspecific analyses in *Mus domesticus* found no association between the angle of curvature of the hook and the level of sperm competition [39-40]. Although it should be borne in mind that it is difficult to make comparison between intra- and interspecific studies, there are two reasons that could explain differences between results. Firstly, *Mus domesticus* often experiences low levels of sperm competition [57, 58]. Secondly, even though these mouse lines were bred under different levels of sperm competition, the variation in hook curvature may not be large enough to detect changes in this trait.

Results of our study also show that higher levels of sperm competition are associated with longer hooks. These results agree with those obtained in previous studies [37, 43]. We also found that as sperm competition increases, there is a decrease in the variation of hook length. The reduction in the variation of sperm dimensions as a consequence of sperm competition has been observed in birds [25, 59], insects [60] and across mammals [31]. The adaptive value of longer hooks is not known, but the fact that this trait is under strong selective pressure suggests that it could be a key determinant of sperm performance in a competitive arena.

The wide diversity of hook morphologies observed in rodents reveals that sperm cells in myomorphs have high plasticity. Geometric morphometric analyses revealed the existence of a pattern of covariation between hook shape and length. Longer hooks were found to be more prominent and to cover most of the ventral side of the head, while shorter ones showed a more sharpened morphology with a more pronounced forward projection. Thus, hook shapes are strikingly variable between closely related species but a certain hook length is associated with a particular hook design. An explanation for such a pattern could relate to the possibility that some cell phenotypes are more stable than others [61]. If sperm cells attain an optimal morphology through evolution, with an optimal performance, then cell design may be the result of a balance

between exploring shape space, by increasing size and remodeling the shape of certain structures, and at the same time remaining functional and competitive. In promiscuous rodent species this process seems to be driven by sperm competition. However, despite the phenotypic variability observed in hook traits, little is known about the developmental constrains and biomechanical implications underlying the evolution of rodent sperm head appendages such as the apical hook.

Finally, a strong association was observed between hook traits and sperm swimming velocity. The relationship between hook angle with both curvilinear velocity and overall sperm velocity were negative, indicating that an increase in hook curvature is translated into enhanced swimming speed. There was also a relationship between hook length and swimming velocity in which sperm cells with a longer hook were able to swim faster. From a hydrodynamic point of view it seems plausible that sperm cells with a retracted hook might be able to swim faster because sperm of muroid rodents exhibit a particular pattern of movement, which can be described as a hatchet-like motion: when the cell moves forward, it "cuts" the fluid with the rostral portion of the hook (Fig. 7). Electron microscopy images showing the fine structure of the hook [62] revealed that cross sections of muroid sperm hooks have triangular shape with sharpened edges. This design together with the pattern of cell movement resemble the bow of a boat and may behave in analogous functional terms where a long and more retracted hook might confer a higher and more hydrodynamically-efficient swimming velocity.

In conclusion, results of this study provide evidence for the influence of sperm competition on sperm head design in muroid rodents. It seems that in this group of species, hook curvature and length are under the influence of postcopulatory sexual selection. In addition, results show that hook length and hook design are strongly interrelated, with hook morphology exhibiting an important impact on sperm swimming velocity.

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FIGURE LEGENDS

FIG. 1. Definition of four hook curvature angles using geometric morphometric landmarks, as examined in this study. **A**) Landmark configuration of the muroid rodent sperm head. **B**) Angle alpha 1. **C**) Angle alpha 2. **D**) Angle alpha 3. **E**) Angle alpha 4. Landmark numbers for each angle are given in each panel.

FIG. 2. Relationship between the hook curvature angle alpha 3 and relative testis mass. Data were plotted representing species grand mean values for alpha 3 and relative testis mass.

FIG. 3. Relationship between relative testes mass and hook length (**A**) and coefficient of variation (CV) of hook length (**B**). Data were plotted representing species grand mean values for relative testis mass, hook length and the coefficient of variation of hook length.

FIG. 4. Relationship between hook length and hook curvature angle alpha 3. Data were plotted representing species grand mean values for hook length and the hook curvature angle alpha 3.

FIG. 5. Shape variation-associated differences in hook length: Short hook (**A**) and long hook (**B**).

Fig. 6. Relationships between curvilinear velocity (VCL) and hook curvature angle alpha 3 (**A**) and hook length (**B**). Data were plotted representing species grand mean values for curvilinear velocity, hook curvature angle alpha 3, and hook length.

FIG. 7. Pattern of sperm head movement showing how the hook may influence sperm progression.

TABLE 1. Phylogenetically-controlled multiple regression analyses of hook curvature angle in relation to body mass and testes mass.[a]

Dependent variable	Predictor	Adjusted R^2	Slope	F	P[b]	Lambda (λ)[c]	Effect size (r)[d]	Effect size (CLs)[b]
Alpha 1	Body mass	-0.08	14.06	0.40	0.52	$0.00^{\dagger,*}$	0.13	(-0.31 to 0.58)
	Testes mass		1.62	0.006	0.94		0.02	(-0.43 to 0.47)
Alpha 2	Body mass	0.10	35.71	3.38	0.08	$1.00^{*,\dagger}$	0.38	(-0.04 to 0.86)
	Testes mass		-9.94	1.07	0.31		0.22	(-0.21 to 0.67)
Alpha 3	Body mass	0.21	25.62	2.29	0.15	$1.00^{*,\dagger}$	0.36	(-0.07 to 0.82)
	Testes mass		-14.76	6.30	**0.02**		0.49	**(0.03 to 0.93)**
Alpha 4	Body mass	0.18	21.57	2.73	0.11	$1.00^{*,\dagger}$	0.34	(-0.08 to 0.82)
	Testes mass		-10.18	3.81	0.06		0.41	(-0.02 to 0.87)

[a]All tests were conducted with 19 degrees of freedom.
[b]*P* values and CLs that indicate statistical significance are shown in bold.
[c]The superscripts following λ value indicate significance levels (†non-significant; $^{*}P<0.05$) in a likelihood ratio tests against models with λ=0 (first position) and λ=1 (second position).
[d]The effect size *r* was calculated from the *F* values; its non-central 95% confidence limits (CLs) are also given. Confidence intervals excluding 0 indicate statistically significant relationships.

TABLE 2. Phylogenetically-controlled multiple regression analyses of hook length and its intra-male coefficient of variation in relation to body mass and testes mass.[a]

Dependent variable	Predictor	Adjusted R^2	Slope	F	P[b]	Lambda (λ)[c]	Effect size (r)[d]	Effect size CLs[b]
Log hook length	Body mass	0.11	-0.08	0.004	0.95	$1.00^{*,\dagger}$	0.01	(-0.44 to 0.45)
	Testes mass		0.12	5.28	**0.033**		0.47	**(0.05 to 0.94)**
CV hook length	Body mass	0.44	4.67	0.003	0.95	$0.85^{\dagger,\dagger}$	0.01	(-0.44 to 0.45)
	Testes mass		-5.97	20.15	**0.0002**		0.72	**(0.44 to 1.34)**

[a]All tests were conducted with 19 degrees of freedom.
[b]*P* values and CLs that indicate statistical significance are shown in bold.
[c]The superscripts following λ value indicate significance levels ([†]non-significant; ***P*<0.05) in a likelihood ratio tests against models with λ=0 (first position) and λ=1 (second position).
[d]The effect size *r* was calculated from the *F* values; its non-central 95% confidence limits (CLs) are also given. Confidence intervals excluding 0 indicate statistically significant relationships.

TABLE 3. Phylogenetically-controlled regression analyses of hook length, hook curvature, and sperm swimming velocity parameters.[a]

Dependent variable	Predictor	Adjusted R^2	Slope	F	P[b]	Lambda (λ)[c]	Effect size (r)[d]	Effect size (CLs)[b]
Hook length	Alpha 3	0.14	-0.03	4.42	**0.04**	1.00*,†	0.42	**(0.004 – 0.90)**
VCL	Alpha 3	0.13	-0.001	3.55	0.07	1.00*,†	0.38	(-0.04 – 0.85)
VCL	Hook length	0.57	0.33	22.30	**<0.0001**	0.00†,†	0.72	**(0.47 – 1.37)**
OSV-PC1	Alpha 3	0.36	-0.06	10.25	**0.005**	0.00†,*	0.63	**(0.23 – 1.27)**
OSV-PC2	Hook length	0.68	4.58	35.36	**<0.0001**	0.34†,†	0.83	**(0.68 – 1.73)**

[a]Species number is n = 22 for the analysis between hook curvature and hook length, n=17 for the regression between sperm swimming velocity parameters and hook curvature and length.
[b]*P* values and CLs that indicate statistical significance are shown in bold.
[c]The superscripts following λ value indicate significance levels (†non-significant; $^{*}P < 0.05$) in a likelihood ratio tests against models with λ=0 (first position) and λ=1 (second position).
[d]The effect size *r* was calculated from the *F* values; its non-central 95% confidence limits (CLs) are also given. Confidence intervals excluding 0 indicate statistically significant relationships.

Figure 1

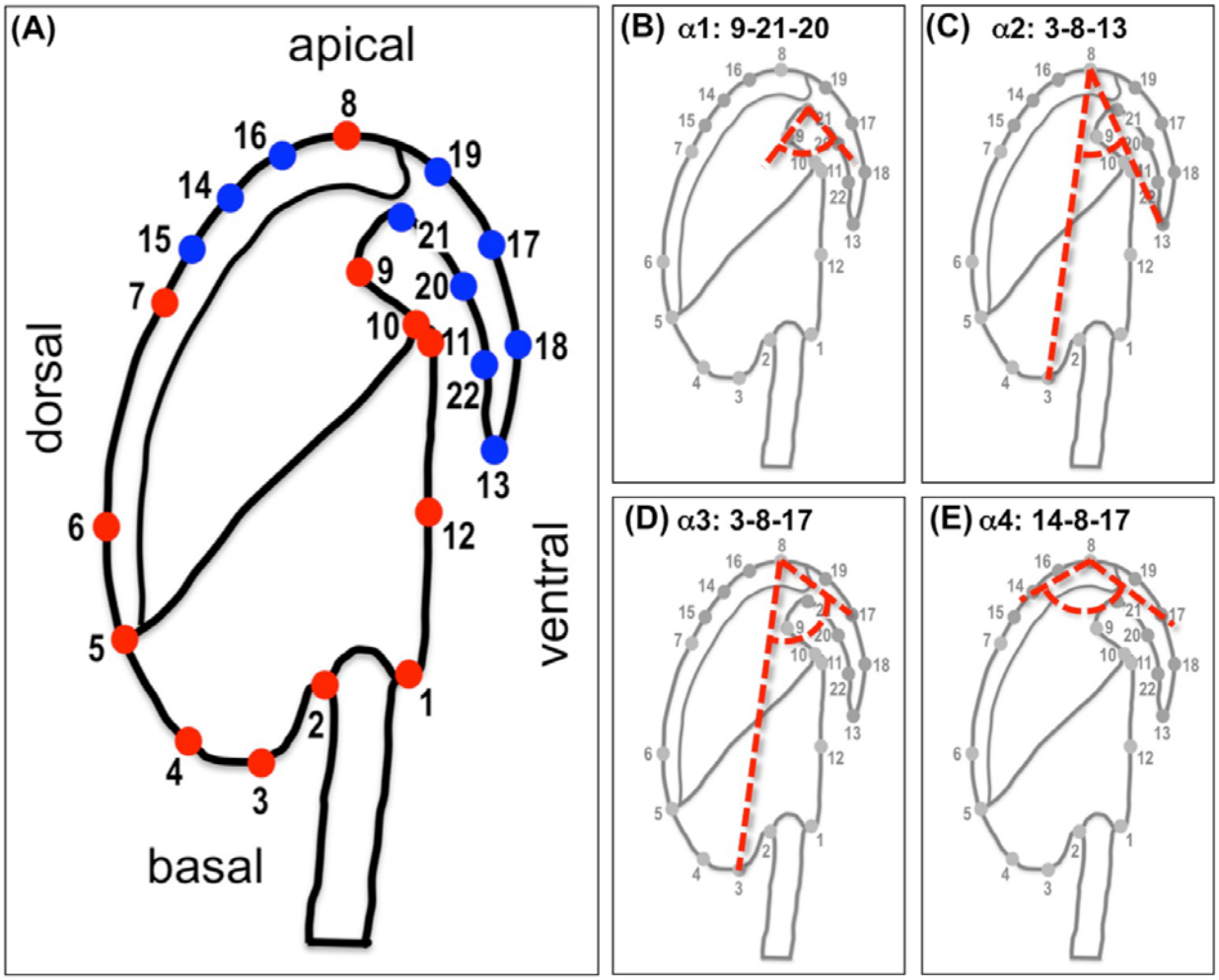

(A)
apical
dorsal
ventral
basal
8
16
19
14
15
21
17
7
9
20
10
11
18
22
13
6
12
5
1
2
4
3
(B) α1: 9-21-20
(C) α2: 3-8-13
(D) α3: 3-8-17
(E) α4: 14-8-17

Figure 2

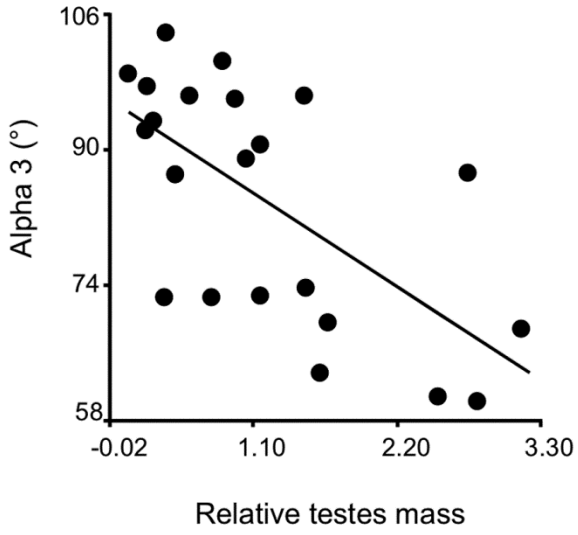


Figure 3

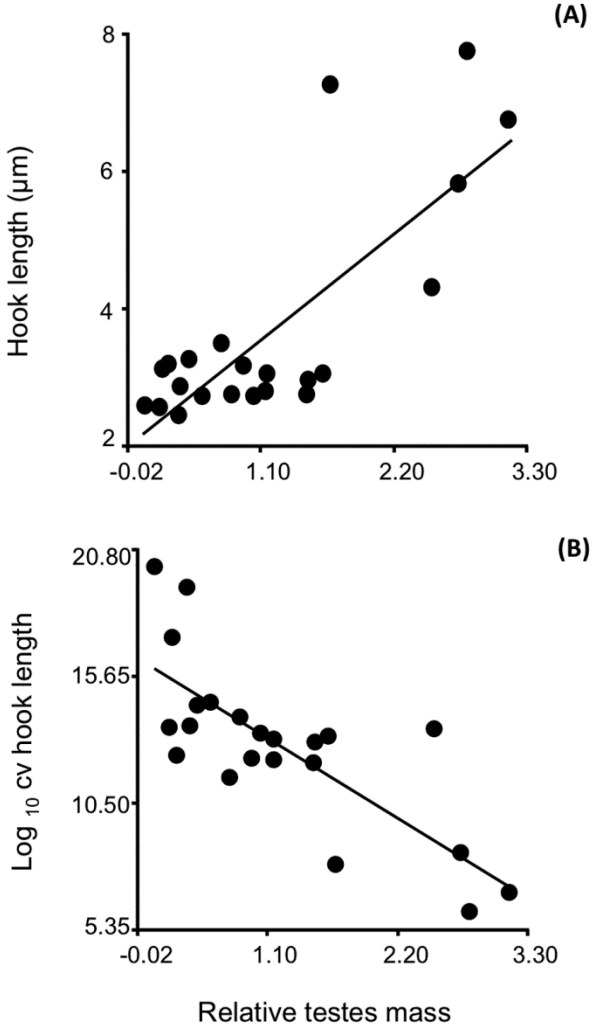

(A)
Hook length (µm)
8
6
4
2
-0.02
1.10
2.20
3.30
(B)
Log 10 cv hook length
20.80
15.65
10.50
5.35
-0.02
1.10
2.20
3.30
Relative testes mass

Figure 4

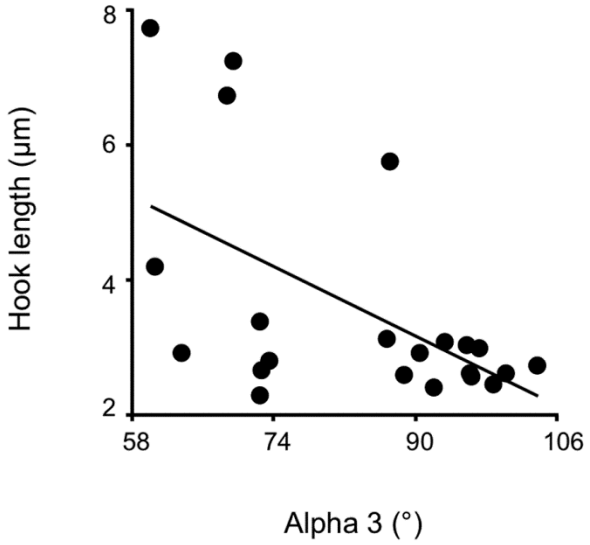


Figure 5

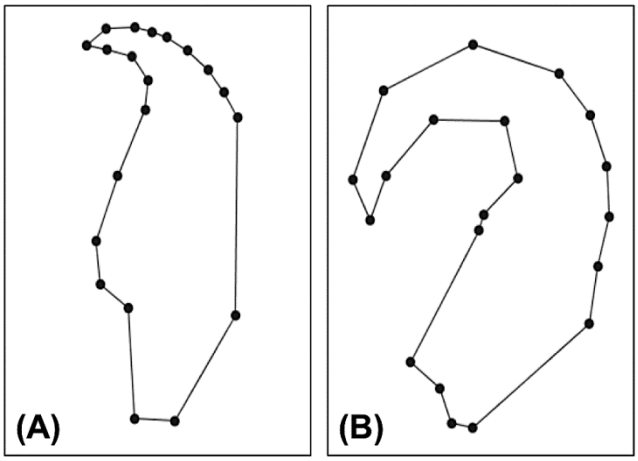


Figure 6

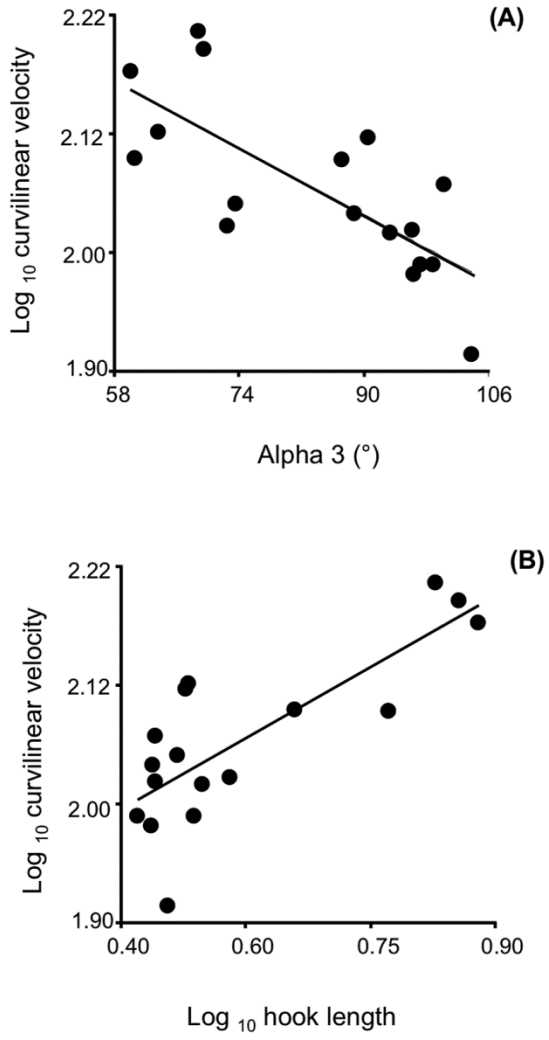
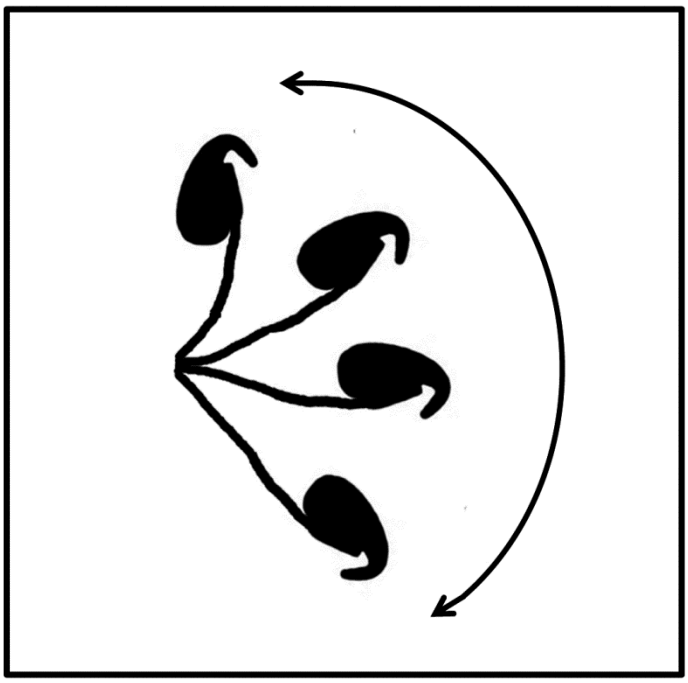

(A)
2.22
2.12
2.00
1.90
Log 10 curvilinear velocity
58
74
90
106
Alpha 3 (°)
(B)
2.22
2.12
2.00
1.90
Log 10 curvilinear velocity
0.40
0.60
0.75
0.90
Log 10 hook length

Figure 7



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SUPPLEMENTAL DATA FOR

Unraveling the Sperm Bauplan: Relations Between Sperm Head Morphology and Sperm Function

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SUPPLEMENTAL FIG. S1. Phylogenetic tree used for analyses.

SUPPLEMENTAL TABLE S1. Data for body mass, testes mass and relative testes mass.

SUPPLEMENTAL TABLE S2. Hook angles and hook length.

SUPPLEMENTAL TABLE S3. Intra-male coefficients of variation for angles of hook curvature and for hook length.

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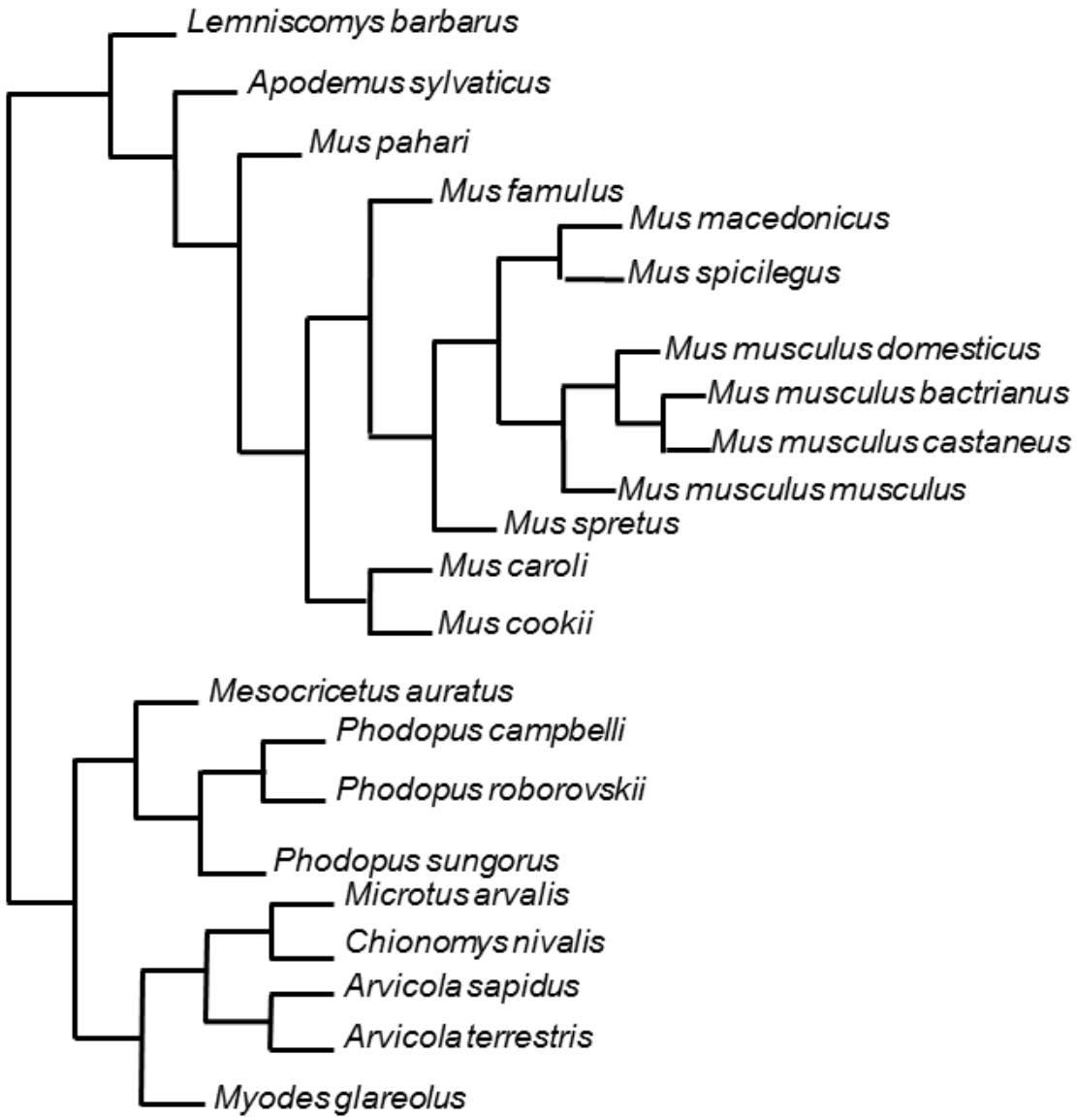

SUPPLEMENTAL FIG. S1 Phylogenetic tree used for analyses.

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SUPPLEMENTAL TABLE S1. Data for body mass, testes mass and relative testes mass.

Species	Body mass (g)	Testes mass (g)	RTM
Apodemus sylvaticus	30.46	1.08	2.52
Arvicola sapidus	217.67	2.23	1.14
Arvicola terrestris	91.56	0.41	0.40
Chionomys nivalis	50.33	1.02	1.61
Lemnicomys barbarus	44.61	0.66	1.15
Mesocricetus auratus	124.99	3.50	2.74
Microtus arvalis	44.26	0.44	0.76
Mus m. bactrianus	18.06	0.17	0.49
Mus caroli	18.07	0.14	0.86
Mus m. castaneus	18.81	0.07	0.13
Mus cookii	23.66	0.30	0.95
Mus m. domesticus	22.05	0.10	0.60
Mus famulus	27.40	0.05	0.25
Mus macedonicus	20.10	0.29	0.32
Mus m. musculus	21.85	0.13	0.42
Mus pahari	33.14	0.12	0.27
Mus spicilegus	18.09	0.42	1.48
Mus spretus	18.16	0.29	1.03
Myodes glareolus	29.26	0.62	1.49
Phodopus campbelli	48.52	1.92	3.16
Phodopus rovorovskii	25.72	1.06	2.82
Phodopus sungorus	46.70	0.99	1.67

Mean values by species for the variables body mass and testes mass are in grams. Relative testis mass (RTM) has been calculated using the Kenagy-Trombulak formula for rodents: testes mass = 0.031 x body mass$^{0.77}$.

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SUPPLEMENTAL TABLE S2. Hook angles and hook length. Average values for the 4 angles of hook curvature measured in this study.

Species	Alpha 1 (º)	Alpha 2 (º)	Alpha 3 (º)	Alpha 4 (º)	Hook length (μm)
Apodemus sylvaticus	105.09	39.18	60.88	127.98	4.38
Arvicola sapidus	89.98	49.14	72.77	127.80	2.87
Arvicola terrestris	91.78	24.99	72.67	118.61	2.50
Chionomys nivalis	75.52	51.07	63.75	121.71	3.13
Lemnicomys barbarus	125.84	72.95	90.58	143.21	3.11
Mesocricetus auratus	119.36	65.64	87.24	134.47	5.90
Microtus arvalis	68.10	39.09	72.66	125.66	3.57
Mus m. bactrianus	32.00	63.05	86.98	145.99	3.32
Mus caroli	117.99	88.10	100.34	157.81	2.82
Mus m. castaneus	113.68	84.67	98.79	158.38	2.66
Mus cookii	29.80	78.85	95.96	151,85	3.24
Mus m. domesticus	115.31	81.79	96.38	156.41	2.78
Mus famulus	25.52	73.29	92.20	153.45	2.62
Mus macedonicus	109.87	75.42	93.34	153.30	3.27
Mus m. musculus	118.54	87.06	103.74	157.49	2.93
Mus pahari	120.89	83.61	97.33	151.97	3.18
Mus spicilegus	110.13	80.02	96.23	150.27	2.82
Mus spretus	86.88	69.85	88.85	152.71	2.79
Myodes glareolus	41.63	42.34	73.74	128.39	3.01
Phodopus campbelli	61.46	27.69	68.96	116.66	6.85
Phodopus rovorovskii	90.77	21.66	60.35	102.95	7.83
Phodopus sungorus	80.71	30.48	69.58	119.83	7.35

Each angle is defined by three landmarks in the sperm head; alpha 1 (9-21-20), alpha 2 (3-8-13), alpha3 (3-8-17) and alpha 4 (14-8-17). Hook length has been measured as the linear distance between landmarks 8 and 13.

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SUPPLEMENTAL TABLE S3. Intra-male coefficients of variation (CV) for angles of hook curvature and for hook length.

Species	CV Alpha 1	CV Alpha 2	CV Alpha 3	CV Alpha 4	CV Hook length
Apodemus sylvaticus	14.56	21.07	14.25	6.16	13.45
Arvicola sapidus	14.26	15.45	11.80	6.57	13.05
Arvicola terrestris	13.26	34.57	17.42	6.53	19.21
Chionomys nivalis	14.82	13.89	10.80	5.77	13.18
Lemnicomys barbarus	7.35	10.84	8.92	5.12	12.23
Mesocricetus auratus	6.93	14.20	6.57	4.53	8.42
Microtus arvalis	18.13	21.34	10.95	6.71	11.52
Mus m. bactrianus	24.58	24.60	12.70	7.44	14.53
Mus caroli	10.45	9.91	7.73	4.93	14.42
Mus m. castaneus	9.78	7.72	6.91	4.44	13.53
Mus cookii	26.49	11.46	8.76	6.15	13.95
Mus m. domesticus	10.67	11.59	8.26	5.38	12.38
Mus famulus	25.29	14.62	12.71	7.48	20.08
Mus macedonicus	10.49	11.05	6.69	1.13	12.29
Mus m. musculus	8.03	9.82	7.02	5.51	13.57
Mus pahari	8.23	11.04	9.37	5.57	17.19
Mus spicilegus	10.09	9.11	7.39	4.74	12.09
Mus spretus	10.57	7.67	6.72	4.85	13.27
Myodes glareolus	24.43	21.00	11.55	6.96	12.91
Phodopus campbelli	13.07	28.88	9.95	5.36	6.81
Phodopus rovorovskii	13.13	23.02	13.33	6.94	6.03
Phodopus sungorus	15.15	28.39	9.97	5.75	7.98