Postprint del artículo publicado en: Proceedings of the Royal Society of London - B 281(1783): 20133359 (2014)

FINAL

Sexual selection on protamine and transition nuclear protein expression in mouse species

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Short title: Sexual selection and protein expression

Key words: sexual selection; sperm competition, protamine, transition nuclear protein, sperm

Abstract

Postcopulatory sexual selection in the form of sperm competition is known to influence the evolution of male reproductive proteins in mammals. The relationship between sperm competition and regulatory evolution, however, remains to be explored. Protamines and transition nuclear proteins are involved in the condensation of sperm chromatin and are expected to affect the shape of the sperm head. A hydrodynamically-efficient head allows for fast swimming velocity and, therefore, more competitive sperm. Previous comparative studies in rodents have documented a significant association between the level of sperm competition (as measured by relative testes mass) and DNA sequence evolution in both the coding and promoter sequences of protamine 2. Here, we investigate the influence of sexual selection on protamine and transition nuclear protein mRNA expression in the testes of eight mouse species that differ widely in levels of sperm competition. We also examined the relationship between relative gene expression levels and sperm head shape, assessed using geometric morphometrics. We found that species with higher levels of sperm competition express less protamine 2 in relation to protamine 1 and transition nuclear proteins. Moreover, there was a significant association between relative protamine 2 expression and sperm head shape. Reduction in the relative abundance of protamine 2 may increase the competitive ability of sperm in mice, possibly by affecting sperm head shape. Changes in gene regulatory sequences thus seem to be the basis of the evolutionary response to sexual selection in these proteins.

1. Introduction

When females mate promiscuously, the sperm of rival males compete for the fertilization of available ova [1]. Postcopulatory sexual selection mediated by sperm competition has a profound influence on male reproductive traits across a wide range of taxa (reviewed in [2-4]). In mammals, key traits affected by sperm competition include sperm quality parameters [5], processes that prepare sperm to interact with the oocyte [6], sperm design (e.g., overall size, head shape and dimensions [7-10]), and sperm swimming velocity [10-12]. Several lines of evidence suggest that sperm head shape is particularly important in competitive situations. For example, the size and curvature of the apical hook of rodent sperm heads is thought to be associated with levels of sperm competition ([9], but see [13]). Likewise, head shape may affect the hydrodynamic efficiency of spermatozoa. Head elongation, which may reduce drag, associates with faster sperm swimming velocity [10]. Faster sperm are more likely to succeed in fertilization [14].

To date, most work on the molecular evolution of male reproductive genes has focused on protein-coding regions [15,16]. A number of studies have found a positive relationship between sequence divergence of these genes and levels of sperm competition, and several such genes show evidence of positive selection in coding regions ([17-21] but see [22,23]). However, a positive correlation between sequence divergence in the promoter region of *protamine 2* and relative levels of sperm competition in house mice and their close relatives [24] suggests that regulatory changes may also contribute to species differences in sperm competitive ability. Surprisingly, despite order of magnitude differences in the absolute and relative expression levels of protamines and associated transition nuclear proteins across eutherian mammals [25], the relationship between sperm competition and gene expression remains largely unexplored.

Protamines and transition nuclear proteins are integral to chromatin remodelling and condensation during the final stages of spermatogenesis. This nuclear reshaping in postmeiotic

spermatids affects the overall shape of the sperm head which, in turn, may influence hydrodynamic efficiency, resulting in an increase in sperm swimming speed and more competitive sperm. Notably, sperm from transition nuclear protein-deficient mice perform poorly in some competitive assays [26]. Whereas protamines (PRM1 and PRM2 in most eutherian mammals) bind directly to DNA in the nucleus of elongating spermatids and mature spermatozoa [27], transition nuclear proteins (TNP1 and TNP2) are involved in intermediate stages in the replacement of histones by protamines [28,29]. Protamines remain associated with sperm chromatin in the oocyte and influence the rate of nuclear decondensation, a trait associated with embryonic survival [30-33].

Protamine and transition protein mRNAs are highly co-expressed in round spermatids [34-37], and the protein products of both gene families exhibit significant overlap in elongating spermatid nuclei [28,38]. TNP1 and TNP2 seem to perform partially redundant functions: only double TNP1/TNP2 mouse knockouts are completely sterile [28]. However, deletion of either transition protein results in incomplete PRM2 processing and defective chromatin condensation [29,39]. This, together with the co-localization of mRNAs and mature proteins, strongly suggests that functional interactions between protamines and transition proteins are necessary for normal sperm development.

In mice and humans, both PRM1 and PRM2 are essential for male fertility [40]. Strikingly, although the relative abundance of PRM1 and PRM2 proteins differs widely across mammals (from 0 to 77% PRM2) [41], disruption of species-specific protamine ratios causes fertility defects comparable to gene knockouts [40,42]. In human males, for example, protamine imbalance can result in reduced sperm concentration and motility, and in abnormal head morphology, an indicator of deficits in chromatin condensation [43-45]. In particular, incomplete processing of the PRM2 precursor is associated with sperm dysfunction [45,46], and PRM2-deficient sperm are characterized by incomplete nuclear condensation and increased DNA

damage [40,46,47], defects that can lead to embryonic mortality [31]. Thus, protamine ratios play a large role in sperm head morphology, a phenotype important for competitive ability both before and during fertilization. This suggests that sexual selection mediated by sperm competition should act on protamine ratios, resulting in an association between species differences in levels of sperm competition and protamine expression.

Here, we investigate the influence of sexual selection on protamine and transition nuclear protein mRNA expression in the testes of eight closely related species in the genus *Mus*. These species exhibit a wide range of relative testes mass, a robust proxy for different levels of sperm competition [2,4], and differ in sperm traits associated with competitive ability [5,7,24,48]. Moreover, evolution of the *Prm2* promoter in seven of the same species is consistent with stronger selection in taxa with higher inferred levels of sperm competition [24]. This provides specific motivation for studying the relationship between protamine expression and sperm competition in *Mus*. Given the functional relationship between protamines and transition proteins, and the role of transition proteins in PRM2 processing, we expected that transition nuclear protein expression should co-vary with species differences in protamine expression. Because protamines and transition nuclear proteins are involved in the condensation of sperm chromatin, and are expected to affect the shape of the sperm head, we also assessed the relationship between gene expression and sperm head shape.

2. Materials and methods

(a) Species

This study included 8 species in the genus *Mus*: *M. caroli, M. castaneus, M. domesticus, M. macedonicus, M. musculus, M. pahari, M. spicilegus,* and *M. spretus* (4 to 5 males per species). This group of species shows diverse levels of sperm competition, as inferred from their differences in relative testes mass (table 1). Large testes in relation to body mass (relative testes

mass) is a strong predictor of high sperm competition levels in many taxa (reviewed in [2,4,49]), and relative testes mass is correlated with genetic paternity (i.e., percentages of multiple paternity) in mammals in general [50], and rodents in particular [51]. Therefore, relative testes mass is used in this study as a robust proxy for sperm competition levels.

Individuals were purchased from the Institut des Sciences de l'Evolution-Montpellier, CNRS-Universite de Montpellier II. Males were kept in our animal facilities in individual cages under standard laboratory conditions in environmentally-controlled rooms (20 - 24ºC) on a 14 h light - 10 h darkness photoperiod, and were provided with food and water ad libitum. All animal handling was done following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

(b) Testes collection and relative testes mass

Animals were sacrificed at an age of 2 to 4 months by cervical dislocation and were immediately weighed and dissected. Testes were removed, weighed, flash-frozen in liquid nitrogen, and stored at -80ºC. All dissection instruments and areas were cleaned with RNase AWAY® (Molecular BioProducts, Thermo Fisher Scientific, San Diego, CA) before use. Relative testes mass was calculated based on the rodent power function, following the method in Kenagy and Trombulak [52].

(c) RNA extraction and cDNA synthesis

RNA was extracted in a sterile vertical laminar flow hood using either the RNeasy Plus kit (Qiagen) or the E.Z.N.A® Total RNA kit I (Omega, Madrid, Spain) following the manufacturer's recommendations. All instruments and surface areas were cleaned with RNase AWAY®. RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Madrid, Spain) and cDNA was synthesized the same day from 10 µg of RNA, using

143 the Superscript III First Strand Synthesis Kit with oligo(dT) (Invitrogen, Barcelona, Spain)
144 according to the manufacturer's recommendations. cDNA concentration and purity were
145 determined using a NanoDrop1000 spectrophotometer and samples were stored at -20ºC.
146
147 **(d) Quantitative PCR (qPCR)**
148 Expression levels for *M. musculus, M. spretus*, *M. spicilegus,* and *M. pahari* were determined at
149 the University of Arizona in Tucson using a MyiQ2 light cycler (Bio-Rad), and expression levels
150 for *M. domesticus, M. castaneus, M. macedonicus*, and *M. caroli* were determined at the Museo
151 Nacional de Ciencias Naturales in Madrid using a CFX96 Real Time System / C1000 Thermal
152 Cycler (Bio-Rad). To check the consistency of results obtained using different cyclers, assays for
153 the standard gene (see below) were run by the same person (LL) with a set of testes samples
154 taken from the same individuals used in both Tucson and Madrid, using exactly the same
155 protocol. Results were consistent across locations (e.g., *Mus musculus* individual 1 (Tucson,
156 right testis): average C_T (± SD) = 12.94 (0.02); *Mus musculus* individual 1 (Madrid, left testis):
157 average C_T (± SD) = 12.89 (0.07)).
158 Primers were designed in Primer3 (v. 0.4.0) to amplify a product between 70 and 150
159 bases across an exon-exon junction. Protamine primers were placed in sequences that are
160 invariant across all species in this analysis. Transition protein primers were placed in sequences
161 that are conserved between *Mus* and *Rattus*, and therefore are unlikely to vary among closely
162 related *Mus* species. Primer sequences and amplicon sizes are provided in supplementary table
163 S1. Each qPCR run included one individual of each species with three technical replicates for the
164 four experimental genes (*Prm1, Prm2, Tnp1,* and *Tnp2*), and two technical replicates for the
165 standard gene (*18SrRNA*). qPCR reactions were run in 96-well plates with an end volume of 16
166 µl per sample containing 8 µl SYBR green Master Mix (Invitrogen), 15 ng of each primer and 50
167 ng/µl of cDNA. The conditions of the thermocycler program consisted of an initial denaturation

of 95ºC for 10 min, 40 cycles of 95ºC for 15 sec and an annealing and elongation stage of 62ºC for 1 min. Melt curve analysis was performed at the end of each run to check for multiple peaks, indicative of non-specific amplification.

(e) Analysis of expression data

Cycle threshold data (C_T) were normalized relative to *18SrRNA* for each plate (ΔC_T). To avoid statistical analysis using a dataset of mixed negative and positive values, data were transformed by adding a constant based on the lowest ΔC_T value. Expression ratios and percentages were calculated from transformed individual ΔC_T values (*M. domesticus* $n = 4$, all other species $n = 5$), and median values were obtained for each species. Because of the expectation that relative expression levels may be of greater functional significance than absolute expression levels (see above), we calculated ratios (*Prm1/Prm2, Tnp1/Tnp2, Prm/Tnp, Prm2/Tnp*) and proportions (*Prm2/Prm, Prm2/*(*Prm+Tnp*), *Prm1/*(*Prm+Tnp*)), where *Prm* refers to the combined expression of *Prm1* and *Prm2*, and *Tnp* refers to the combined expression of *Tnp1* and *Tnp2*. To obtain a measure of variability between individuals and species, as well as for individual genes, the coefficient of variation (CV = standard deviation/mean) was calculated.

(f) Phylogenetic generalized least squares (PGLS) analysis

Species data may not be free of phylogenetic association because shared character values may result from common ancestry rather than independent evolution, and thus may not be truly independent. To control for this phylogenetic inertia, we used phylogenetic generalized least squares (PGLS) analyses [53] to test for relationships between species differences in total and relative protamine and transition protein expression, and relative testes mass. PGLS analysis was implemented in COMPARE 4.6b [54], using a phylogenetic tree based on Lundrigan et al. [55] and Gómez Montoto et al. [5] (supplementary figure S1).

193

194 **(g) Geometric morphometrics analysis of sperm head shape**

195 Geometric morphometrics methods were used to quantify head shape variation based on a set of
196 landmarks that correspond to the spatial position of particular anatomical traits [56,57]. A total of
197 20 bidimensional landmark coordinates were gathered from spermatozoa of seven of the eight
198 species used in the gene expression analysis (*M. caroli, M. castaneus, M. domesticus, M.*
199 *macedonicus, M. musculus, M. spicilegus, M. spretus*; $n = 5$ males/species). Landmark data were
200 processed as described previously [58]. All morphometric analyses were conducted with
201 MorphoJ [59]. An independent contrast for morphometric shape data [60] was conducted to
202 check for phylogenetic signal in the sperm head shape data set. This test simulates the null
203 hypothesis of total absence of phylogenetic signal by a permutation procedure. The *P*-value was
204 not significant ($P = 0.102$) for the null hypothesis of independence, which indicates a lack of
205 phylogenetic signal and, therefore, that phylogenetic correction was not needed for this analysis.
206 Canonical variate analysis (CVA) [61] was used to explore the relationship between
207 sperm head shape and relative protamine expression. Species were grouped into three categories
208 based on well-defined differences in relative protamine expression: low, intermediate and high
209 expression ratios (table 1, supplementary table S2; see Results section for details). The CVA
210 produces a set of canonical variates that are uncorrelated within and among groups and account
211 for the maximum amount of among-group variance relative to within-group variance. As a result
212 of the CVA, distances in the original space are transformed to Procrustes distances. These
213 Procrustes distances for between-category comparisons were used to test for significant
214 differences in sperm head shapes between species with low, intermediate and high protamine
215 expression ratios.

216

217 ## 3. Results

(a) Expression of protamines and transition nuclear proteins

Median expression levels for each gene and species are shown in table 1. The ranges of expression medians, and the coefficient of variation (CV) for each gene and species are provided in supplementary table S2. Within species, expression levels were positively correlated in all pairwise comparisons among genes (supplementary figure S2 and supplementary table S3), suggesting that there may be functional constraint to maintain consistent relative expression levels of these genes and/or common regulatory control. The median expression level for individual genes varied by a factor of approximately threefold among species (supplementary table S2). *Tnp1* was expressed at a slightly higher level than *Tnp2* although both showed the same CV. Likewise, *Prm2* was expressed at a slightly higher level than *Prm1* but there was no difference in CV (table 1, supplementary table S2).

The ratios and proportions of expression levels for different genes are shown in supplementary table S4. These relative levels of expression were much more constant among species (supplementary table S4) than expression levels of individual genes (cf. supplementary table S2). The ratio of total protamines to total transition nuclear proteins was close to one in half the species and above one in the other four species, revealing higher overall expression levels of protamines. Ratios between *Tnp1* and *Tnp2* were generally above one, in agreement with higher expression levels of *Tnp1* in comparison to *Tnp2* (see above). The reverse was true for protamines, with ratios of *Prm1/Prm2* below one (supplementary table S4).

(b) Relationships between relative testes mass and gene expression

We tested for associations between relative testes mass and patterns of protamine and transition protein expression, both for individual genes and for ratios of expression levels among genes.

The correlation between relative testes mass and *Prm1/Prm2* or *Prm2/Prm* was not significant when all eight species were considered (figure 1*a*, table 2). However, we noted that

M. pahari appears to be an outlier in this analysis. *M. pahari* is basal to the other species included in this study and belongs to a different subgenus (*Coelomys*) [62].When the analysis was restricted to the seven species in the subgenus *Mus*, there was a significant positive relationship between relative testes mass and *Prm1/Prm2* (α = 15.5, CI 95% (slope) = 1.67 to 4.25, correlation = 0.89) (figure 1*a*, table 2) and a significant negative relationship between relative testes mass and *Prm2/Prm* (α = 15.5, CI 95% (slope) = -0.14 to -0.06, correlation = 0.80) (table 2). In contrast, there was no relationship between testes mass and transition protein ratios (data not shown).

Significant negative associations with relative testes mass were found for *Prm2/Tnp* (α = 1.56, CI 95% (slope) = -4.64 to -0.03, correlation = -0.63)(figure 1*a*, table 2) and *Prm2/*(*Prm+Tnp*)(α = 6.05, CI 95% (slope) = -0.19 to -0.02, correlation = -0.72) (table 2). In contrast, there was no association between relative testes mass and *Prm1/Tnp, Prm1/(Prm+Tnp)* or *Prm/Tnp* (data not shown), or between relative testes mass and any of the four genes when analyzed separately (supplementary table S5). Thus, significant relationships between testes mass and the expression of sperm condensation proteins are driven mainly by the relative expression of *Prm2.*

Together, these results indicate that species with higher inferred levels of sperm competition express proportionately less *Prm2* in relation to total transition protein, and in relation to total protamine and transition protein combined. Within the subgenus *Mus*, species with higher sperm competition have a higher *Prm1/Prm2* expression ratio and therefore a lower *Prm2/Prm* proportion.

(c) Relationships between protamine expression and sperm head shape

Geometric morphometrics was employed to quantify differences in head shape between the seven species in the subgenus *Mus*. Species were categorized as having high, intermediate or low

268 protamine expression ratios, and Procrustes distances (*D*) calculated from canonical variate
269 analysis were used to test for between-category differences in sperm head shape.

270 Sperm head shapes were significantly different between species with high, intermediate
271 and low *Prm1/Prm2* ratios (high vs. intermediate: $D = 0.08$, $P = 0.0002$; high vs. low: $D = 0.1$, P
272 $= 0.0001$; intermediate vs. low: $D = 0.05$, $P = 0.05$; figure 2). The same between-category
273 differences in sperm head shape were obtained for *Prm2/Prm* ratio (high vs. intermediate: $D =$
274 0.05, $P = 0.0001$; high vs. low: $D = 0.1$, $P = 0.0001$; intermediate vs. low: $D = 0.08$, $P = 0.0001$).
275 These results support the idea that sperm head shape is influenced by relative protamine
276 expression.
277

278 ## 4. Discussion

279 Despite the long-standing debate over the relative contribution of coding vs. regulatory changes
280 to adaptive evolution [63-65], mounting empirical evidence demonstrates that regulatory
281 evolution can play a major role in adaptive divergence, particularly between closely related
282 lineages (e.g. [65-71]). In this study we compared protamine and transition nuclear protein
283 mRNA expression in the testes of eight species in the genus *Mus* that share recent common
284 ancestry but differ widely in inferred levels of sperm competition. We found that species that
285 experience higher levels of sperm competition express less *protamine 2* in relation to both
286 transition nuclear proteins, and to *protamine 1*. This strongly suggests that species differences in
287 relative expression levels of these key spermiogenesis genes are influenced by variation in the
288 strength of post-copulatory sexual selection. The fact that this pattern is driven by the relative
289 expression of *protamine 2* is consistent with evidence that the promoter region of this gene is
290 evolving under sexual selection in *Mus* [24]. Importantly, we found that species that differ in
291 ratios of *protamine 2* expression, both in relation to *protamine 1* and in relation to total
292 protamines, also differ in sperm head shape. This suggests that regulatory changes contribute to

293 modifications of sperm phenotype that could, ultimately, influence sperm's competitive ability.
294 Taken together, the results of this study support the proposition that selection on regulatory
295 regions can fine-tune adaptive phenotypes on short evolutionary timescales [72]. We discuss
296 these results in relation to previous work on the evolution of sperm chromatin condensation
297 genes in mammals, and the genetics and functional consequences of sperm competition in
298 rodents.
299
300 **(a) Protamines and sperm competition: evolution at two levels**
301 Sperm chromatin condensation genes, including protamines, are thought to be among the fastest
302 evolving male reproductive proteins in eutherian mammals (e.g. [73,74]). There is ample
303 evidence from primates and rodents that selection contributes to this rapid rate of change
304 [16,21,75,76] and sperm competition is often invoked as the driving force [15]. However, how
305 particular substitutions might enhance sperm competitiveness remains untested, and it has been
306 suggested that selection for protein stability is an equally parsimonious explanation for
307 protamine coding sequence evolution in primates [77]. Notably, in case-control studies of human
308 males, associations between infertility and coding region SNPs in either *Prm1* or *Prm2* are rare
309 (e.g. [78-80]), whereas men with imbalanced PRM1/PRM2 ratios are consistently subfertile or
310 sterile (reviewed in [81]). Thus, while the functional consequences of protamine coding sequence
311 substitutions are largely unknown, changes in protamine expression have a demonstrated impact
312 on male fertility, and therefore might co-vary with the strength of post-copulatory sexual
313 selection across species.
314 In the *Mus* clade comprising house mice and their close relatives, there is evidence for
315 weak positive selection on *Prm2* coding sequence in the three species with the highest inferred
316 levels of sperm competition (*M. spicilegus, M. spretus* and *M. macedonicus*), whereas divergence
317 in the promoter region is positively correlated with relative testes mass, and with sperm

swimming speed, across the entire clade [24]. Here, using a subset of the same species, we show that the relative abundance of *Prm2* mRNA in the testes is negatively correlated with relative testes mass. These findings suggest that nucleotide substitutions in the *Prm2* promoter region influence expression, and that high levels of sperm competition act to decrease the relative abundance of *Prm2* in the testes.

We emphasize, however, that our understanding of the relationship between protamine 2 regulation and sperm competition in *Mus* is far from complete. First, the functional relationship between promoter evolution and expression is not straightforward: species with higher *Prm2* promoter divergence express less *Prm2* only in relation to transition nuclear proteins and *Prm1*. Despite substantial interspecific differences in the expression levels of all four genes, there was no relationship between relative testes mass and individual gene expression. Likewise, although the *Prm1* promoter region is highly variable in *Mus*, there is no relationship between divergence and levels of sperm competition [24]. A plausible explanation for these patterns is that sexual selection for reduced PRM2 is counter-balanced by natural selection to maintain the relative proportions of protamines and transition nuclear proteins within a functional range. Potential mechanisms include compensatory evolution in the promoter regions of interacting sperm chromatin condensation proteins, or a single regulatory modifier shared among genes. In mice, as in humans, *Prm1, Prm2* and *Tnp2* are tightly clustered in the genome. Thus, an enhancer element common to all three genes is a formal possibility. Comparative analysis of intergenic regions in the *Prm1/Prm2/Tnp2* cluster, together with the *Tnp1* and *Tnp2* promoter regions, will help to discriminate these non-mutually exclusive alternatives.

Second, the correlation between mRNA expression levels and protein abundance is often imperfect [82]. Quantification of sperm chromatin condensation proteins in mature spermatozoa will provide a direct measure of species differences in their relative abundance. Finally, evidence for selection on *Prm2* coding sequence in *M. spicilegus, M. spretus* and *M. macedonicus* is

intriguing, because it suggests that high levels of sperm competition can drive coding and regulatory evolution in tandem [24]. However, whether positively-selected *Prm2* amino acid substitutions in these species affect sperm phenotypes related to competitive ability remains to be determined.

(b) The relative abundance of protamine 2: functional implications for sperm phenotypes

Why should high levels of sperm competition favour reduction in the relative abundance of PRM2? While the phenotypic effects of inter-specific differences in protamine ratios are largely unstudied, there is some evidence that sperm from species that either lack PRM2, or produce very little PRM2 relative to PRM1, exhibit slower DNA decondensation in the oocyte [32,41]. Sperm with more compact heads may have higher competitive ability [10], and sperm with incomplete DNA compaction often have over-sized or less streamlined heads [34]. Thus, it is plausible that high levels of sperm competition select for higher DNA compaction, and thus proportionately less PRM2. Evaluation of this hypothesis will require comparative analyses of sperm chromatin compaction in relation to head morphology, the proportion of PRM2, and the strength of sexual selection mediated by sperm competition. Notably, the finding that relative abundance of *Prm2* is associated with differences in sperm head shape is an important first step towards revealing the functional relationship between protamine expression and sperm head morphology. Future studies will investigate the hydrodynamic consequences of these *Prm2*-associated differences in sperm head shape.

(c) Conclusions

An important role of comparative studies such as this is to identify patterns that generate testable hypotheses [83]. Here, we show that species of mice with higher inferred levels of sperm competition express less *protamine 2* in relation to *protamine 1* and transition nuclear proteins.

Based on this pattern, together with evidence for sexually selected divergence in the promoter region of *protamine 2* [24], we propose that reduction in the relative abundance of *protamine 2* enhances sperm competitive ability in mice by influencing sperm head shape, and that regulatory evolution plays a key role in this evolutionarily rapid response to selection.

Acknowledgements

Lena Lüke holds a studentship from the Spanish Research Council (JAEpre-CSIC) cofinanced by the European Social Fund (ESF). This work was supported by the Spanish Ministry of Economy and Competitiveness (grant CGL2011-26341) and was developed in part during a short stay at the University of Arizona with funding from CSIC ("Estancia Breve"). This work was also funded by NSF and NIH grants to MWN. We thank François Bonhomme and Annie Orth for facilitating access to animals used in this study.

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Figure Legends

Figure 1. Relationships between relative testes mass and relative protamine 2 expression. (a) Protamine ratio (*Prm1/Prm2*): the dashed line corresponds to analyses with N = 8 mouse species, and the correlation is not significant. The open circle identifies *Mus pahari*, a species that behaves as an outlier in these analyses. The solid line corresponds to analyses with N = 7 species in which *M. pahari* is not included, and this correlation is statistically significant. (b) Ratio of *Prm2* to total *Tnp* (*Prm2/Tnp*). Results of statistical analyses are given in Table 2.

Figure 2. Procrustes distances (*d*) and *P* values for canonical variate analyses examining head shape in relation to *Prm1/Prm2* ratio. Three groups of species were defined according to their ratios of protamine expression: high (*M. macedonicus, M. spicilegus*), intermediate (*M. musculus, M. caroli, M. spretus*) and low (*M. castaneus, M. domesticus*)(see Table 1). Morphometric data were taken from 35 individuals of 7 species. Procrustes distances different from zero indicate shape differences between groups. Wireframe graphics show the shape associated to each group categorized according to its *Prm1/Prm2* ratio.

Table 1. Relative testes mass was calculated as described [52] followed by a calculation of the median for the species. Gene expression data are normalized, transformed median values. Species were ordered by relative testes mass (ascending).

species	relative testes mass	*Prm1* (ΔC_T)	*Prm2* (ΔC_T)	*Tnp1* (ΔC_T)	*Tnp2* (ΔC_T)	*Tnp1/ Tnp2*	*Prm1/ Prm2*	*Prm/ Tnp*	*Prm2/ Tnp*	*Prm2/ Prm*	*Prm2/ (Prm+Tnp)*
Mus castaneus	0.27	3.16	4.29	2.96	3.38	0.83	0.67	1.24	0.75	0.60	0.33
Mus pahari	0.27	3.80	3.69	3.01	2.26	1.13	1.01	1.38	0.68	0.50	0.29
Mus domesticus	0.32	2.11	3.22	1.88	2.32	0.81	0.65	1.27	0.77	0.61	0.34
Mus musculus	0.44	2.87	3.72	3.27	2.98	1.14	0.76	1.06	0.61	0.57	0.29
Mus caroli	0.46	5.83	7.28	6.71	6.18	1.07	0.78	1.00	0.56	0.56	0.28
Mus spretus	0.87	1.90	2.31	2.32	1.70	1.38	0.82	1.05	0.58	0.55	0.28
Mus macedonicus	0.95	3.54	3.49	2.48	2.14	1.05	0.99	1.49	0.74	0.50	0.30
Mus spicilegus	1.51	4.61	4.60	4.76	3.92	1.16	0.98	1.05	0.53	0.50	0.26
Coefficient of variation (CV)	0.69	0.37	0.36	0.46	0.46	0.18	0.17	0.15	0.14	0.08	0.09

Table 2. Relationship between relative testes mass and relative protamine or transition protein expression. Analyses were carried out with all species and excluding Mus pahari (see text). CI- and CI+ indicate the confidence intervals for the regression slope, lnL=log likelihood estimate of alpha, alpha = measure of evolutionary constraints acting on phenotypes, corr = the correlation value (r). Bold CI values indicate statistical significance.

	excluding *Mus pahari* (n=7)		relationships for all species (n=8)			
	Prm1/Prm2	*Prm2/Prm*	*Prm2/Tnp*	*Prm2/(Prm+Tnp)*	*Prm1/Prm2*	*Prm2/Prm*
CI-	**1.67**	**-0.14**	**-4.64**	**-0.19**	-0.47	-0.12
CI+	**4.25**	**-0.06**	**-0.03**	**-0.02**	3.68	0.01
lnL	8.15	8.14	5.20	6.05	4.52	4.63
alpha	15.50	15.50	1.56	1.66	5.62	5.37
corr	0.89	-0.80	-0.63	-0.72	0.53	-0.54

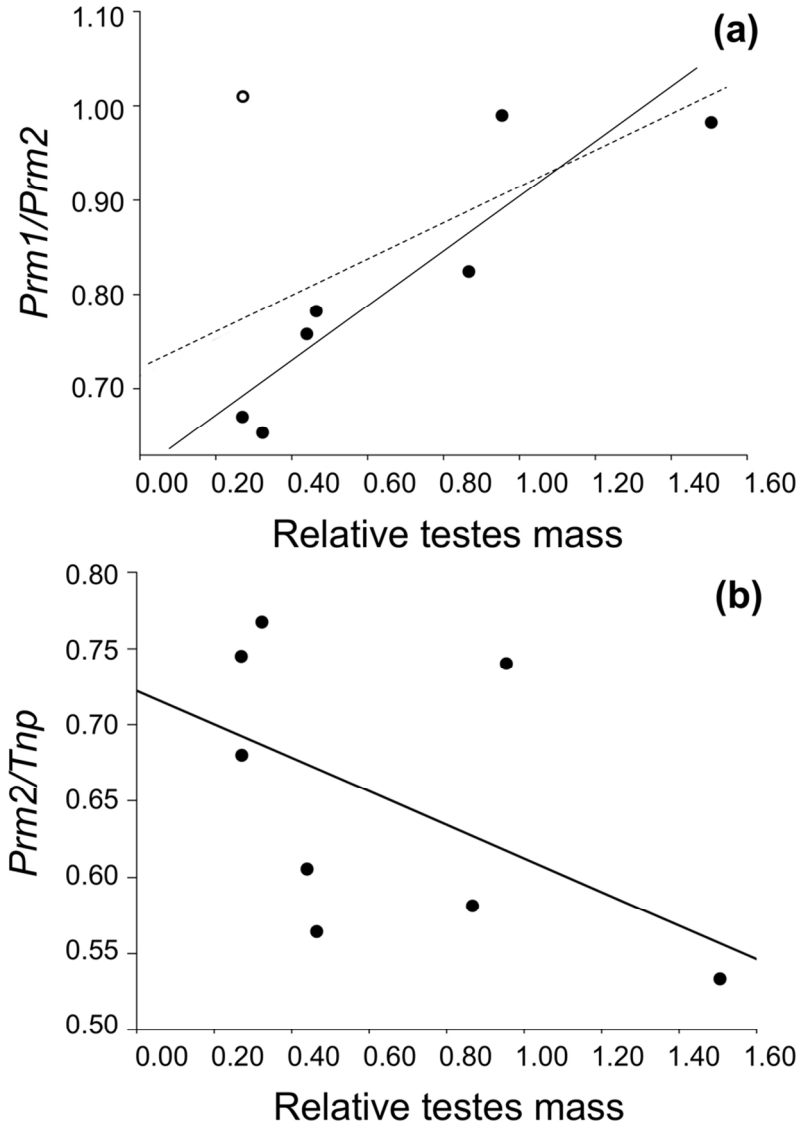


Figure 1
90x128mm (300 x 300 DPI)

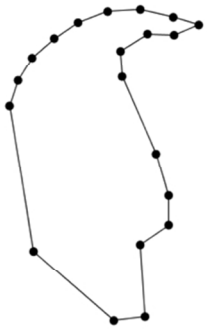
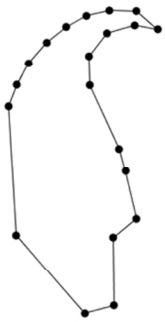
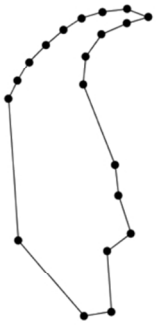
Prm1/Prm2 ratio		High	Intermediate	Low
				
Prm1/Prm2 ratio	Range	0.98 – 0.99	0.76 - 0.82	0.65 - 0.67
	Intermediate	*D* = 0.08 *P* = 0.0002	-	-
	Low	*D* = 0.10 *P* = 0.0001	*D* = 0.05 *P* = 0.05	-

Figure 2
90x73mm (300 x 300 DPI)