

Sexual selection towards a protamine expression ratio optimum in two rodent groups?

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Post-copulatory sexual selection is thought to influence the evolution of genes involved in reproduction. However, the detection of straightforward effects has been proven difficult due to the complexity and diversity of reproductive landscapes found in different taxa. Here, we compare the possible effect of relative testes mass as a sperm competition proxy on protamine genotype (protamine 1/protamine 2 ratio) and the link to sperm head phenotype in two rodent groups, mice, and voles. In mice, protamine expression ratios were found to increase from low values toward a 1:1 ratio in a positive association with testes mass, and relative sperm head area. In contrast, in voles, decreasing protamine expression ratios were found in species with larger testes but, surprisingly, they range from high values, again toward a 1:1 ratio, and showing a negative correlation with relative sperm head area. Altogether, we found differences in the way protamines seem to be selected and involved in adaptations of the sperm head in voles and mice. However, sexual selection driven by sperm competition seems to exhibit a common evolutionary pattern in both groups toward an equilibrium in the expression of the two protamines.

KEY WORDS: protamines, protamine ratio, sexual selection, sperm competition, rodents.

For genes that regulate reproductive function, post-copulatory sexual selection appears to be the main driver of evolutionary change. The degree of female promiscuity differs greatly among species that leads to a high variation in sperm competition risk and the opportunity for cryptic female choice as well as the adaptive responses to it (Parker 1970; Eberhard 1996). The level of sperm competition (Parker 1970) could, therefore be an important driver of adaptation of sperm form and function (reviewed in Birkhead and Moller 1998; Simmons 2001; Birkhead et al. 2009). Rodents and primates, in particular, show a wide range of sperm competition levels across a broad range of species and a particular diversity in sperm head phenotypes (Cummins and

Woodall 1985; Roldan et al. 1992; Pitnick et al. 2009). Previous studies suggest that protamines play a specific role in adaptations of sperm head phenotype in response to sperm competition (Lüke et al. 2014a,b; Lüke et al. 2016a,b).

Protamines are small, arginine-rich sperm-specific proteins that replace histones in the sperm nucleus (Oliva and Dixon 1991; Balhorn 2007). During histone-to-protamine replacement the spermatid genome is globally inactivated, condensed, and protected, resulting in a strongly reduced nucleus size affecting the shape of the sperm head (Balhorn 2007; Balhorn and Balhorn 2011). Two protamines are found in mammals, protamine 1 (*PRM1*, PRM1) and protamine 2 (*PRM2*, PRM2). *PRM1* is

expressed in all mammals, while *PRM2* is known to be expressed in most rodents, primates, and a subset of other mammalian species (Oliva 2006; Balhorn 2007). Both are essential for male fertility (Cho et al. 2001; Schneider et al. 2016). In men, the ratio between *PRM1* and *PRM2* (the so-called protamine ratio) seems to be important, and alterations in this ratio are seen in infertile patients with abnormal sperm (de Yebra et al. 1998; Carrell and Liu 2001; Steger et al. 2008; García-Peiró et al. 2011). In horses, the protamine ratio correlates with sperm morphology and female fertility, and a diminished protamine ratio associates with sperm defects (Paradowska-Dogan et al. 2014). Notably, the physiological protamine ratio seems to be stable within species but varies between them (Corzett et al. 2002).

Our previous studies explored the relationship between sexual selection on protamines and sperm head phenotype. Sperm competition seems to drive protamine coding sequence evolution in cricetid rodents, and to a change in *PRM1* arginine content across eutherian mammals. Both, protamine coding sequence evolution in cricetid rodents and arginine content across mammals, associate with changes in sperm head phenotype (Lüke et al. 2014a,b; Lüke et al. 2016a,b). Mouse species, which are very closely related to one another, lack differences in protamine coding sequences. Here, the effect of sexual selection is evident on the protamine expression ratio which, in turn, associates with differences in sperm head shape (Lüke et al. 2014b).

A large-scale evolutionary study of *PRM1* and *PRM2* across mammals showed that sexual selection seems to drive protamine evolution in different ways in different taxa. This was proposed to be a result of adaptations to differing female reproductive tract environments and sperm cell architecture among other factors. As a result, the detection of the effects of sexual selection is complicated in large-scale analyses (Lüke et al. 2016a).

To explore this further, we compared the relationship between sexual selection on protamines and sperm head phenotype in two groups, on a lower taxonomic level. As a proxy for the level of sperm competition, we used testes mass. An increase in testes mass is a nearly universal response to high levels of female promiscuity. It has been shown to be related to levels of genetic paternity in mammals and is therefore believed to reflect the level of sperm competition (Birkhead and Møller 1998; MacLeod and MacLeod 2009; Soulsbury 2010). Importantly, unlike multiple paternity data, testes mass measures are available for a large number of species, so it is widely used as a reliable index of sperm competition levels.

We chose two rodent families: mice and voles. These two groups provide the advantage of exhibiting a similar range in sperm competition levels but marked differences in sperm head morphology. Additionally, the reproductive phenotypes and phylogenetic relationships in these species are well characterized (Varea-Sánchez 2014). Based on the differing selection patterns

between mammalian taxa and the differences in sperm head phenotype between mice and voles, we hypothesized that the effect of sperm competition level on the protamine expression ratio and its association with sperm head phenotype could vary between these two groups.

Results

PROTAMINE EXPRESSION

Protamine gene expression levels obtained by quantitative PCR (see Materials and Methods) for the different species are presented in Table 1. Expression ratios had a range of 0.93 to 1.00 for mice and 1.01 to 1.31 for voles (Fig. 1A). Mice showed a significantly lower protamine expression ratio than voles on average ($t_{7,2} = 4.5, p < 0.05$) (Fig. 1A, Table 1).

SPERM HEAD PHENOTYPE

To explore possible associations with sperm head phenotype, we chose to use a single variable that is comparable between the groups. Previous studies in cricetid rodents have shown that changes in protamine coding sequence were associated with an increase in both sperm head length and width, thus sperm head size (Lüke et al. 2014a). We therefore chose sperm head area as a single relevant variable. Because total sperm length varies greatly among mouse and vole species, sperm head area was analyzed relative to total sperm length. Relative sperm head area did not differ between voles and mice ($t_{10,54} = -0.24, p = 0.81$) (Table 1).

RESIDUAL/CORRECTED TESTES MASS, A PROXY OF SPERM COMPETITION LEVELS

The association of testes mass to levels of sperm competition in many taxa and its relation to levels of genetic paternity makes testes mass a widely used and reliable proxy for level of sperm competition (Birkhead and Moller 1998; Gomendio et al. 1998; Birkhead et al. 2009; Soulsbury 2010; Lüpold et al. 2020). For regression analysis between testes mass and other variables, we included body mass (log) as the first predictor and testes mass (log) as the second predictor in a phylogenetically corrected multiple regression (“corrected testes mass”). To allow for easier interspecies comparisons and graphical visualization of results, we also estimated the residuals from log-log regressions between body mass and testes mass (from now on referred to as “residual testes mass”). The two groups of species exhibited high diversity but similar ranges of residual testes mass (mice: range = -0.6 to 0.22 , mean = -0.2 ; voles: range = -0.46 to 0.37 , mean = -0.1 ; Table 1). Consequently, these groups are adequate for a comparative evolutionary study based on sperm competition as a driving force.

Table 1. Summary of residual testes mass and protamine expression data. Residual testes mass was taken from a linear regression analysis using species mean testes mass as dependent variable and species mean body mass as predictor. Sperm head area measure is relative to total sperm length. Gene expression data are normalized against *18S rRNA* expression.

	Residual testes mass	<i>Prm1</i> (ΔC_T)	<i>Prm2</i> (ΔC_T)	<i>Prm1/Prm2</i>	Relative sperm head area
<i>Microtus duodecimcostatus</i>	-0.595	22.750	19.695	1.159	0.205
<i>Microtus cabrerai</i>	-0.546	23.105	20.223	1.143	0.238
<i>Arvicola terrestris</i>	-0.447	22.090	16.876	1.311	0.154
<i>Arvicola sapidus</i>	-0.157	22.774	19.719	1.154	0.184
<i>Microtus arvalis</i>	-0.048	22.718	22.477	1.011	0.214
<i>Myodes glareolus</i>	0.201	25.592	23.705	1.079	0.257
<i>Chionomys nivalis</i>	0.223	24.254	22.490	1.079	0.288
Mean:	-0.195	23.326	20.741	1.134	0.220
<i>Mus pahari</i>	-0.459	18.647	18.662	0.999	0.283
<i>Mus castaneus</i>	-0.394	17.221	18.495	0.931	0.180
<i>Mus domesticus</i>	-0.320	16.541	17.684	0.935	0.191
<i>Mus musculus</i>	-0.209	17.444	18.333	0.952	0.233
<i>Mus caroli</i>	-0.099	19.914	21.463	0.928	0.223
<i>Mus macedonicus</i>	0.164	18.251	18.234	1.001	0.227
<i>Mus spretus</i>	0.218	16.718	17.093	0.978	0.238
<i>Mus spicilegus</i>	0.374	19.265	19.350	0.996	0.223
Mean:	-0.091	18.000	18.664	0.965	0.225

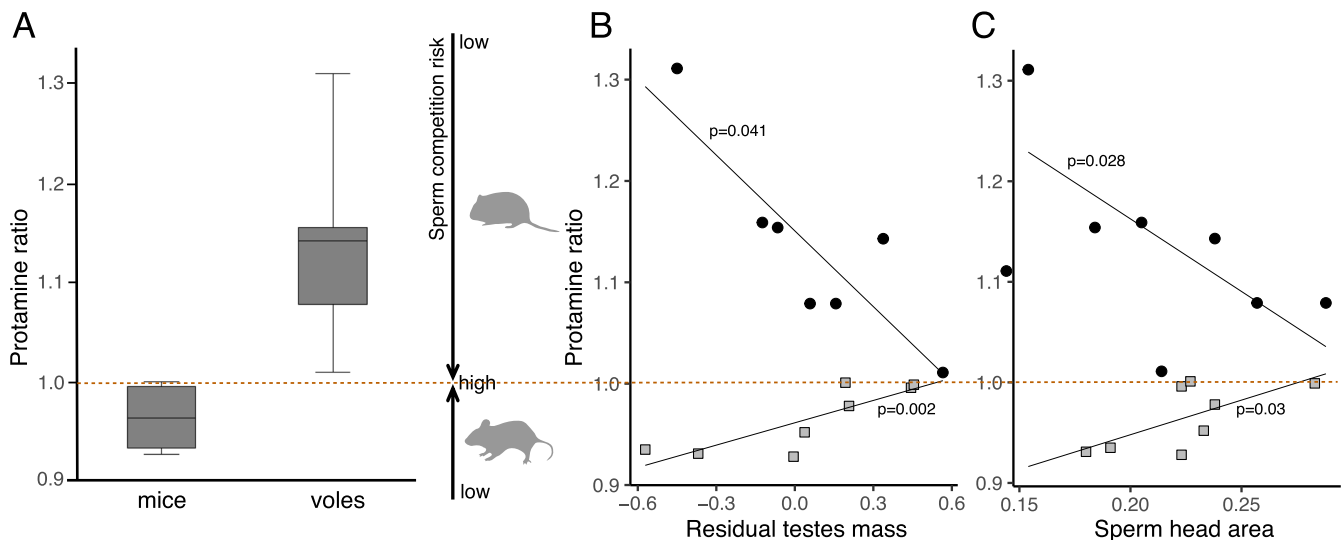


Figure 1. Associations with protamine expression ratio in mice and voles. (A) Comparison of ranges of protamine expression ratio between voles and mice. (B) Scatterplot representing the relationship between residual testes mass and protamine gene expression ratio. Mouse data are represented by grey squares, vole data by black dots. (C) Scatterplot representing the relationship between sperm head area relative to total sperm length and protamine gene expression ratio. Mouse data are represented by grey squares, vole data by black dots.

RELATIONSHIPS BETWEEN SPERM COMPETITION, PROTAMINE RATIO, AND SPERM HEAD PHENOTYPE

Our rationale was to test for an effect of sperm competition level on the protamine expression ratio and, in turn, an effect of protamine expression ratio on relative sperm head area in both

groups. To control for phylogenetic inertia, we used phylogenetic generalized least squares (PGLS) analyses (Felsenstein 1985) when testing for relationships between corrected testes mass, protamine expression ratio, and relative sperm head area. Detailed PGLS results are shown in Table 2.

Table 2. Phylogenetic generalized least squares results. Values in superscripts following the λ value indicate significance levels (ns: $p > 0.05$; * $p < 0.05$) for likelihood ratio tests against models with $\lambda = 0$ (first superscript) and $\lambda = 1$ (second superscript). Abbreviations: n : number of species in analysis.

		N	Slope	t	p	sig	adj R^2	λ
Arvicolinae	Prm ratio ~ log body mass + log testes mass	7	0.21	1.81	0.14			
			−0.19	−2.96	0.041	*	0.53	1 ^{ns,ns}
	Relative sperm head area ~ log body mass + log testes mass	7	−0.69	−2.97	0.041			
			0.33	1.73	0.157		0.53	0 ^{ns,ns}
Murinae	Prm ratio ~ log body mass + log testes mass	8	0.26	7.51	0.001			
			0.13	6.12	0.002	**	0.9	0 ^{ns,*}
	Relative sperm head area ~ log body mass + log testes mass	8	1.50	6.03	0.016			
			0.42	3.16	0.058		0.85	0.7 ^{ns,ns}
	Relative sperm head area ~ Prm ratio	8	2.9	2.75	0.033	*	0.48	1 ^{ns,ns}

p : p -value. adj R^2 : adjusted R^2 . Sig: level of significance (* $p < 0.05$, ** $p < 0.01$).

In mice, we found a positive correlation between corrected testes mass and protamine expression ratio (PGLS: $p = 0.002$, $R^{2\text{adj}} = 0.9$; Fig. 1B, Table 2) and a positive correlation between the protamine expression ratio and relative sperm head area (PGLS: $p = 0.033$, $R^{2\text{adj}} = 0.48$; Fig. 1C, Table 2). In these species, multiple PGLS regression showed a significant association with the first predictor, body mass (Table 2). When visualizing these data, it becomes clear that this association is driven by *Mus pahari*, which shows a comparatively larger body mass (Fig. S2). In voles, we found a negative correlation between corrected testes mass and the protamine expression ratio (PGLS: $p = 0.041$, $R^{2\text{adj}} = 0.53$; Fig. 1B, Table 2) and a negative correlation between the protamine expression ratio and relative sperm head area (PGLS: $p = 0.028$, $R^{2\text{adj}} = 0.58$; Fig. 1C, Table 2).

Finally, we tested for a direct association between corrected testes mass and relative sperm head area. Neither group showed a significant association (voles PGLS: $p = 0.157$, $R^{2\text{adj}} = 0.53$; mice PGLS: $p = 0.059$, $R^{2\text{adj}} = 0.85$; Table 2).

Discussion

Large-scale evolutionary analyses of protamine sequences found differing patterns of sexual selection on protamines among groups of mammalian taxa (Lüke et al. 2016a,b). To explore these differences at a lower taxonomic level (i.e., among species that are more closely related), we examined the relationship between sexual selection in the form of sperm competition on protamines

and sperm head phenotype in voles and mice. Studies have shown that sperm head dimensions respond to high levels of sperm competition and that they affect sperm swimming velocity (Gómez-Montoto et al. 2011; Varea-Sánchez 2014). Evidence from this and previous studies suggests that sperm head phenotype in rodents is affected at least in part by selective pressures acting on protamine expression levels, specifically on the protamine expression ratio (Lüke et al. 2014a). This association is likely based on the important role of protamines in chromatin compaction and nucleus remodeling (Balhorn 2007). In both mice and voles, we found evidence for high sperm competition levels favoring a protamine expression ratio closer to 1, which is additionally related to sperm head size. However, all vole species examined here express more *Prm1* than *Prm2* leading to a protamine expression ratio greater than 1, as opposed to mice which show ratios below 1. This leads to opposing correlation patterns between these two groups. Thus, high sperm competition levels seem to drive selection towards equal expression of the two protamines in both families. Under the pressure of sperm competition, the protamine ratio may be optimized to influence sperm head phenotype, possibly to become more streamlined and hydrodynamically efficient, and increase sperm swimming velocity. Empirical studies in rodents have demonstrated that head dimensions, especially head area, are critical for swimming velocity and trajectory (Varea-Sánchez 2014). We found an association between protamine ratio and relative sperm head area matching the opposing patterns found between protamine ratio and sperm

competition proxy. However, we did not find a direct association between sperm competition proxy and relative sperm head area. When considering that the most well-known function of protamines is to strongly condense the sperm nucleus it is not surprising that we found correlations of the protamine ratio with a measure of sperm head size. It is possible that DNA condensation is most efficient with a balanced 1:1 protamine 1 and 2 proportion. In any case, their effect on sperm head size might not be a direct cause of the apparent selective pressure toward equal expression. The relationships seem to be far more complex. Protamines are believed to influence sperm head phenotype to reduce drag under the pressure of sperm competition, thus increasing sperm velocity (Lüke et al. 2014b; Gómez-Montoto et al. 2011; Varea-Sánchez 2014). However, which type of sperm head is the most hydrodynamically efficient is still not known. The complex interactions between sperm hook and nucleus sizes, and sperm shape, together with sperm metabolism and flagellar beating pattern, has to be considered in order to understand the advantages of certain sperm head phenotype for sperm swimming (Malo et al. 2006; Gómez-Montoto et al. 2011; Tourmente et al. 2013). Our results suggest that a simple connection between protamine ratio and sperm head size does not suffice as an explanation for the selection of an expression equilibrium between the two protamines under strong sperm competition. An in-depth, comparative study of protamine-protamine interaction could shed light on why a balance between the two protamines might lead to a more competitive sperm phenotype. Since the optimal protamine expression ratio under high levels of sperm competition seems to be similar for both mice and voles, the question that arises is why voles and mice would show such different ranges of protamine expression ratios. Since sperm competition-driven selection favors such a clear optimum, other selective forces should be involved in producing these vast differences between species in the first place. Besides condensing the nucleus and protecting paternal chromatin, protamine expression levels and the protamine expression ratio might be connected to the level of histone retention. In human males there seems to be an inverse correlation between the protamine expression ratio and the degree of histone retention in sperm chromatin (Hammoud et al. 2009). Sperm histones convey epigenetic information of importance to early embryonic development and possibly to the maintenance of paternal imprinting (Brykczynska et al. 2010; Hammoud et al. 2011; Yamaguchi et al. 2018). It is therefore conceivable that selective pressures due to sexual conflict, for example, drive the protamine expression ratio, because of its potential association with histone retention. This would be an interesting new avenue for evolutionary comparative studies. But for this, we first need more detailed knowledge of protamine function, the functional differences between PRM1 and PRM2 and how these proteins are involved in maintaining paternal epigenetic information.

Relative testes mass has been shown to be a reliable proxy for sperm competition levels in numerous studies. However, it is still an incomplete and indirect measure of the level of post-copulatory sexual selection. The level of female promiscuity is directly related to the strength of post-copulatory sexual selection (Tregenza and Wedell 2000; Birkhead and Pizzari 2002), so data on number of mating partners during one receptive period, or multiple paternity in litters, could give us a more direct index of levels of post-copulatory sexual selection and should be included in future studies. However, these data would give us a combined measure of two mechanisms that might operate in conflict. Post-copulatory sexual selection comprises both sperm competition and cryptic female choice. Female promiscuity increases both mechanisms that drive selection of reproductive genes and phenotypes although not necessarily in the same way (Birkhead and Pizzari 2002). Even though sperm competition is believed to be the main selective driver of sperm form and function, cryptic female choice is likely to play a part. Untangling the effects of sperm competition and cryptic female choice is complex. One approach suggested by Firman et al. (2017) is a comparison of multiple paternity expectations based on sperm competitive phenotypes with the actual paternity distribution in the litter. Deviations from the expected distribution could be considered as an index for the level of cryptic female choice. Comparing the effects of sperm competition levels and cryptic female choice would give us a clearer picture of how post-copulatory sexual selection drives protamine evolution. In addition to studying existing diversity, an experimental approach would greatly add to our understanding. Experimental evolution studies with mouse populations held under conditions favoring different levels of sperm competition have been successfully used to study the effect on sperm phenotype (Firman and Simmons 2011; Godwin et al. 2017). This approach will be useful to confirm if sperm competition in fact drives a protamine expression ratio optimum.

Conclusions

Protamines are crucial to male fertility and the protection of the paternal genome, but their function appears to be flexible enough to allow for adaptations to strong selective pressures. Even though protamine ratios differ across mammals (from 0 to 77% PRM2) (Corzett et al. 2002). Alteration in the species-specific ratio has major effects on male fertility (Cho et al. 2001; Haueter et al. 2010) and it seems to be connected to histone retention (Hammoud et al. 2009). Understanding how and why different protamine ratios have evolved through different selective pressures would allow us to understand how different selective pressures trade off and why an imbalance in protamine expression can lead to changes in sperm phenotype and male infertility. Here,

we found that an equilibrium between *Prm1* and *Prm2* expression seems to be favored in species with high sperm competition levels, as inferred from their relative testes mass, in both mice and voles. This however raises the question of why the protamine ratios differ so drastically between species suggesting different, even opposing selective pressures acting on protamines. Further studies disentangling the effects of sperm competition and cryptic female choice, as well as experimental evolution studies, are needed to confirm this pattern. Understanding the impact protamines have on formation and function of the sperm head and their potential role in the retention of epigenetic information for the next generation will be crucial to our understanding of reproductive biology and the evolution of reproductive traits.

Materials and Methods

SPECIES

This study includes data from eight species of the genus *Mus* in the group of mice: *M. caroli*, *M. castaneus*, *M. domesticus*, *M. macedonicus*, *M. musculus*, *M. pahari*, *M. spicilegus*, and *M. spretus* (4–5 males per species) and seven species of the subfamily Arvicolinae in the group of voles: *Arvicola sapidus*, *Arvicola terrestris*, *Myodes glareolus*, *Chionomys nivalis*, *Microtus arvalis*, *Microtus cabrerai*, *Microtus duodecimostatus* (4–6 males per species). For *Mus* expression data and body mass and testes mass data were taken from previous studies (Lüke et al. 2014b). Sperm head area measurements for all species were taken from previous studies (Varea-Sánchez et al. 2014). Individuals belonging to Arvicolinae were trapped in the field during the breeding season at different locations in Spain, with permissions from the Comunidad Autónoma de Madrid and Junta de Castilla y León (Gómez-Montoto et al. 2011). Males were kept in our animal facilities in individual cages under standard laboratory conditions in environmentally-controlled rooms (20–24°C) 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 RD53/2013, which conforms to European Union Regulation 2010/63.

TESTES AND SPERM COLLECTION AND SPERM PHENOTYPE

Animals were sacrificed at an age of 2–4 months by cervical dislocation and were weighed and dissected. Testes were dissected, weighed and then flash-frozen in liquid nitrogen, and stored at –80°C. Before use, all instruments and areas were cleaned with RNase AWAY® (Molecular BioProducts, Thermo Fisher Scientific, San Diego, CA). Sperm head area and total sperm measurements for all species were taken from previous studies (Varea-Sánchez et al. 2014). Total sperm length (TSL) varies greatly

among species. When analyzing head size in terms of drag or hydrodynamics the length of the flagellum should be considered (Humphries et al. 2008). Therefore, sperm head area was calculated relative to TSL.

RNA EXTRACTION AND cDNA SYNTHESIS

RNA extraction was performed under a sterile vertical laminar flow hood using the E.Z.N.A® Total RNA kit I (Omega, Madrid, Spain) following the manufacturer's recommendations. Instruments and surfaces were cleaned with RNase AWAY® before RNA extraction. 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 the Superscript III First Strand Synthesis Kit (Invitrogen, Barcelona, Spain) according to the manufacturer's recommendations. cDNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer.

QUANTITATIVE PCR

Expression levels for *Mus* species were available from previous studies (Lüke et al. 2014b). Expression levels for Arvicolinae species were analyzed using a CFX96 Real Time System / C1000 Thermal Cycler (Bio-Rad). Primers were designed in Primer3 (version 0.4.0) to amplify a product between 70 and 150 bases across an exon-exon junction. Primer sequences are provided in Table S1. In order to assure comparability with the mouse dataset, we used the same methodology (Lüke et al. 2014b). The experiments were done in the same lab, on the same machine and by the same person. The same control gene (*18S rRNA*) was used. In each quantitative PCR (qPCR) run, we included one individual per species and three technical replicates for the two experimental genes (*Prm1*, *Prm2*), and two technical replicates for the standard gene (*18S rRNA*). qPCR reactions were run in 96-well plates with an end volume of 16 µl per sample containing 8 µl SYBR green Master Mix (Invitrogen), 15 ng of each primer and 50 ng/µl of cDNA. The thermocycler program consisted of an initial denaturation of 95°C for 10 min, 40 cycles of 95°C for 15 s and an annealing and elongation stage of 62°C for 1 min. Melt curve analysis was performed for each run.

ANALYSIS OF EXPRESSION DATA

Cycle threshold data (CT) were normalized relative to *18S rRNA* for each plate (Δ CT). To avoid statistical analysis using a dataset of mixed negative and positive values, data were transformed by adding a constant based on the lowest Δ CT value in the joined dataset (*Mus* and Arvicolinae). Expression ratios and percentages were calculated from transformed individual Δ CT values (between 4 and 5 individuals per species), the protamine expression ratio was calculated (*Prm1/Prm2*), and mean values were obtained for each species.

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 (Felsenstein 1985) to test for relationships between relative protamine expression, and corrected testes mass. PGLS analysis was performed using CAPER version 1.0.1 (Orme et al. 2018) package for R (version 3.6.0; R core team 2019), using a phylogenetic tree based on Fabre et al. (2012) (Fig. S1). PGLS analysis estimates lambda as a measure for the phylogenetic signal in the trait data. If lambda is estimated 0, then it can be inferred that the traits show no phylogenetic signal. With a lambda of 1, Brownian motion (for example genetic drift) is inferred. To correct for the effect of body mass on testes mass we included body mass as the first predictor, and testes mass as the second predictor in PGLS regressions (corrected testes mass, which is used as a proxy for sperm competition).

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AUTHOR CONTRIBUTIONS

L.A. participated in the design of the study, generated data, carried out evolutionary and statistical analysis, and drafted the manuscript. D.O.G. generated data. M.T. and M.V.-S. generated data and participated in statistical analyses. E.R.S.R. participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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Supplementary material