BIOLOGY OF REPRODUCTION (2014) **90**(3):67, 1–8
Published online before print 12 February 2014.
DOI 10.1095/biolreprod.113.115956

Evolution of Protamine Genes and Changes in Sperm Head Phenotype in Rodents[1]

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ABSTRACT

Little is known about the genetic basis of evolutionary changes in sperm phenotype. Postcopulatory sexual selection is associated with differences in protamine gene sequences and promoters and is a powerful force acting on sperm form and function, although links between protamine evolution and sperm phenotype are scarce. Protamines are involved in sperm chromatin condensation, and protamine deficiency negatively affects sperm morphology and male fertility, thus suggesting that they are important for sperm design and function. We examined changes in protamine genes and sperm phenotype in rodents to understand the role of sexual selection on protamine evolution and sperm design. We performed a genotype-phenotype association study using root-to-tip dN/dS (nonsynonymous/synonymous substitutions rate ratio) to account for evolutionary rates and phylogenetic generalized least squares analyses to compare genetic and morphometric data. Evolutionary rates of protamine 1 and the protamine 2 domain cleaved off during chromatin condensation correlated with head size and elongation. Protamine 1 exhibited restricted positive selection on some functional sites, which seemed sufficient to preserve its role in head design. The cleaved-protamine 2, whose relaxation is halted by sexual selection, seems to ensure small, elongated heads that would make sperm more competitive. No association existed between mature-protamine 2 and head phenotype, suggesting little involvement during chromatin condensation and a likely role maintaining the condensed state. Our results suggest that evolutionary changes in protamines could be related to complex developmental modifications in the sperm head. This represents an important step toward understanding the role of changes in gene coding sequences in the divergence of germ cell phenotype.

evolutionary rates, positive selection, protamines, sperm head, sperm phenotype

INTRODUCTION

Postcopulatory sexual selection, in the form of sperm competition, is an evolutionary force known to drive rapid adaptation of reproductive traits [1–4] and that has also been linked to rapid diversification of coding sequences of the so-called reproductive proteins [5, 6]. Reproductive proteins include those that act following copulation and that mediate gamete usage, storage, signal transduction, and fertilization [5]. Sperm competition occurs when females mate with more than one male during their receptive period, resulting in rival ejaculates competing for fertilization of the ova [1]. The diversity of male reproductive traits and the adaptive significance of differences in sperm form and function have been analyzed using comparative methods and, more recently, experimental evolution studies [4]. However, the genetic basis of evolutionary changes in sperm phenotype has, so far, received very limited attention in mammals.

The most widely recognized phenotypic response to an increased level of sperm competition is an increase in sperm numbers [2, 4], which enhances a male's chance of fertilization [2, 7, 8]. High sperm numbers may be achieved by an increase in the size of the testes relative to body mass (relative testes mass). Such an increase in relative testes mass is associated with sperm competition in a variety of taxa [2, 4, 9], including mammals [10], and is widely used as a proxy for sperm competition. Another important sperm trait found to be driven by sperm competition in mammals is sperm design (i.e., sperm dimensions and head shape) [11–14]. Sperm design is known to influence sperm function by affecting sperm swimming velocity in many taxa (reviewed in [15]). In mammals, sperm head shape and size may infuence hydrodynamic efficiency of the sperm cell and thereby affect swimming velocity [13, 16]. Sperm that have more elongated heads and heads that are smaller in relation to the length of the flagellum exhibit higher swimming velocity. A smaller and more elongated head is believed to produce less drag during swimming and is therefore thought to positively influence sperm velocity [17]. In rodents, spermatoza from many species display a hook in their rostral region; hook shape and size may also have an impact on sperm swimming velocity [18]. Altogether, the evidence available indicates that sperm head shape and size are important factors in sperm competition because they strongly influence how fast sperm may swim toward the ova.

Proteins of the reproductive system that affect crucial phenotypic traits are thought to experience rapid divergence in their gene sequences [5, 6]. Evidence for a link between elevated evolutionary rate and sexual selection is, however, limited. Studies that examined this relationship tested for correlations between evolutionary rate of the gene sequence and a proxy for sperm competition, that is, relative testes mass, number of mating partners, and level of sexual dimorphism (in primates) in different groups of species. Only four studies have found positive relationships between gene sequence divergence (evolutionary rate) and levels of sperm competition: SEMG2 [19], SVS2 [20], ADAM2 and ADAM18 [21], and zonadhesin [22]. Other studies suggest that in some genes positive selection may not be responsible for fast evolutionary rates but, instead, that such fast evolutionary rate may be due to a relaxation of functional constraints acting on gene sequences.

[1]Supported by the Spanish Ministry of Economy and Competitiveness (grant number CGL2011-26341 to E.R.S.R.). L.L. holds a studentship from the Spanish Research Council (JAEpre-CSIC) which is cofunded by the European Social Fund (ESF). A.V. has a studentship from the FPI programme whereas M.T. holds a "Juan de la Cierva" postdoctoral fellowship, both from the Spanish Ministry of Economy and Competitiveness.
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Received: 13 November 2013.
First decision: 16 December 2013.
Accepted: 31 January 2014.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

These studies identified negative relationships between gene sequence divergence and sperm competition [23, 24].

Protamines are nuclear proteins involved in sperm chromatin condensation. They replace histones and transition proteins during the process of sperm chromatin condensation that takes place in spermiogenesis [25]. In some eutherian mammals, two types of protamines are found: protamine 1 (PRM1) and protamine 2 (PRM2). While PRM1 is present in all mammals, PRM2 is only detected in sperm of primates, most rodents, and a subset of other placental mammals [26, 27]. In contrast to the *Prm1* gene, the *Prm2* gene codes for a precursor (hereafter, PRM2 precursor), which is processed in elongated spermatids by successive proteolytic cleavages. This results in the removal of about 40% of the protein N-terminal region (the removed domain is hereafter referred to as cleaved-PRM2) [25, 26]. The PRM2 form resulting after full cleavage (hereafter, mature-PRM2) consists of 63 amino acids in the mouse. The mature-PRM2 shows very similar structural and functional properties to PRM1 and is proposed to be the result of gene duplication, while cleaved-PRM2 shows no resemblance and might be of retroviral origin [24, 28].

It has been claimed that protamines are the fastest evolving reproductive proteins, with sexual selection being the underlying selective force [29]. Evidence of positive selection in PRM1 has been detected in primates [29, 30], but recent studies have revealed some contrasting results in other mammalian species. In closely related mouse species (genus *Mus*), no evidence of positive selection was found for *Prm1* and only weak positive selection for *Prm2* [31]. In a group of more diverse rodents (voles and hamsters), *Prm1* is conserved with signs of localized positive selection but no evidence of being sexually selected [24]. On the other hand, the *Prm2* gene sequence of voles and hamsters was found to be under relaxation, leading to degradation of the gene. This process is halted by sexual selection removing deleterious mutations in species with higher levels of sperm competition [24]. In closely related species, among which conservation of coding regions may exist, the evolution of gene regulatory regions may be an early stage in speciation. Among protamines, an association was found between the divergence of *Prm2* promoters and levels of sperm competition of mouse species [31]. Divergence in the *Prm2* promoter was also associated with sperm swimming velocity [31], which suggests that changes in regulatory regions could increase the efficiency of DNA condensation in the sperm head, thus, affecting head shape and size and, in turn, sperm performance.

The importance of protamines in sperm chromatin condensation and the influence of protein expression on sperm function have been shown in mouse and human models [26, 32–34]. Alterations in sperm protamine content can have major negative effects on sperm concentration, motility and sperm head morphology in men [33]. Haploinsufficiency of protamines in mice results in sperm morphological abnormalities, DNA damage, and decreases in sperm motility [35]. In particular, PRM2 deficiency has a negative impact on chromatin packaging and sperm head morphology [32]. Incorrect condensation of sperm chromatin results in larger heads as well as head abnormalities [36]. This further supports the idea that protamines are important in sperm head formation and function.

To understand the effect of sperm competition on reproductive trait evolution, both molecular changes and the phenotypic response should ideally be analyzed together. Here we analyze the effect of protamines on sperm head size by examining a possible association between divergence in gene sequences and various sperm head dimensions. In order to compare genetic and morphometric data, we employed methods used in previous studies to analyze genotype-phenotype correlations, that is, the phylogenetic generalized least squares (PGLS) approach (to account for phylogenetic bias in the correlation) using phylogenetic data and root-to-tip dN/dS (nonsynonymous/synonymous substitutions rate ratio, a measure of the evolutionary rate of gene sequences for each species) [37, 38]. Using these approaches, genetic-morphometric associations have been uncovered in studies of microcephaly genes and brain size [37], evolution of RUNX2 and face length [37], and the evolution of ASPM in both an increase and decrease of brain size [39].

We studied a group of rodent species belonging to the family Cricetidae (subfamilies Arvicolinae and Cricetinae) [24]. These species have a wide range of relative testes mass and, therefore, inferred levels of sperm competition and, unlike murids, they show a diverse range of sperm head sizes and a higher level of divergence in the protamine coding sequences. To incorporate the structural and functional differences of the two main PRM2 domains (cleaved-PRM2 and mature-PRM2) we analyzed their respective coding sequences separately to take into account possible differences in how they may affect sperm head size. The *Prm2* gene sequence in this group of species was shown to be under relaxation, which is halted in species with higher levels of sperm competition [24]. Species with high levels of sperm competition therefore show a lower evolutionary rate. Consequently, we expected a negative association between the evolutionary rate of *Prm2* and sperm head size. In addition, although PRM1 was not shown to be under postcopulatory sexual selection in these cricetid rodents, it was still expected to influence sperm head size based on its functional similarity with PRM2 and their joint action in sperm nucleus condensation; therefore, this gene was also included in the study.

MATERIALS AND METHODS

Ethics

All the procedures were carried out following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. The research protocol was approved by the Ethics Committee of the Spanish Research Council (CSIC). Wild specimens were captured with permits from the Comunidad de Madrid and the Junta de Castilla-León, Spain.

Animals

The study included 12 species of the family Cricetidae, eight of which belong to the subfamily Arvicolinae (*Arvicola sapidus, Arvicola terrestris, Clethrionomys glareolus, Chinomys nivalis, Microtus arvalis, Microtus cabrerae, Pitymys duodecimostatus, Pitymys lusitanicus*) and four to the subfamily Cricetinae (*Mesocricetus auratus, Phodopus sungorus*, *Phodopus campbelli, Phodopus roborovskii*) [40]. This group of species has experienced rapid evolutionary radiation and diversification [40], and shows different levels of sperm competition, as suggested by their differences in relative testes mass [24]. Individuals belonging to Arvicolinae were trapped in the field during the breeding season at different locations in Spain [41]. Individuals belonging to Cricetinae stem from laboratory strains purchased from commercial suppliers and were unrelated [24]. We obtained the gene sequences of at least four individuals per species to generate a consensus sequence. For assessments of sperm traits, sample size varied between three and eight individuals for each species. For all the species, size variance (coefficient of variation, CV) was much lower within species than between species (e.g., *Pitymys lusitanicus* ($n = 3$): CV head length = 0.04, CV sperm length = 0.04; all species: CV head length = 0.15, CV sperm length = 0.3). Males were kept in our animal facilities in individual cages under standard laboratory conditions in environmentally controlled rooms (20°C–24°C) on a 14L:10D photoperiod and provided with food and water ad libitum.

Protamine Sequences

Prm1 sequence for *P. sungorus* and *P. roborovskii* and *Prm1* and *Prm2* sequences for *M. auratus* were obtained from the literature [20, 42]. All the other nucleotide sequences were obtained through PCR amplification and sequencing.

DNA Isolation and Gene Amplification

Genomic DNA was extracted from frozen tissues using the E.Z.N.A Tissue DNA kit (Omega, Madrid, Spain) following the manufacturer's recommendations. Protamine sequences were amplified by polymerase chain reaction (PCR). PCR mixtures were prepared in a 50 μl volume containing PCR Gold buffer 1× (Roche, Barcelona, Spain), 2.5 mM $MgCl_2$ (Roche), 0.8 mM dNTPs mix supplying 0.2 mM of each deoxynucleotide triphosphate (Applied Biosystems, Barcelona, Spain), 0.3 mM of forward and reverse primers (Applied Biosystems), 2 units of Taq Gold DNA polymerase (Roche), and 20–100 ng/μl of genomic DNA template. All the PCRs were performed in a Veriti thermocycler (Applied Biosystems). The conditions of the thermocycler program consisted of 35–45 cycles with an initial denaturation of 95°C for 30–40 sec, an annealing stage at 52°C–62°C (depending on template and primers) for 40 sec, and an elongation stage at 72°C for 30–50 sec (depending on gene length). PCR primers were designed on the basis of protamine genomic sequences of other closely related rodent species accessible in the literature or in National Center for Biotechnology Information GeneBank. All the alignments were performed in BioEdit [43], and the most conserved segments within untranslated regions were chosen. When protamines of one or more individuals of each closely related group were sequenced, new specific primers on the basis of these sequences were designed to ensure efficient PCR performance. The primer sequences can be found in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org). PCR products were purified by using the E.Z.N.A. Cycle Pure kit (Omega). In cases in which additional nonspecific bands were obtained after separation in a 1.5% agarose gel, bands of about 600 bp size for *Prm2* and about 300 bp size for *Prm1* were extracted with E.Z.N.A. Gel Extraction Kit (Omega). The purified products were sequenced (Secugen S.L., Madrid, Spain).

Alignments and Trees

Processing of the sequenced fragments was done using the sequence viewer and alignment editor BioEdit [43]. The fragments were reduced to a consensus sequence and trimmed to a coding sequence. These sequences combined with database sequences were aligned on the basis of their amino acid sequences and retranslated using ClustalW implemented in BioEdit [43]. As well as an input alignment, we produced an input tree to calculate the sequence evolution of *Prm1* and both domains of *Prm2* for Cricetidae. *Mus musculus* was used as the outgroup. The phylogenetic tree was built based on information gathered from the literature [31, 40, 41, 44–46] (Supplemental Fig. S1).

Evolutionary Rates (Root-to-Tip dN/dS)

The dN/dS is an indicator of selective pressure at the protein level, with dN/dS = 1 indicating neutral evolution, dN/dS < 1 purifying selection, and dN/dS > 1 diversifying positive selection [47]. To estimate the rates of sequence evolution, we used the application Codeml implemented in PAML 4 [48, 49] through the ETE toolkit [50]. The dN/dS value was generated based on the input tree and input alignment. To obtain species-specific dN/dS values to analyze the relationship between evolutionary rate and sperm head size for each species, we used the free branch model (PAML 4's Codeml) and calculated an dN/dS value for each species by addition of dN values and dS values from the root to the terminal species branch and taking the ratio of the sum to obtain the root-to-tip dN/dS value.

The association between morphometric and genetic data demands the calculation of evolutionary rates that take into account not only selective pressure acting on the terminal branch (i.e., classic free branch model, Codeml in PAML4), but also the accumulated selective pressure on the sequence during its evolution to the tip of the branch (root-to-tip dN/dS) in the selected group of taxa. Calculating an evolutionary rate in this way, values obtained become more comparable with measured phenotypical data because the latter also represent the accumulated evolution rather than being the result of changes solely on the terminal branch [24, 37].

Relative Testes Mass and Sperm Measurements

Animals were killed by cervical dislocation, weighed, and dissected immediately to remove and weigh both testes. Relative testes mass was calculated based on the rodent power function described previously [51] and used as in our previous study [24]. Mature sperm were collected from both epididymides and vasa deferentia as described [41] and suspended in a Hepes-buffered modified Tyrode medium under air [52]. Sperm dimensions were measured in sperm smears stained first with eosin-nigrosin and subsequently with Giemsa as described previously [41]. Spermatozoa were examined at 1000× under bright field, and 200 sperm cells per male were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

PGLS Analysis

Associations between genetic and morphometric traits should also take into account that such traits are not independent from their phylogenetic history. The PGLS approach [53] has been shown to be a powerful tool to detect associations of this kind [54], and it has been used in earlier studies in combination with the root-to tip dN/dS method showing genetic-morphometric associations [24, 37–39]. We performed the PGLS analyses using the program COMPARE 4.6b [55]. The PGLS in COMPARE uses a single parameter, alpha (α), which can be interpreted as the fit of the comparative data with a specific evolutionary model. When $\alpha = 0$, phenotypic change (i.e., change in signal parameters) and phylogenetic distance are linearly related and, thus, the phylogenetic effect is large. When $\alpha > 0$, then phenotypic change and phylogenetic distance are exponentially related, and phylogenetic effects on trait evolution are unimportant, that is, the phylogenetic effect is very low. PGLS trait regressions using a large α are identical to standard, non-phylogenetically corrected regressions [55].

RESULTS

Sperm Measurements and Relative Testes Mass

Sperm head dimensions were analyzed in 12 species of the family Cricetidae. Measurements showed that in these species head length (HL) ranged from 4.75 μm to 8.59 μm (mean ± SEM = 6.94 ± 1.06 μm), head width (HW) varied from 2.86 μm to 4.66 μm (mean ± SEM = 3.44 ± 0.58 μm), and head elongation (HL/HW ratio) ranged from 1.54 to 2.99 (mean ± SEM = 2.06 ± 0.43). Total sperm length was also measured, and it ranged from 62.69 μm to 189.26 μm (mean ± SEM = 111.30 ± 34.01 μm). Because total sperm length varies greatly among these species, and drag resulting from head size should be analyzed taking into account the length of the flagellum [17], relative HL and HW were each calculated as percentages of total sperm length (hereafter, relative HL and relative HW). Calculated values ranged from 4.53% to 8.02% (mean ± SEM = 6.52% ± 0.34%) for relative HL and 1.51% to 4.94% (mean ± SEM = 3.37% ± 0.32%) for relative HW. Relative testes mass, which is used as a proxy of sperm competition levels, varied from 0.18 to 3.61 (mean ± SEM = 1.30 ± 1.10).

Relative Testes Mass and Sperm Head Size

To examine possible links between sperm competition on sperm head size in this dataset, we tested for correlations between species relative testes mass and sperm head measurements. We corrected for phylogenetic effects using the PGLS tool of the program COMPARE 4.6b and a phylogenetic tree reconstructed based on information gathered from the literature (Supplemental Fig. S1). The relative testes mass of cricetid species showed significant negative relationships with relative HL ($\alpha = 15.5$, CI [confidence interval] 95% (PGLS slope) = −1.02 to −0.12, correlation = −0.62) and relative HW ($\alpha = 15.5$, CI 95% (PGLS slope) = −1.10 to −0.23, correlation = −0.68) (Fig. 1, A and B, and Table 1). In addition, it showed a significant positive relationship with head elongation (HL/HW) ($\alpha = 15.5$, CI 95% (PGLS slope) = 0.78 to 2.98, correlation = 0.72) (Fig. 1C and Table 1). There were no significant correlations between relative testes mass and uncorrected HL and HW (Supplemental Table S2).

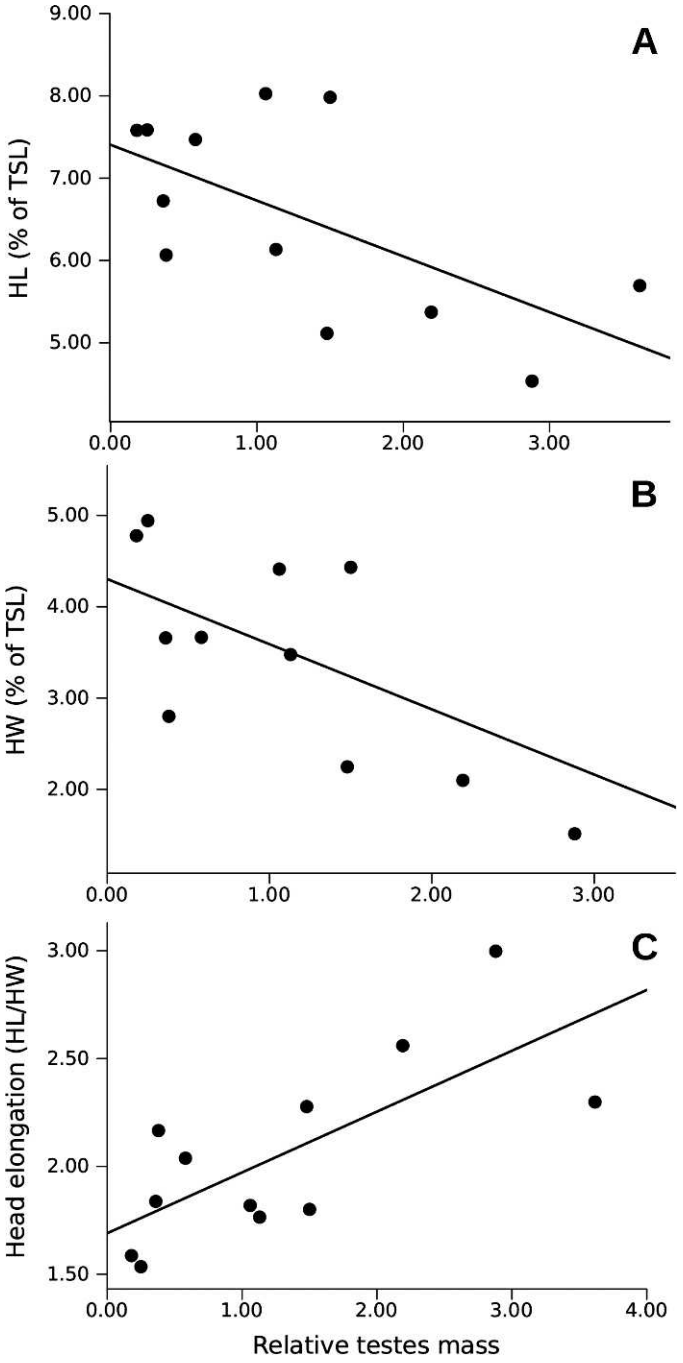

FIG. 1. Scatterplots of significant PGLS relationships between head measurements and relative testes mass. Scatterplots shown for relationships between relative sperm head length (HL) and relative testes mass (**A**), relative sperm head width (HW) and relative testes mass (**B**), and head elongation and relative testes mass (**C**). TSL, total sperm length. Dots represent species and regression lines represent least square slopes without phylogenetic correction. **A**) Slope = −0.68, $R^2 = 0.39$. **B**) Slope = −0.71, $R^2 = 0.48$. **C**) Slope = 0.28, $R^2 = 0.53$.

Evolutionary Rate of Divergence (Root-to-Tip dN/dS) and Sperm Head Size

Root-to-tip dN/dS values of branch analysis (Codeml in PAML4) ranged from 0.12 to 1.17 (mean = 0.58 ± 0.30) for *Prm1*, from 0.19 to 0.51 (mean = 0.33 ± 0.096) for cleaved-*Prm2* and from 0.28 to 1.66 (mean = 1.00 ± 0.55) for mature-*Prm2*. To test the relationship of protamine evolution with sperm head measurements, we correlated the species dN/dS value calculated from the root with the species sperm head measurements. We corrected for phylogenetic effects using the PGLS tool of the program COMPARE 4.6b.

TABLE 1. Relationships of cricetid relative testes mass with head size.

PGLS estimates	Sperm HL (%)[a]	Sperm HW (%)[a]	HL/HW[b]
CI−[c]	**−1.02**	**−1.10**	**0.78**
CI+[c]	**−0.12**	**−0.23**	**2.98**
lnL[d]	−3.82	−2.87	−2.22
Alpha[e]	15.50	15.50	15.50
Corr[f]	−0.62	−0.68	0.72

[a] Presented as percentage of total sperm length.
[b] The ratio between sperm HL and HW, which is an indicator of sperm head elongation.
[c] Confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance) after PGLS analyses.
[d] The maximum likelihood estimate of alpha.
[e] The measure of evolutionary constraints acting on phenotypes; a value of 15.5 is the maximum value calculated by the program (COMPARE 4.6b).
[f] The correlation coefficient.

Prm1 root-to-tip dN/dS values for the different species showed significant positive relationships with both relative HL ($\alpha = 8.82$, CI 95% (PGLS slope) = 0.16 to 0.29, correlation = 0.90) and relative HW ($\alpha = 8.59$, CI 95% (PGLS slope) = 0.16 to 0.31, correlation = 0.88) (Fig. 2, A and B, and Table 2). On the other hand, *Prm1* root-to-tip dN/dS values showed a significant negative relationship with head elongation (HL/HW) ($\alpha = 2.78$, CI 95% (PGLS slope) = −0.73 to −0.14, correlation = −0.67) (Fig. 2C and Table 2). There was a significant relationship between the evolutionary rate of *Prm1* and uncorrected HW, but not with uncorrected HL (Supplemental Table S3).

Cleaved-*Prm2* root-to-tip dN/dS values showed significant positive relationships with relative HL ($\alpha = 7.19$, CI 95% (PGLS slope) = 0.03 to 0.10, correlation = 0.77) and with relative HW ($\alpha = 7.64$, CI 95% (PGLS slope) = 0.03 to 0.11, correlation = 0.76) (Fig. 2, D and E, and Table 2). Furthermore, cleaved-*Prm2* root-to-tip dN/dS values had a significant negative relationship with head elongation (HL/HW) ($\alpha = 7.99$, CI 95% (PGLS slope) = −0.28 to −0.08, correlation = −0.74) (Fig. 2F and Table 2). In contrast, mature-*Prm2* exhibited no significant relationship between root-to-tip dN/dS values and head measurements (Table 2). Relationships with uncorrected HL and HW were nonsignificant for cleaved-*Prm2* and mature-*Prm2* (Supplemental Table S3).

DISCUSSION

In this study, we examined genetic-morphometric associations by analyzing differences in protamine gene sequences and sperm head size and elongation with the aim of understanding the role of sperm competition on reproductive trait evolution. Earlier studies have successfully used this approach to assess genotype-phenotype associations in brain size and face length [37–39] and, to the best of our knowledge, this is the first study to explore this association in germ cells. We found significant associations between the evolutionary rates (root-to-tip dN/dS values) of *Prm1* and cleaved-*Prm2* gene sequences and sperm head morphometry, while no significant relation was found for the mature domain of *Prm2*. The evolutionary rates of both *Prm1* and the cleaved domain of *Prm2* show positive relations with relative HL and relative HW, and negative relations with head elongation (i.e., HL/HW ratio). These parameters of sperm morphometry are also

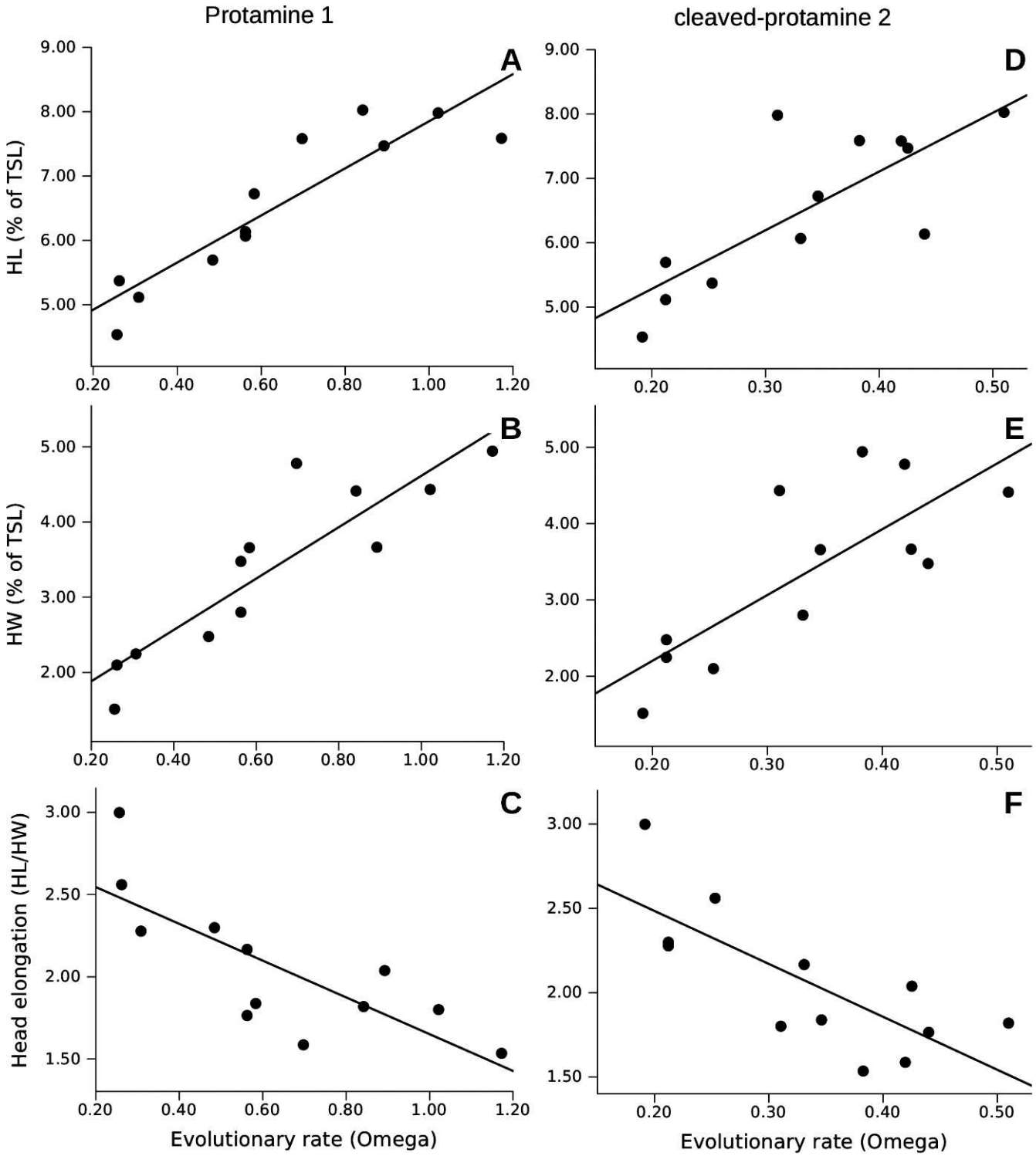

FIG. 2. Scatterplots of significant PGLS relationships between head measurements and protamine evolutionary rates (root-to-tip dN/dS). Scatterplots shown for relationships between relative sperm head length (HL) and evolutionary rate of *Prm1* (**A**), relative sperm head width (HW) and evolutionary rate of *Prm1* (**B**), head elongation and evolutionary rate of *Prm1* (**C**), relative sperm HL and evolutionary rate of cleaved-*Prm2* (**D**), relative sperm HW and evolutionary rate of cleaved-*Prm2* (**E**), and head elongation and evolutionary rate of cleaved-*Prm2* (**F**). TSL, total sperm length. Dots represent species and regression lines represent least square slopes without phylogenetic correction. **A**) Slope = 3.65, $R^2 = 0.82$. **B**) Slope = 3.41, $R^2 = 0.79$. **C**) Slope = −1.12, $R^2 = 0.60$. **D**) Slope = 9.12, $R^2 = 0.61$. **E**) Slope = 8.62, $R^2 = 0.61$. **F**) Slope = −3.14, $R^2 = 0.57$.

significantly associated with the species relative testes mass, which represent inferred levels of sperm competition [10]. Thus, these results report for the first time evidence of a relationship between differences in protamine coding sequences and sperm head shaping.

An analysis of protamine promoter sequences in mouse species found that divergence of *Prm2* promoters, which may result in differences in protamine expression, is positively correlated with sperm velocity [31]. Such a study suggested a possible effect of protamines on head shape and, hence, on the

TABLE 2. Relationships of protamine evolutionary rates with head size.

PGLS estimates	Sperm HL (%)[a]	Sperm HW (%)[a]	HL/HW[b]
Prm1			
CI−[c]	**0.16**	**0.16**	**−0.73**
CI+[c]	**0.29**	**0.31**	**−0.14**
lnL[d]	19.44	18.54	15.03
Alpha[e]	8.82	8.59	2.78
Corr[f]	0.90	0.88	−0.67
Cleaved-*Prm2*			
CI−[c]	**0.03**	**0.03**	**−0.28**
CI+[c]	**0.10**	**0.11**	**−0.08**
lnL[d]	27.48	27.40	26.88
Alpha[e]	7.19	7.64	7.99
Corr[f]	0.77	0.76	−0.74
Mature-*Prm2*			
CI−[c]	−0.02	−0.06	−0.70
CI+[c]	0.41	0.39	0.43
lnL[d]	8.19	7.82	7.11
Alpha[e]	1.32	1.07	0.59
Corr[f]	0.49	0.40	−0.15

[a] Presented as percentage of total sperm length.
[b] The ratio between sperm HL and HW, which is an indicator of sperm head elongation.
[c] Confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance) after PGLS analyses.
[d] The maximum likelihood estimate of alpha.
[e] The measure of evolutionary constraints acting on phenotypes (COMPARE 4.6b).
[f] The correlation coefficient.

sperm hydrodynamic efficiency that, in consequence, may influence sperm velocity. A direct relationship between sequence divergence and sperm head shape or size in this group of closely related mouse species was not apparent [30] perhaps because subtle changes in sperm head phenotype could not be identified with the methods employed in that study.

We found positive relationships between *Prm1* and cleaved-*Prm2* divergence and relative HL and HW in cricetid rodents, a group that started diverging 16–18 million years ago [40], in contrast to a more recent emergence of mouse species, which is thought to have started about 5 million years ago [56]. A higher sequence divergence for both *Prm1* and cleaved-*Prm2* correlates with longer and wider and, therefore, bigger heads in relation to total sperm length. The relationships of sperm head elongation (HL/HW) with sequence divergence were negative for both *Prm1* and cleaved-*Prm2*. A lower sequence divergence coincided with more elongated heads. Analyses of possible associations between sperm head size and elongation with relative testes mass, which we used to infer the levels of sperm competition, revealed a different association between these parameters. Species exhibiting high relative testes mass (i.e., higher inferred sperm competition levels) showed smaller, more elongated sperm heads relative to total sperm length. This agrees with earlier comparative studies in which it was found that more elongated and smaller heads in relation to flagellum length are favored by sperm competition in mammals [13].

Elongation of the sperm head as well as lower relative sperm head size are thought to reduce drag on the sperm cell, increasing its hydrodynamic efficiency and therefore its swimming speed [17, 57]. Because swimming speed is a major factor in fertilization success [58, 59] an improvement of the hydrodynamic efficiency should be strongly favored by postcopulatory sexual selection. The correlations of evolutionary rates of *Prm1* and cleaved-*Prm2* with sperm head size and elongation, and the significant role played by head size and elongation in making sperm more competitive, support the hypothesis that protamines may influence head shape and sperm's hydrodynamic properties and sperm competitive capacity in general.

It is noteworthy that we did not find significant correlations between sequence divergence of mature-*Prm2* and sperm head size and elongation although there were strong relationships between cleaved-*Prm2* and sperm phenotype. Both PRM1 and mature-PRM2 appear to share the role of condensing DNA, whereas the function of cleaved-PRM2 is largely unknown. The uncleaved PRM2 precursor binds to DNA and is cleaved over a period of several days until only the mature-PRM2 is left bound to the DNA [60, 61]. Because DNA condensation has been found to coincide temporally with the start of protamine translation and posttranslational processing [62–64], it is reasonable to envisage that the cleaved-PRM2 domain may have a more important function during the actual process of DNA condensation than the mature-PRM2 domain. Previous studies have focused mainly on mature-PRM2, but our results suggest a more important role for cleaved-PRM2 in the process of DNA condensation and head shaping, thus warranting further studies of the role of cleaved-PRM2 and the evolution of this domain.

Our previous work on the same group of cricetid species found that both cleaved-*Prm2* and mature-*Prm2* are affected by relaxation [24]. Sexual selection was found to halt the relaxation of the *Prm2* gene, as shown by a negative relationship between sequence divergence and relative testes mass. Furthermore, *Prm1* was found to be functionally conserved with directed positive selection on specific functional sites, but it was not influenced by sexual selection [24]. In the present study, we observed that more divergent coding sequences of *Prm1* and cleaved-*Prm2* were associated with proportionately bigger and less elongated sperm heads, traits that may be less favorable in competitive situations, while higher levels of sperm competition associated with relatively smaller and more elongated sperm heads. Our current results thus suggest possible reasons why sexual selection may act to halt relaxation in *Prm2*. Higher sequence divergence in cleaved-*Prm2* seems to be related to an enlargement and reduction of elongation of the sperm head. Thus, divergence in cleaved-*Prm2* might have a negative effect on the hydrodynamic efficiency of the sperm cell, and therefore sexual selection appears to halt relaxation in *Prm2* to preclude such a decrease in hydrodynamic efficiency. Previous results showing no evidence of sexual selection on *Prm1* [24] seem to be at odds with our current results linking changes in head size and elongation with increased *Prm1* sequence divergence. However, in our previous study, *Prm1* was shown to be functionally conserved, allowing rapid changes in very specific functional sites. This functional conservation may be sufficient to ensure a hydrodynamically efficient sperm head shape in competitive situations. Because of its function and efficiency in DNA condensation, *Prm1* can be expected to affect sperm head size and elongation even though it does not appear to be influenced by sexual selection.

In conclusion, this study presents the first evidence for a potential link between divergence in protamine coding sequences and sperm head size and elongation. Because a strong correlation between sperm head phenotype and the PRM2 domain that is cleaved off during sperm chromatin condensation was found, it could be argued that this cleaved-PRM2 domain may have a role in regulating the process of DNA condensation. On the other hand, the role of mature-PRM2 (i.e., the PRM2 domain remaining after cleavage and which stays attached to DNA) might be restricted to maintenance of the condensed state of DNA in the differen-

tiated mature sperm. Lower sequence divergence in *Prm1* and cleaved-*Prm2* may be important for proportionately smaller and more elongated sperm heads, which seems to be favored by sperm competition in this group of rodents. This evidence supports previously hypothesized involvement of protamines in sperm head shaping [31]. Additionally the proposed negative influence of cleaved-*Prm2* divergence on sperm head shaping offers a possible explanation for sexual selection acting to halt relaxation of this sequence in rodents.

ACKNOWLEDGMENT

We appreciate the collaboration of Juan José Luque-Larena and his team in the field work. We thank María Varea Sánchez and Laura Gómez Montoto for their help with the sperm measurements.

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