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POSTCOPULATORY SEXUAL SELECTION INCREASES ATP CONTENT IN RODENT SPERMATOZOA

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Abstract

Sperm competition often leads to increases in sperm numbers and sperm quality, and its effects on sperm function are now beginning to emerge. Rapid swimming speeds are crucial for mammalian spermatozoa, since they need to overcome physical barriers in the female tract, reach the ovum and generate force to penetrate its vestments. Faster velocities associate with high sperm competition levels in many taxa and may be due to increases in sperm dimensions, but they may also relate to higher ATP content. We examined if variation in sperm ATP levels relates to both sperm competition and sperm swimming speed in rodents. We found that sperm competition associates with variations in sperm ATP content and sperm-size adjusted ATP concentrations, which suggests proportionally higher ATP content in response to sperm competition. Moreover, both measures were associated with sperm swimming velocities. Our findings thus support the idea that sperm competition may select for higher ATP content leading to faster sperm swimming velocity.

Introduction

Postcopulatory sexual selection may take place when females mate with multiple males during a single receptive period. This generates opportunities for competition between rival ejaculates for fertilization of one or more ova, i.e., sperm competition (Parker 1970), and cryptic female choice (Thornhill 1983). Competition between sperm from different males to reach the vicinity of the ovum and be the first to engage in fertilization has led to a number of evolutionary adaptations in sperm phenotype in many taxa, such as higher sperm numbers in sperm reserves (reviewed in: Birkhead and Møller 1998; Gomendio et al. 1998; Birkhead and Pizzari 2002; Parker and Pizzari 2010) and higher proportions of spermatozoa that are motile, viable, morphologically normal, possess an intact acrosome and that respond to signals emitted by the ovum (García-González and Simmons 2005; Gomendio et al. 2006; Gómez Montoto et al. 2011a, Rowe and Pruett-Jones 2011). Sperm competition has also been associated with increases in sperm dimensions (Gomendio and Roldan 1991, Tourmente et al. 2009, 2011a,b; reviewed in Gomendio and Roldan 2008), and modifications in sperm head shape (Immler et al. 2007, Tourmente et al. 2011a), which may have important consequences for sperm movement.

In addition, sperm competition has been positively linked to sperm swimming velocity (Kleven et al. 2009; Fitzpatrick et al. 2009; Tourmente et al. 2011a; Gómez Montoto et al. 2011b) which is positively related with fertilization success (Pizzari and Parker 2009). In mammals, sperm are required to swim through barriers in the female tract, such as the uterine cervix or the utero-tubal junction, and along the oviduct to the site of fertilization (Suarez 2008). Thus, the ability of sperm to achieve and sustain high swimming speeds can have a significant impact on male fitness. Comparative studies have shown that increases in sperm dimensions or changes in sperm head shape in

response to sperm competition allow sperm cells to achieve faster swimming speeds (Gomendio and Roldan 1991, 2008; Malo et al. 2006; Gillies et al. 2009; Lüpold et al. 2009; Fitzpatrick et al. 2009; Tourmente et al. 2011a; Gómez Montoto et al. 2011b). However, the underlying cellular mechanisms allowing sperm to swim faster remain unclear.

Spermatozoa may swim faster if they have a higher ATP content (rat: Jeulin and Soufir 1992; carp: Perchec et al. 1995). Sperm energy metabolism is a key factor in sperm function because sustained motility, active protein phosphorylation, and ion regulation generate exceptionally high energetic demands in spermatozoa relative to other cell types (Miki 2007; Garrett et al. 2008). In mammalian sperm, ATP availability is essential for multiple cellular and biochemical processes that are required for successful fertilization, such as capacitation (Visconti et al. 1995; Travis et al. 2001), exocytosis of the acrosomal granule (Fraser and Quinn 1981) and both activated and hyperactivated motility (Fraser and Quinn 1981; Miki 2007). A reduction of internal ATP levels decreases sperm motility, flagellum beating frequency and sperm velocity (Ford 2006; Storey 2008). ATP can be generated either through oxidative phosphorylation by mitochondria located in the midpiece, or by glycolysis in the principal piece (Ford 2006; Ruiz-Pesini et al. 2007; Storey 2008). The latter has been claimed to be the predominant pathway for ATP generation in the mouse (Miki et al. 2004; Mukai and Okuno 2004), perhaps because long sperm, which are typical of muroid rodents, precludes efficient transport of mitochondria-generated ATP down the flagellum (cf. Ford 2006), although this issue remains the topic of considerable debate (Storey 2008).

Spermatozoa move forward as a result of thrust generated by the flagellum, a cell component containing the axoneme whose microtubules are associated with large

ATPases (dyneins). Motility is directly dependent upon the availability of energy obtained through ATP hydrolysis (Ford 2006; Ruiz-Pesini et al 2007; Storey 2008) since ATP utilization by dyneins in motility generation accounts for a high proportion of the total ATP consumption (e.g., ~75% in bull sperm) (Rikmenspoel 1965; Halangk et al. 1985).

To the best of our knowledge, no information exists on whether inter-specific variation in sperm ATP levels are associated with differences in sperm competition levels. Therefore, the aim of this study was to quantify ATP content in spermatozoa from muroid rodents differing in levels of sperm competition. These species were chosen because an earlier comparative study demonstrated that muroid species reflect a wide range of sperm competition levels (Gómez Montoto et al. 2011a). Moreover, this broad range of sperm competition levels allows us to begin to draw more general conclusions regarding the evolutionary implications of sperm competition for traits underlying sperm performance. We hypothesized that higher sperm competition levels would be associated with greater ATP content, and that these high ATP levels would be associated with a greater proportion of motile sperm and faster swimming speeds.

Material and Methods

ANIMALS, MORPHOLOGICAL MEASURES AND SPERM RECOVERY

Adult males from nine species of muroid rodents were studied. Males of *Mus pahari, M. musculus*, *M. spretus, M. minutoides* and *Phodopus sungorus* come from wild-derived colonies which have been kept in captivity for only a few generations in our animal facilities. Males of *Apodemus sylvaticus*, *Chionomys nivalis*, *Myodes glareolus*, and *Microtus arvalis* were trapped in the field during their breeding season (April - June). Animals were maintained under standard conditions (14 h light - 10 h darkness,

22 - 24ºC), with food and water provided *ad libitum*. Each male to be used in this study was housed alone (i.e., in individual cages) for at least a month before sampling to eliminate the possibility that males had a different perceived risk of sperm competition. Samples from all species were collected during spring-summer, which is the reproductive season of the species included in our work, to avoid potential biases due to seasonality. All procedures followed Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

Males (n = 5, except for *M. glareolus* in which n = 4, and *M. arvalis* and *M. minutoides* in which n = 6) were sacrificed by cervical dislocation and weighed immediately. Testes were then removed and weighed. Mature sperm were collected from the caudae epididymides and vasa deferentia by placing tissue in a Petri dish containing Hepes-buffered modified Tyrode's medium (mT-H) (Shi and Roldan 1995) prewarmed to 37º C, and allowing sperm to swim out for a period of 5 min. The volume of medium used was adjusted to provide a concentration of ~20 $x10^6$ sperm/ml, according to previous estimations of total sperm numbers for these species (Gómez Montoto et al. 2011a). Sperm suspensions were maintained at 37º C at all times.

ASSESSMENT OF SPERM MOTILITY AND VELOCITY

Immediately after sperm swim-out, we quantified the percentage of motile spermatozoa and sperm swimming velocity. The percentage of motile sperm was assessed subjectively to the nearest 5% under phase-contrast microscopy. To determine sperm velocity, an aliquot of sperm suspension was placed in a pre-warmed microscopy chamber with a depth of 20 μm (Leja, Nieuw-Vennep, Netherlands) and filmed at 40x using a phase contrast microscope connected to a digital video camera. Sperm curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s) and straight line

velocity (VSL, μm/s) were assessed using a computer assisted sperm analyzer (Sperm Class Analyzer, Microptic SL, Barcelona, Spain). Species values for each velocity parameter were obtained by averaging values of individuals of the same species. Since velocity measures tend to be highly correlated (Gómez Montoto et al 2011b) we sought to obtain an overall variable to integrate the velocity information. Thus, species averages of the three velocity parameters (log_{10}-transformed) were used to perform a principal component analysis (PCA), which extracted two eigenvectors that summarized multivariate velocity variation across all species. Loadings and correlation of the three sperm velocity traits with principal components are available in Table S1. The first principal component (PC1) accounted for 89.4% of the variability on sperm velocity while the second principal component (PC2) only accounted for a 10.6%. The species values for each of the three sperm velocity parameters (VCL, VSL, and VAP) showed a significant positive correlation with PC1 and no correlation with PC2. Thus, we elected PC1 values for each species (hereafter referred to as "overall sperm velocity") as our integrated sperm velocity measure.

DETERMINATION OF ATP CONTENT

ATP concentration was determined using a luciferase-based ATP bioluminescent assay kit (Roche, ATP Bioluminescence Assay Kit HS II). A 100 μl aliquot of (previously diluted) sperm suspension was mixed with 100 μl of Cell Lysis Reagent, vortexed and incubated at room temperature for 5 min. The resulting cell lysate was centrifuged at 12,000 g for 2 min, and the supernatant (sample) was recovered and immediately frozen in liquid N_2. Bioluminescence was measured in triplicate in 96-well plates using a luminometer (Varioskan Flash, Thermo Fisher Scientific Inc.). A total of 50 μl of Luciferase reagent was added to 50 μl of sample (via auto-injection), and, following a 1

s delay, light emission was measured over a 10 s integration period. Standard curves were constructed from measurements obtained from solutions containing known concentrations of ATP, diluted in mT-H and Cell Lysis Reagent (in a proportion equivalent to that of the samples). To estimate the number of spermatozoa in the samples, an aliquot of the original sperm suspension was fixed in 0.1% formaldehyde solution and sperm counted using a modified Neubauer chamber. ATP concentration was expressed as nmol per 10^6 cells. Additionally, because bigger cells might contain greater quantities of ATP, and sperm size differs between these species (see Table S2), we calculated the amount of ATP per unit of sperm length for each species ("length-adjusted ATP concentration"; amol/µm). To do this, we calculated the amount (amoles) of ATP per sperm cell, and divided it by the mean total sperm length for each species. Total sperm length, measured from the most apical point of the sperm head to the last observable portion of the end piece, was assessed in sperm smears stained with Giemsa (Gómez Montoto et al. 2011a,b). Smears were examined at 1000x under bright field: images of 30 cells per male were captured using a digital camera (Digital Sight DS-5M, Nikon, Tokyo, Japan) and image software for microscopy (NIS-Elements F v.2.20, Nikon). Sperm length was obtained for each sperm cell using ImageJ v.1.41 Software (National Institutes of Health, Bethesda, MD, USA).

DATA ANALYSIS

We chose to use relative testes size as a measure of the level of sperm competition of each species. Since testes size relative to body mass is a reliable indicator of investment in sperm production, this trait is considered to be a very good proxy of sperm competition levels in many taxa (Briskie and Montgomerie 1992; Jennions and Passmore 1993; Gage 1994; Stockley et al. 1997; Gomendio et al. 1998; Birkhead and

Møller 1998; Byrne et al. 2002; Brown and Brown 2003). Moreover, a recent comparative study in mammals (Soulsbury 2010) found that levels of multiple paternity correlate very well with relative testes size. Relative testes size appears to be a particularly reliable indicator of sperm competition risk in muroid rodents. An interspecific study in the genus *Apodemus* (Bryja et al. 2008) found a strong relationship between relative testes size and the proportion of multiple paternity. Additionally, studies on *Peromyscus maniculatus* (Long and Montgomerie 2005) and *Mus domesticus* (Firman and Simmons 2008) showed that population-specific sperm competition levels were positively correlated with relative testes size.

To test the effects of sperm competition on percentage of motile sperm, overall sperm velocity and both absolute and length-adjusted ATP concentration, multiple linear regressions were performed using sperm traits as dependent variables and body mass and testes mass as predictors of sperm traits. Since the two independent variables were related to each other (non-orthogonal), a sequential (type I) sum of squares was used, adding the predictors to the model in the following order: body mass, testes mass. Additionally, the effects of ATP amount per cell and length-adjusted ATP concentration on sperm velocity parameters were tested by means of single linear regressions using ATP concentrations as predictors and sperm traits as dependent variables. All variables were $\log_{10}$-transformed, except for percentages of motile sperm which were arcsine-transformed.

Because species trait values may be similar as a result of phylogenetic association rather than selective evolution (Felsenstein 1985; Harvey and Pagel 1991), all regressions were performed using phylogenetic generalized least-squares (PGLS) analyses (Freckleton et al. 2002). PGLS incorporates phylogenetic interdependency among the data points by including the phylogenetic structure within a standard linear

model as a covariance matrix that assumes a predetermined evolutionary model. Then, the branch lengths of the phylogenetic tree are altered (using a scaling parameter) to optimize the fit between the statistical model under test (i.e., the relationship between traits) and the predetermined evolutionary model. In our study, we used PGLS to estimate (via maximum likelihood) a phylogenetic scaling parameter lambda (λ) of the tree's branch lengths that fits evolution proceeding via Brownian motion. If λ values are close to 0, the variables are likely to have evolved independently of phylogeny, whereas λ values close to 1 indicate strong phylogenetic association of the variables. The maximum likelihood value of λ (ML λ) was compared against models with fixed λ=1 and λ=0 by means of a log-likelihood (LL) ratio test which used the following formula: LL ratio = $2 * (LL_{ML\,\lambda} - LL_{fixed\,\lambda})$. Additionally, we calculated the effect size *r* from *F*-values (Rosenthal 1991; Rosenthal 1994; Rosnow and Rosenthal 2003) obtained from the PGLS model; effect sizes > 0.5 were considered large (Cohen 1988). Non-central confidence limits (CLs) for r, which indicate statistical significance if 0 is not contained within the interval (Smithson 2003), were also calculated.

All statistical analyses were performed using a code developed by R. Freckleton for R (v2.15.2; R Foundation for Statistical Computing 2012), which uses the APE (Paradis et al. 2004), MVTNORM (Genz and Bretz 2009), and MASS (Venables et al. 2002) packages. *P* values were considered statistically significant at $\alpha < 0.05$. The phylogenetic reconstruction for species analysed in this study (Fig. S1) was inferred from a phylogenetic hypothesis by Fabre et al. (2012), which was based on 11 nuclear and mitochondrial genes. For graphical representations (Fig. 1,2) relative testis size was calculated using Kenagy and Trombulak's rodent specific regression equation: relative testes size = testes mass/0.031*body mass$^{0.77}$ (Kenagy and Trombulak 1986).

Results

Sperm parameters were assessed immediately upon recovery of spermatozoa from the epididymides. Mean values (± standard error) for body mass, testes mass, relative testes mass and sperm parameters are shown in Table S2. The percentage of motile sperm and overall sperm velocity were significantly related to relative testis size (Figs. 1A, B; Table 1). ATP levels (expressed as amoles of ATP per cell) were also significantly related to relative testes (Fig. 1C; Table 1). Because there are differences in total sperm length of spermatozoa among these species (Table S2), which would impact on cell volume, the ATP content per sperm cell (amol/cell) was corrected taking into account sperm length (hereafter, "length-adjusted ATP concentration"). When this correction was performed, a significant positive relation between length-adjusted ATP concentration (amol/μm) and relative testis size was also obtained (Fig. 1D; Table 1). Thus, the higher the inferred sperm competition level, the higher the ATP content per sperm cell length unit.

ATP is required to propel spermatozoa. We therefore examined if higher ATP levels were associated with a higher proportion of sperm motility and faster swimming speeds. In agreement with this idea, analyses revealed a significant positive relation between both ATP amount per cell and length-adjusted ATP concentrations with the percentage of motile sperm (Table 2). In addition, a positive and significant relation was found between absolute ATP amount per cell and length-adjusted ATP concentrations with overall sperm velocity (Figs. 2A, B; Table 2) supporting the idea that more ATP per length unit results in faster sperm.

Discussion

The results of our study clearly show that species with higher inferred levels of sperm competition have significantly higher amounts of ATP in their spermatozoa, and that the ATP content is significantly related to the proportion of motile sperm and sperm swimming velocity. These findings suggest that the increase in swimming velocity related to sperm competition is, at least partially, determined by an increase in the amount of ATP present in spermatozoa.

We found that ATP levels in sperm cells (expressed as amoles of ATP per cell) revealed a clear positive relation to relative testes size, which is a reliable proxy of sperm competition levels in mammals (Gomendio et al. 1998; Long and Montgomerie 2005; Bryja et al. 2008; Firman and Simmons 2008; Soulsbury 2010). Thus, we infer that in species with high sperm competition levels (i.e. those with relatively larger testes), spermatozoa contain more ATP. Furthermore, ATP amount was positively associated with the proportion of motile sperm and swimming velocity. A relation between sperm ATP content and the proportion of motile sperm has long been recognized for mammalian sperm (Mann 1945a,b) but it is only more recently that a link has been established between sperm ATP content and sperm swimming velocity (Jeulin and Soufir 1992; Burness et al. 2004). Intra-specific studies in fishes have revealed that males who experience higher levels of sperm competition (i.e., sneakers) have higher concentrations of ATP in sperm (Atlantic salmon: Vladić and Järvi 2001; Vladić et al. 2002; bluegill: Burness et al. 2004) although this is not always the case (grass goby *vs.* black goby: Locatello et al. 2007). ATP content has also been related to sperm fertilizing capacity in fish (salmon: Vladić et al. 2002), birds (domestic fowl and turkey: Wishart et al. 1982) and mammals (laboratory mouse: Narisawa et al. 2002; bull: Garrett et al. 2008).

Since sperm cells vary in size between species, we reasoned that, such differences in size should be taken into account to assess possible variations in sperm ATP concentration. However, although information on sperm length is available for various species (Tourmente et al. 2011a), data on sperm volume is scarce and it is not readily calculated for these cells (Du et al. 1994; Yeung et al. 2002). Thus, we used total sperm length to estimate ATP concentration. When the ratio between sperm ATP amount and sperm length was taken into account, a significant relationship was found between the (length-adjusted) ATP concentration and relative testes mass. The relevance of this result is underscored by the finding that length-adjusted ATP concentration of sperm cells was positively related to the percentage of motile sperm and, more importantly, to sperm swimming velocity. Thus, species with higher sperm competition levels have proportionally more ATP, as well as higher sperm swimming velocity.

These findings suggest that since more ATP per sperm length unit (i.e., length-adjusted ATP concentration) translates into higher swimming speeds, an increase in sperm length would need (at least) a proportional increase in ATP content to achieve a higher velocity than a shorter sperm. Without this proportional increase the available ATP per length unit of flagellum may be insufficient to support sperm motility. Furthermore, mammalian species with short sperm would have to invest a relatively lower amount of energy per sperm cell in order to increase sperm velocity than species with long sperm.

In conclusion, our inter-specific analysis provides the first evidence suggesting that, in rodent sperm, sperm competition results not only in enhanced sperm ATP levels but also in a higher ATP concentration. This high ATP content, in turn, associates with higher sperm swimming speeds. Further work on rodent sperm metabolism and

physiology will be required to understand mechanisms underlying ATP production and how they impact sperm swimming ability. Mammalian spermatozoa are capable of using endogenous substrates as well as exogenous sources present in seminal plasma or in the female reproductive tract to synthesize ATP (Ford 2006; Ruiz-Pesini et al. 2007; Storey 2008). However, it is not yet clear what is the relative contribution of the glycolytic and respiratory pathways to ATP generation in rodent species. In addition, it is possible that the importance of these pathways changes during the life of spermatozoa. ATP production may rely on one pathway to sustain sperm motility and survival in the female tract and on a different one during capacitation and hyperactivated motilty, the last steps before spermatozoa interact with female gametes. In this context, morphological variations in absolute and relative sizes and volumes of the sperm's midpiece and principal piece, which have been found to be influenced by sperm competition (Tourmente et al. 2011; Gomendio et al. 2011), may contribute to differences in the energy-producing machinery. A better characterization of factors affecting sperm bioenergetics will undoubtedly help in our understanding of how sperm competition influences sperm function.

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FIGURE LEGENDS

Figure 1. Relation between relative testes size, motility, swimming velocity and ATP concentration in rodent spermatozoa from species differing in sperm competition levels. Relations between relative testes size (*sensu* Kenagy and Trombulak 1986) and (A) percentage of motile sperm, (B) overall sperm velocity, (C) ATP amount per sperm (amol/sperm), and (D) length-adjusted ATP concentration in spermatozoa (amoles/µm of sperm). Overall sperm velocity represents the first component of a principal components analysis that included curvilinear velocity (µm/s), average path velocity (µm/s) and straight-line velocity (µm/s). Black symbols: Muridae; white symbols: Cricetidae. Species abbreviations: ASY: *Apodemus sylvaticus*; CNI: *Chionomys nivalis*; MAR: *Microtus arvalis*; MGL: *Myodes glareolus*; MMI: *Mus minutoides*; MMU: *Mus musculus*; MPA: *Mus pahari*; MSP: *Mus spretus*; PSU: *Phodopus sungorus*.

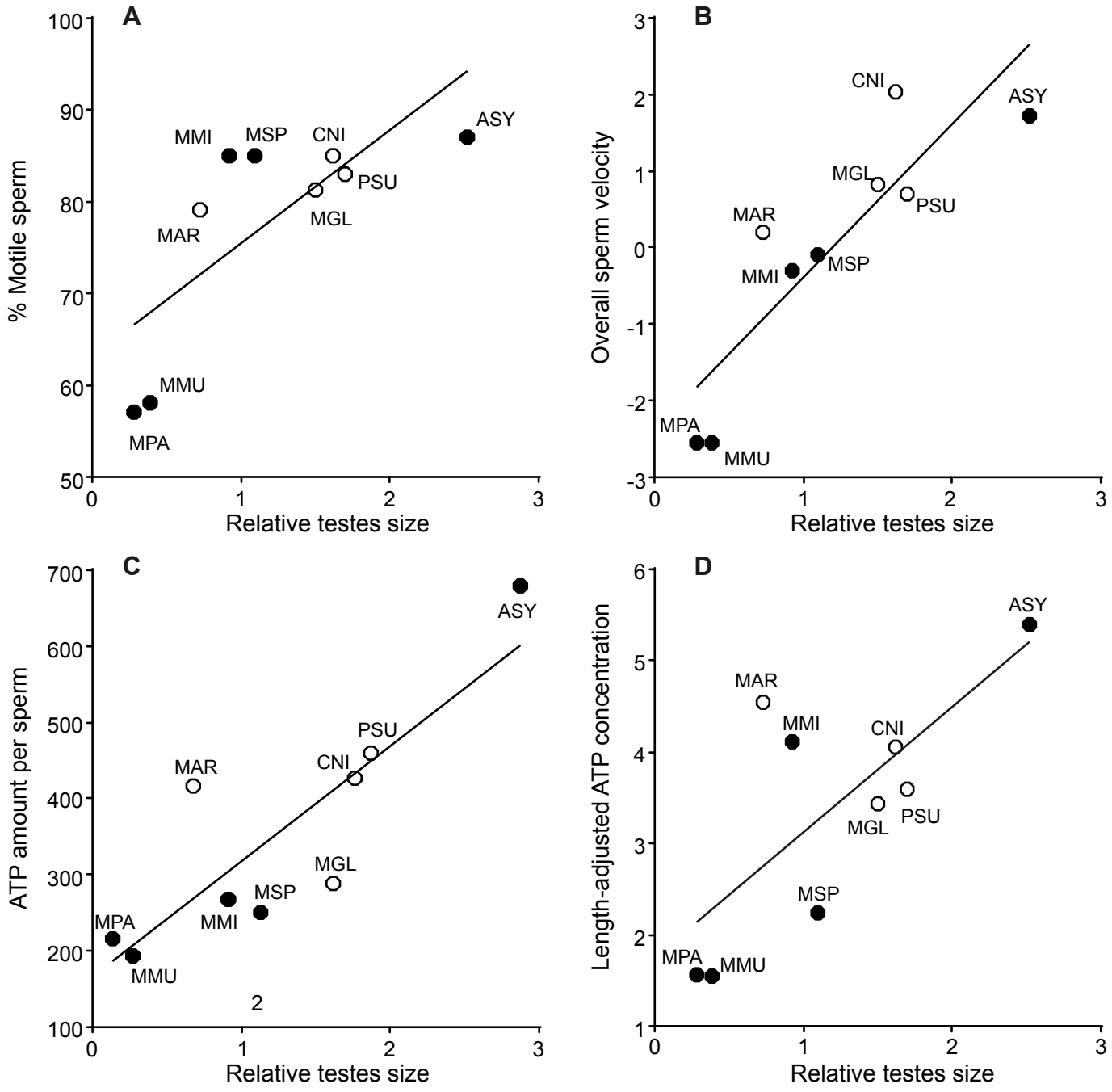
Figure 2. Relation between absolute and length-adjusted ATP concentration and sperm velocity. (A) Relation between ATP amount per sperm (amoles/sperm) and overall sperm velocity, and (B) relation between length-adjusted ATP concentration (amoles/µm of sperm) and overall sperm velocity. Overall sperm velocity represents the first component of a principal components analysis that included curvilinear velocity (µm/s), average path velocity (µm/s) and straight-line velocity (µm/s). Black symbols: Muridae; white symbols: Cricetidae. Species abbreviations: see Figure 1.

Supporting Information

Figure S1. Phylogenetic relationships for the species analyzed in this study. Relationships were inferred from Fabre et al. (2012). Branches corresponding to each species are shaded according to relative testes size (higher RTS values are shaded darker).

Supplementary Table S1. Loadings and correlation of sperm traits with principal components of sperm quality and velocity in Muroid rodent species.

Supplementary Table S2. Body mass, testes mass and sperm parameters for 9 muroid rodent species.


A
% Motile sperm
Relative testes size
ASY
MMI
MSP
CNI
PSU
MGL
MAR
MMU
MPA
B
Overall sperm velocity
Relative testes size
CNI
ASY
MGL
PSU
MAR
MSP
MMI
MPA
MMU
C
ATP amount per sperm
Relative testes size
ASY
PSU
CNI
MAR
MGL
MSP
MMI
MPA
MMU
D
Length-adjusted ATP concentration
Relative testes size
ASY
MAR
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MPA
MMU

Figure 1

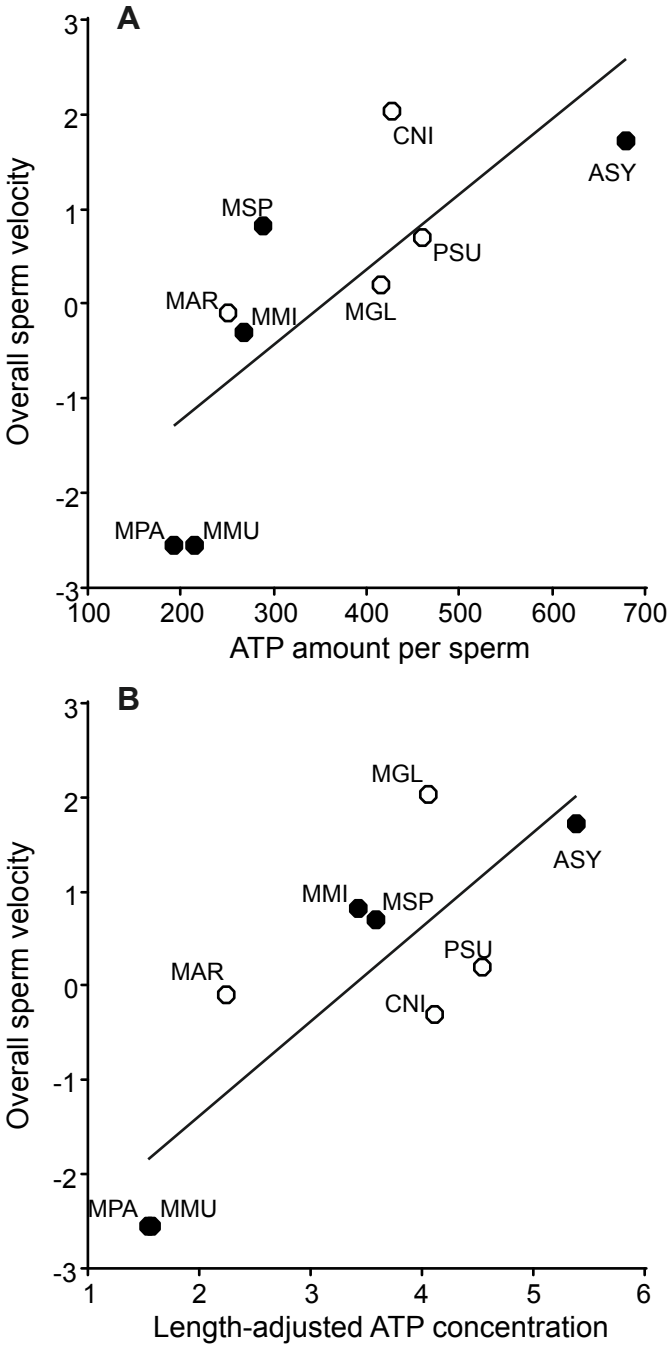
A
Overall sperm velocity
ATP amount per sperm
CNI
ASY
MSP
PSU
MAR
MMI
MGL
MPA
MMU
B
Overall sperm velocity
Length-adjusted ATP concentration
MGL
ASY
MMI
MSP
PSU
MAR
CNI
MPA
MMU

Figure 2

Table 1. Relationship between testes mass relative to body mass and percentage of motile sperm, overall sperm velocity (PCA), ATP amount per cell or length-adjusted ATP concentration in spermatozoa. Phylogenetically controlled multiple regression analyses (PGLS). AICc: Corrected Akaike information criterion. *λ* LR: Log-likelihood ratios for *λ* against models with *λ*=0 and *λ*=1 respectively. Effect size *r*, calculated from the *F*-values and its non-central 95% confidence limits (CLs) are presented. Confidence intervals excluding 0 indicate statistically significant relationships. Statistically significant *P*-values, *λ* LRs and CL are in bold. Overall sperm velocity represents the first component of a principal components analysis that included curvilinear velocity (μm/s), linear velocity (μm/s), and average path velocity (μm/s).

Dependent variable	Independent variable	Slope	R^2	*P*	*F*	AICc	λ value	λ LR	Effect size	CL(-)	CL(+)
Sperm motility	Body mass	-0.4019	0.88	0.4295	0.7173	-18.32	0.0001	0.00, **4.07**	0.3268	-0.4609	1.1394
	Testes mass	0.3749		**0.0007**	41.6517				0.9349	**0.8960**	**2.4963**
Overall sperm velocity	Body mass	-3.4024	0.92	0.3576	0.9923	23.55	0.9999	1.87, 0.00	0.3767	-0.4039	1.1964
	Testes mass	4.5874		**0.0002**	68.6656				0.9590	**1.1329**	**2.7332**
ATP amount per sperm	Body mass	-0.1960	0.73	0.1157	3.3772	-5.27	0.0001	0.00, 1.34	0.6001	-0.1068	1.4935
	Testes mass	0.4270		**0.0119**	12.6679				0.8238	**0.3683**	**1.9686**
Length-adjusted ATP concentration	Body mass	-0.4680	0.67	0.9402	0.0061	-1.84	0.0001	0.00, 0.31	0.0319	-0.7683	0.8321
	Testes mass	0.5051		**0.0131**	12.1186				0.8178	**0.3501**	**1.9504**

Table 2. Relationship between ATP amount per cell or length-adjusted ATP concentration and percentage of motile sperm, and overal sperm velocity. Phylogenetically controlled multiple regression analyses (PGLS). AICc: Corrected Akaike information criterion. *λ* LR: Log-likelihood ratios for *λ* against models with *λ*=0 and *λ*=1 respectively. Effect size *r*, calculated from the *F*-values and its non-central 95% confidence limits (CLs) are presented. Confidence intervals excluding 0 indicate statistically significant relationships. Statistically significant *P*-values, *λ* LRs and CL are in bold. Overall sperm velocity represents the first component of a principal components analysis that included curvilinear velocity (μm/s), linear velocity (μm/s), and average path velocity (μm/s).

Dependent variable	Independent variable	Slope	R^2	*P*	*F*	AICc	λ value	λ LR	Effect size	CL(-)	CL(+)
Sperm motility	ATP amount per sperm	0.4959	0.48	**0.0391**	6.4085	-11.57	0.0001	0.00, **7.92**	0.6913	**0.0503**	**1.6507**
Sperm motility	Length-adjusted ATP concentration	0.0739	0.62	**0.0113**	11.6327	-14.54	0.0001	0.00, **7.22**	0.7901	**0.2716**	**1.8720**
Overall sperm velocity	ATP amount per sperm	7.5341	0.66	**0.0046**	16.8278	28.71	0.0001	0.00, **5.16**	0.8404	**0.4223**	**2.0226**
Overall sperm velocity	Length-adjusted ATP concentration	7.0876	0.62	**0.0025**	21.2044	27.19	0.0001	0.00, **5.87**	0.8671	**0.5210**	**2.1213**