BIOLOGY OF REPRODUCTION (2015) **93**(3):64, 1–13
Published online before print 8 July 2015.
DOI 10.1095/biolreprod.114.127621

Performance of Rodent Spermatozoa Over Time Is Enhanced by Increased ATP Concentrations: The Role of Sperm Competition[1]

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ABSTRACT

Sperm viability, acrosome integrity, motility, and swimming velocity are determinants of male fertility and exhibit an extreme degree of variation among closely related species. Many of these sperm parameters are associated with sperm ATP content, which has led to predictions of trade-offs between ATP content and sperm motility and velocity. Selective pressures imposed by sperm competition have been proposed as evolutionary causes of this pattern of diversity in sperm traits. Here, we examine variation in sperm viability, acrosome integrity, motility, swimming velocity, and ATP content over time, among 18 species of closely related muroid rodents, to address the following questions: (a) Do sperm from closely related species vary in ATP content after a period of incubation? (b) Are these differences in ATP levels related to differences in other sperm traits? (c) Are differences in ATP content and sperm performance over time explained by the levels of sperm competition in these species? Our results revealed a high degree of interspecific variability in changes in sperm ATP content, acrosome integrity, sperm motility and swimming velocity over time. Additionally, species with high sperm competition levels were able to maintain higher levels of sperm motility and faster sperm swimming velocity when they were incubated under conditions that support sperm survival. Furthermore, we show that the maintenance of such levels of sperm performance is correlated with the ability of sperm to sustain high concentrations of intracellular ATP over time. Thus, sperm competition may have an important role maximizing sperm metabolism and performance and, ultimately, the fertilizing capacity of spermatozoa.

ATP, cellular metabolism, gamete biology, rodents (rats, guinea pigs, mice, sperm competition, sperm motility, voles), sperm transport

INTRODUCTION

Sperm viability [1], sperm motility [2–6], and sperm velocity [7, 8] are key determinants of male fertility. During storage in the epididymis, spermatozoa are immotile or barely twitching [9]. Upon ejaculation, when sperm cells come in contact with secretions from male accessory glands and the female reproductive tract, or when they are suspended in incubation media, spermatozoa become motile in a process known as activation. Active motility is important for spermatozoa when negotiating several barriers in the female tract. After being deposited in the vagina, cervix, or uterus, sperm cells will actively swim along the cervix and the uterotubal junction, or only the latter [10]. Active motility also is required for sperm association with the oviductal epithelium in the lower region of the oviductal isthmus, an event that seems to determine the survival of spermatozoa [11]. Thus, the proportion of sperm showing forward motility (% motile sperm) becomes crucial for fertility because a high percentage of motile sperm would increase the chances of fertilization. The duration of sperm motility (i.e., longevity) varies between mammalian species, from a few hours (muroid rodents) to several days (dog, horse) or even months (in some bats) [12], and seems to be related to the interval between mating and the occurrence of ovulation followed by fertilization [13]. In addition, the velocity at which sperm swim appears also to be crucial in the final stages leading to fertilization, when spermatozoa need to reach the vicinity of the ovum [6]. The fastest sperm may be the first to reach the site of fertilization and may be more efficient at penetrating the ovum vestments.

The integrity of the acrosome (the large secretory granule located over the sperm nucleus) is also required at several stages in the sperm's life. Only acrosome-intact sperm are capable of attaching to the oviductal wall and penetrating the oocyte vestments (cumulus oophorus and zona pellucida) [10], and only acrosome-reacted spermatozoa can bind to the oolema [14]. Under physiological conditions, the acrosome reaction is elicited by biochemical signals from the cumulus-oocyte complex and is carefully synchronized with the ovum [15, 16]. However, the loss of the acrosome can occur spontaneously in the absence of an ovum-derived signal (e.g., by damage to the plasma membrane and outer acrosomal membrane), and spermatozoa can remain viable for a short time after the occurrence of this process [17]. Thus, a spontaneous, unsynchronized and premature loss of the acrosome is detrimental for sperm transport and interaction with the ovum.

Mammalian spermatozoa move forward due to the propulsive force generated by the axoneme in the flagellum, whose

[1]Supported by grants CGL2011-26341 to E.R.S.R., CSD2007-00020 and SAF2010-20256 to E.R., and CGL2012-35348 to J.J.L.-L., and a Juan de la Cierva Fellowship JCI-2011-10381 to M.T., all from the Spanish Ministry of Economy and Competitiveness. A JAE-Tec (CSIC) training studentship supported P.V.M.
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Received: 14 December 2014.
First decision: 12 January 2015.
Accepted: 6 July 2015.

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

microtubules are associated with dyneins (ATPases). Thus, sperm motility relies on the continuous supply of ATP [18–20], which is mainly produced either through oxidative phosphorylation by mitochondria located in the midpiece or by glycolysis in the principal piece [18–20]. Several studies support the notion that ATP utilization by dyneins in motility generation accounts for a high proportion of the total sperm ATP consumption [21–24]. Numerous intraspecific in vitro studies showed a close association between sperm internal ATP levels and sperm motility, flagellum beat frequency, and swimming velocity in different mammalian species (mouse [25], rat [26], goat [27]). In muroid rodents, comparative studies revealed that the interspecific variability in sperm motility and velocity was strongly associated to differences in the total content of sperm ATP in freshly collected spermatozoa [28–30].

In addition to motility-related functions, ATP availability in sperm is essential for multiple cellular and biochemical processes that are required for successful fertilization, such as active protein phosphorylation, ion transport [31, 32], and capacitation [33, 34]. Moreover, the maintenance of transmembrane ion gradients that ensure the integrity of the plasma membrane (cell homeostasis), and the stability of the acrosomal vesicle (which relies on low intracellular Ca^{2+} levels) are both dependent on the action of ATP-fueled ion pumps [35–42].

Considering the relations among sperm motility, swimming velocity, and ATP content, some authors have predicted the existence of a trade-off between sperm longevity and sperm velocity [7, 43]. According to these authors, faster swimming sperm would consume more ATP per time unit than slow swimming ones, resulting in an earlier depletion of ATP reserves and decreased longevity. This hypothesis has received support from studies in externally fertilizing species [5, 44] in which sperm are able to produce ATP only by catabolizing endogenous substrates. On the other hand, sperm from internally fertilizing species (such as mammals) may encounter an ample variety of metabolic substrates in the milieu of the female reproductive tract. Moreover, while the relative contribution of different metabolic pathways to total ATP content seems to vary among species and remains the topic of considerable debate [20, 45], it is clear that mammalian sperm are capable of avoiding ATP depletion by using the substrates available in fluids of the female tract [19, 46, 47], thus, making the existence of a trade-off between sperm swimming velocity and longevity less probable.

A final point of analysis are the evolutionary causes for the ample diversity found in sperm traits among closely related species among which postcopulatory sexual selection via sperm competition has been proposed to be an important factor. Sperm competition is a phenomenon that arises from female promiscuity in which sperm from more than one male would compete to fertilize a set of ova [48]. This evolutionary scenario may promote various adaptations in sperm traits leading to an increase in sperm competitiveness and thus the proportion of paternity acquired by a given male. Muroid rodents seem to be a good example of such evolutionary scenario because the observed variability in sperm velocity [30], sperm motility [29], and sperm ATP content [28] among species is likely to result from adaptations to postcopulatory selection. Thus, species with larger testes in relation to body mass (i.e., relative testes size, a proxy for sperm competition level) have higher proportions of motile sperm, faster swimming sperm, and higher sperm ATP content in sperm ready to be transferred to the female tract (i.e., in sperm collected from the epididymis) [28].

In the present study, we evaluated the variation in sperm viability, acrosome integrity, motility, swimming velocity, and ATP content over time among 18 species of closely related muroid rodents. We compared values of sperm traits immediately after sperm collection and after a 3 h period of in vitro incubation in a noncapacitating medium (i.e., mT-H, defined below) [49]. These conditions support sperm survival, with maintenance of motility and good velocity, but without promoting sperm capacitation. The period of incubation was chosen based on previous evidence [13] in order to ensure that a significant percentage of spermatozoa would remain motile after incubation, even in the species with lower motility so that a minimum and a maximum could be recorded. Several questions were addressed: (a) Do sperm from closely related species vary in ATP content after a period of incubation in relation to ATP levels at the start of incubation (i.e., equivalent to ATP levels present upon release from the epididymis/vas deferens)? (b) If so, is the variation in ATP levels related to differences in percentage of viable, acrosome-intact, and motile sperm and to differences in sperm velocity parameters? (c) Are differences in ATP content and sperm performance over time explained by the levels of sperm competition in these species? In other words, would sperm competition promote not only an increase of sperm ATP content in recently collected spermatozoa, which would reflect provisioning during sperm formation and epididymal maturation, but also a sustained ATP synthesis over time such as the one that may take place in the female tract (as recorded after a period of incubation in vitro). And, finally, (d) would ATP content relate to sperm parameters such as acrosome integrity, sperm motility, and sperm velocity?

MATERIALS AND METHODS

Animals, Sperm Collection, and Incubation

Adult males from 18 species of muroid rodents were studied. Males of *Mastomys natalensis* (n = 8), *Micromys minutus* (n = 5), *Mus caroli* (n = 7), *M. macedonicus* (n = 6), *M. minutoides* (n = 10), *M. musculus castaneus* (n = 6), *M. musculus domesticus* (n = 4), *M. musculus musculus* (n = 18), *M. pahari* (n = 10), *M. spicilegus* (n = 13), *M. spretus* (n = 18), *Phodopus campbelli* (n = 5), *P. roborovskii* (n = 6), and *P. sungorus* (n = 7) came from wild-derived colonies that have been kept in captivity for only a few generations in our animal facilities. Males of *Apodemus sylvaticus* (n = 5), *Chionomys nivalis* (n = 5), *Myodes glareolus* (n = 4), and *Microtus arvalis* (n = 6) were trapped in the field during the breeding season (April–June). Animals were maintained under standard conditions (14L:10D, 22°C–24°C) with food and water available ad libitum. Each male to be used in this study was housed alone (i.e., in individual cages) for at least 2 wk before sampling. Samples from all males were collected during spring-summer, which is the reproductive season of these species, to avoid possible biases due to seasonality. Preliminary analyses revealed that there were no differences between males from wild-derived colonies and males of the same species trapped in the field with regards to sperm parameters. The study was approved by the Ethics Committee of the Spanish National Research Council (CSIC). All animal handling was done following the Society for the Study of Reproduction's specific guidelines and standards for experimental animals use, and the Spanish Animal Protection Regulation RD53/2013, which conforms to European Union Regulation 2010/63.

Males were euthanized by cervical dislocation and weighed immediately. Testes were then removed and weighed. Mature sperm were collected from the distal portion of the caudae epididymides and adjoining vasa deferentia. The contents of the vas deferens were pushed toward the epididymal cauda, which was then excised after removing all blood vessels, fat, and surrounding connective tissues. The cauda was then placed in a Petri dish containing Hepes-buffered modified Tyrode medium (mT-H) (pH = 7.4, osmolality = 295 mOsm kg^{-1}) [49] prewarmed to 37°C. Three to five incisions were performed in the distal region of the excised cauda, and sperm were allowed to swim out for a period of 5 min. Care was taken to ensure that sperm collection and time elapsed until parameters were measured was similar for all species. The culture medium mT-H mimics the composition of the mouse oviductal fluid [49] and supports sperm survival, but not capacitation. The volume of medium used was adjusted to provide a concentration of $\sim 20 \times 10^6$ sperm/ml, according to

previous estimations of total sperm numbers for these species [29]. Each resulting sperm suspension was transferred to a plastic tube, and sperm parameters (detailed in the following subsections) were assessed immediately. This time will be referred to as 0 h. Subsequently, sperm suspensions were incubated for 3 h at 37°C in mT-H under air, after which samples were taken and sperm parameters were assessed again. The duration of incubation (3 h) was selected based on preliminary observations of maintenance of sperm motility in vitro in a subset of the species. This period of incubation resulted in an effect of time on motility but without reaching complete sperm immobilization in the species with low sperm survival. Moreover, because fertilization takes place a few hours after copulation in muroid species for which data are available [13, 50–54], our selected incubation time is rather similar to physiological time frames.

Sperm Motility, Viability, Acrosomal Integrity, and Velocity

Species values for each sperm parameter were obtained by averaging the values of individuals of the same species (Supplemental Table S1; all supplemental data are available online at www.biolreprod.org). Percentage of motile sperm (sperm motility) was assessed by examining 10 μl of a previously diluted sperm suspension (concentration $\sim 2 \times 10^6$ sperm ml^{-1} mT-H) placed between a prewarmed slide and a coverslip at 100× magnification under phase-contrast optics. The percentage of motile sperm was estimated subjectively by at least two independent, experienced observers; estimations from the different observers were averaged and rounded to the nearest 5% value. Sperm viability and acrosome integrity were assessed in sperm smears stained first with eosin-nigrosin and subsequently with Giemsa [55]. Briefly, 5 μl sperm suspension and 10 μl eosin-nigrosin solution were mixed on a glass slide placed on a stage at 37°C, and 30 sec later, the mix was smeared and allowed to air-dry. Smears were stained with Giemsa solution and mounted with DPX, a mixture of distyrene, a plasticizer, and xylene. Slides were examined at 1000× under bright field, and 200 spermatozoa per male were examined to evaluate sperm viability and integrity of the acrosome. Viable spermatozoa were those excluding eosin (from the eosin-nigrosin stain). Acrosome integrity was reported as the percentage of sperm with intact acrosomes (observed through Giemsa staining), excluding the cells that showed damaged or missing acrosomes.

To assess sperm swimming velocity, a diluted aliquot of sperm suspension (concentration $\sim 1 \times 10^6$ sperm ml^{-1} mT-H) was placed in a prewarmed microscopy chamber with a depth of 20 μm (Leja) and filmed using a phase contrast microscope (4× objective with pseudonegative phase) connected to a digital video camera. Data of sperm curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec) and straight-line velocity (VSL, μm/sec) were obtained using a computer-aided sperm analyzer (Sperm Class Analyzer; Microptic SL). The software was set with maximum pixel size 250 μm, minimum pixel size 3 μm, connectivity 20, contrast 600, and brightness 60. All the video captures were compared to their overlaying analyzed tracks and rectified if required. Because velocity measures tend to be highly correlated [30], we obtained an overall variable to integrate the velocity information by performing a principal component analysis (PCA) using the species averages of the three $\log_{10}$-transformed velocity parameters. The PCA extracted two eigenvectors that summarized multivariate velocity variation across all the species. Loadings and correlation of the three sperm velocity parameters with principal components are shown in Supplemental Table S2. The first principal component (PC1) accounted for 79% of the variability of sperm velocity at time 0 h and 90% at time 3 h, whereas the second principal component only accounted for 21% and 10% at 0 and 3 h, respectively. The species values for each of the three sperm velocity parameters (VCL, VSL, and VAP) were significantly correlated with PC1 at time 0 h and 3 h. VCL was the only parameter significantly correlated with second principal component, and only at 0 h. Thus, we elected PC1 values for each species as our integrated sperm velocity measure (hereafter referred to as overall sperm velocity, OSV).

Sperm ATP Content and Length-Adjusted ATP Concentration

Sperm ATP content was measured using a luciferase-based ATP bioluminescent assay kit (ATP Bioluminescence Assay Kit HS II; Roche). A 100 μl aliquot of 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 $12\,000 \times g$ for 2 min, and the supernatant was recovered and frozen in liquid N_2. Bioluminescence was measured in triplicate in 96-well plates using a luminometer (Varioskan Flash; Thermo Fisher Scientific Inc.). In each well, 50 μl of luciferase reagent were added to 50 μl of sample (via autoinjection), and, following a 1 sec delay, light emission was measured over a 10 sec integration period. Standard curves were constructed using solutions containing known concentrations of ATP diluted in mT-H and Cell Lysis Reagent in proportions equivalent to that of the samples. Sperm numbers in each sample were estimated by fixing a diluted aliquot of the sperm suspension (concentration $\sim 4 \times 10^5$ sperm ml^{-1} mT-H) in a 0.1% formaldehyde solution before lysis and counting the sperm present in a modified Neubauer chamber. ATP content was expressed as amol $sperm^{-1}$ (i.e., 10^{-18} mol $sperm^{-1}$) Because larger cells might contain greater quantities of ATP due to increased internal volume, differences in cell size were taken into account when assessing possible differences in sperm ATP concentration. Because the volume is proportional to length for cylindrically shaped objects, we calculated the length-adjusted ATP concentration (amol μm^{-1}) as a proxy of ATP concentration. This measure was calculated as the ratio between the amount of ATP per sperm for each species and its total sperm length, which was measured as described previously [28]. Total sperm length was quantified in sperm smears stained with Giemsa, which were examined at 1000× under bright field. Images of 30 cells per male were captured using a digital camera (Digital Sight DS-5M; Nikon) and image software for microscopy (NIS-Elements F v.2.20; Nikon). Sperm length was obtained for each sperm cell using ImageJ v.1.45s Software (National Institutes of Health).

Data Analysis

All the variables were $\log_{10}$-transformed prior to ratio calculations, proportional differences calculations, and statistical analysis except for percentages of motility, viability, and acrosomal integrity, which were arcsine-transformed. Single linear regressions were performed to test the effects of length-adjusted ATP concentration on sperm acrosome integrity, motility, and velocity parameters, using length-adjusted ATP concentrations as predictors and each of the three sperm traits as dependent variables.

To assess associations with sperm competition levels, we used relative testes size as proxy. Testes size relative to body mass is a reliable indicator of investment in sperm production and is considered to be a very good measure of sperm competition levels in many taxa [56–63]. Furthermore, relative testes size appears to be a particularly reliable indicator of sperm competition risk in mammals in general [63], and muroid rodents in particular [64–66], because studies in species of these groups have found strong relationships between relative testes size and the proportion of multiple paternity. Thus, we selected this measure to estimate the level of sperm competition of species in our study.

Multiple linear regressions were performed to test the effects of sperm competition on sperm motility, acrosome integrity, viability, ATP content per cell, length-adjusted ATP concentration, and sperm velocity, using each sperm trait as dependent variable and body mass and testes mass as predictors. Because one major aim of this study was to analyze the influence of sperm competition on the ability of sperm to preserve acrosome integrity and to sustain motility and swimming velocity over a period of time (and thus assess survival), the analyses described above were performed using data from time 0 h and from time 3 h separately. In addition, differences between beginning and end of incubations were calculated as proportional variation (Δ) = (value at 3 h − value at 0 h)/(value at 0 h) and analyzed in relation to testes mass relative to body mass as proxy of sperm competition.

All regressions were performed using phylogenetic generalized least-squares analyses (PGLS) [67] because species trait values may be similar as a result of phylogenetic association rather than independent selective evolution [68, 69]. PGLSs incorporate 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. PGLS estimates (via maximum likelihood) a phylogenetic scaling parameter lambda (λ) of the tree's branch lengths that fits evolution proceeding via Brownian motion. In our study, the length of all branches was set to 1. 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. Additionally, we calculated the effect size r from t-values obtained from the PGLS model and the noncentral confidence limits (CLs) for the z-transformed value of r [70]. The CLs value indicates that the effect size is statistically significant if 0 is not contained within the interval [71].

All statistical analyses were performed using the CAPER v0.5 [72] package for R (v3.0.1; R Foundation for Statistical Computing 2013). P values were considered statistically significant at $\alpha < 0.05$. Residual testes mass was calculated as the residual of a log-log linear regression of testes mass on body mass ($P = 0.0012$, $R^2 = 0.49$) and used exclusively to illustrate the results. The phylogenetic reconstruction used in the PGLS analyses was inferred from previous studies [73, 74] and is presented in Supplemental Figure S1.

RESULTS

Sperm Parameters and Changes During Sperm Incubation

Values for sperm parameters are given in Supplemental Table S1. The percentage of viable cells at 0 h was relatively high in all species, ranging between 70% (*M. musculus castaneus*) and 99% (*P. campbelli* and *M. minutoides*) and did not show a marked decrease after 3 h of incubation (mean decrease = 2%, Fig. 1A).

On the other hand, all the remaining sperm traits exhibited a relatively higher decrease after 3 h of incubation. Acrosome integrity at 0 h ranged from 62% in *M. pahari* to 99% in *M. glareolus* and exhibited a mean decrease of 24% at 3 h (Fig. 1B) with values ranging from 40% in *M. musculus musculus* to 83% in *M. minutoides*. Percentage of motile cells followed a similar pattern, ranging (at 0 h) from 63% in *M. musculus musculus* to 88% in *P. roborovskii* and decreasing a mean 25% after 3 h of incubation (range at 3 h: 30% in *M. musculus castaneus* to 80% in *P. campbelli*) (Fig. 1C). VCL (range: 88–163 μm s^{-1}), VSL (range: 49–110 μm s^{-1}), and VAP (range: 63–126 μm s^{-1}) presented decreases of 11%, 22%, and 20%, respectively. OSV (Fig. 1D), a variable that summarizes VCL, VSL, and VAP as a result of a PCA, showed a mean decrease of 23%. These results validate the choice of a 3 h period of incubation because it ensures a high percentage of sperm survival while allowing for a moderate decrease in sperm motility and velocity. Sperm ATP content (Fig. 1E) showed a high degree of interspecific variability at 0 h, ranging from 130 amol ATP $cell^{-1}$ in *M. musculus domesticus* to 680 amol ATP $cell^{-1}$ in *A. sylvaticus*. These species also represented the extreme values of length-adjusted ATP concentration (Fig. 1F): 1.05 and 5.39 amol ATP μm^{-1}, respectively. Both variables showed a decrease of 30% after 3 h of incubation in mT-H.

Overall, the results described above show that sperm traits (with the exception of viability) tend to decrease after 3 h of incubation in mT-H (a noncapacitating medium). The values for sperm traits in freshly extracted sperm were strongly correlated with their values after 3 h of incubation (Table 1). That is, species that showed high sperm viability, acrosome integrity, motility, velocity (Supplemental Fig. S2), and ATP content at 0 h tended to remain at the high end of the distribution at 3 h, while species that showed low values on these traits at 0 h remained at the low end of the distribution at 3 h (Table 1). One species, *M. minutoides*, exhibited an exceptionally marked decrease in OSV after the 3 h incubation period (Fig. 1D and Supplemental Fig. S2). Because the data point represented by this species had a disproportionate statistical leverage and was not representative of the general trend (Supplemental Table S3), we opted not to include this species in analyses dealing with sperm velocity at 3 h.

The decrease in sperm traits after the period of incubation did not exhibit the same magnitude in all species. Thus, we calculated the proportional variation between 0 h and 3 h (Δ 0 h − 3 h, see *Materials and Methods* for details of calculations) for sperm viability, acrosome integrity and motility, and analyzed their relationships with its equivalent for OSV to test for the possible existence of trade-offs between traits over the time of incubation. In the case a trade-off existed between high sperm velocity and the proportional variation of each trait under consideration, a negative relationship would be expected, that is, a low proportional decrease in sperm velocity would be associated to high proportional decreases in sperm viability, acrosome integrity, and motility. We did not find any significant associations between proportional variation of sperm velocity and sperm viability (Fig. 1G and Table 1) and acrosome integrity (Fig. 1H and Table 1). Remarkably, we found a significant positive association between the proportional variation of sperm velocity and sperm motility (Fig. 1I and Table 1). This suggests that species that can maintain a higher proportion of their initial sperm velocity after 3 h of incubation are also able to maintain a higher proportion of their initial sperm motility.

Relationship Between ATP Content and Viability, Acrosome Integrity, Motility, and Velocity

The percentage of viable cells was not significantly correlated with length-adjusted sperm ATP concentration at 0 h or at 3 h (Fig. 2, A and B, and Table 2), neither its proportional decrease was related to that of ATP content (Fig. 2C and Table 2). On the other hand, our analysis revealed significant positive associations between length-adjusted ATP content and acrosome integrity (Fig. 2, D and E, and Table 2) both in freshly collected sperm and after 3 h. However, the relation between the proportional 3 h decrease of these two parameters was nonsignificant (Fig. 2F and Table 2).

Previous studies have shown that because ATP is required to propel spermatozoa, higher ATP levels tend to be associated with a higher proportion of sperm motility and faster swimming speeds in spermatozoa collected from the epididymis and vas deferens [28]. In agreement with earlier results, significant increases in percentage of motile sperm (Fig. 2G) and overall sperm motility (Fig. 2J) were associated with higher length-adjusted ATP concentrations in the present, larger dataset (Table 2). The positive relationship between increasing length-adjusted ATP concentration and percentage of motile sperm (Fig. 2H) and sperm velocity (Fig. 2K) remained significant after 3 h of incubation (Table 2). In addition, the proportional variation on length-adjusted ATP concentration after incubation (Δ 0 h − 3 h) predicted the variation in percentage of motile sperm (Fig. 2I and Table 2) and OSV (Fig. 2L and Table 2).

Relationships Between Relative Testes Mass and Sperm Parameters

Data for body mass and testes mass used to test the effect of relative testes mass on sperm traits are presented in Supplemental Table S1. The analysis of freshly collected sperm (0 h) revealed that the percentage of viable cells, which was very similar among species (Fig. 1A), showed no association with relative testes mass (Table 3). In contrast, significant positive relationships between relative testes mass and the values of all other sperm parameters emerged when they were assessed immediately upon collection (i.e., at 0 h). Thus, the percentage of sperm with intact acrosomes (Fig. 3A and Table 3), percentage of motile sperm (Fig. 3D and Table 3), OSV (Fig. 3G and Table 3), ATP content per sperm (Table 3), and length-adjusted ATP concentration (Fig. 3J and Table 3) showed a significant positive association with relative testis size. In addition, two of the three sperm velocity measures (VCL and VAP) that made up our OSV measure increased significantly with relative testes mass (Supplemental Fig. S3, A, D, and G, and Supplemental Table S3).

In agreement with results observed at 0 h, the percentage of viable cells was not significantly related to relative testes mass after 3 h of incubation in mT-H (Table 3), and the positive relationships between relative testes mass and sperm parameters observed at 0 h were also detected after a 3 h incubation period (Table 3). The percentage of sperm with intact acrosome exhibited a positive trend, with marginal statistical significance (Fig. 3B and Table 3). The percentage of motile sperm (Fig. 3E

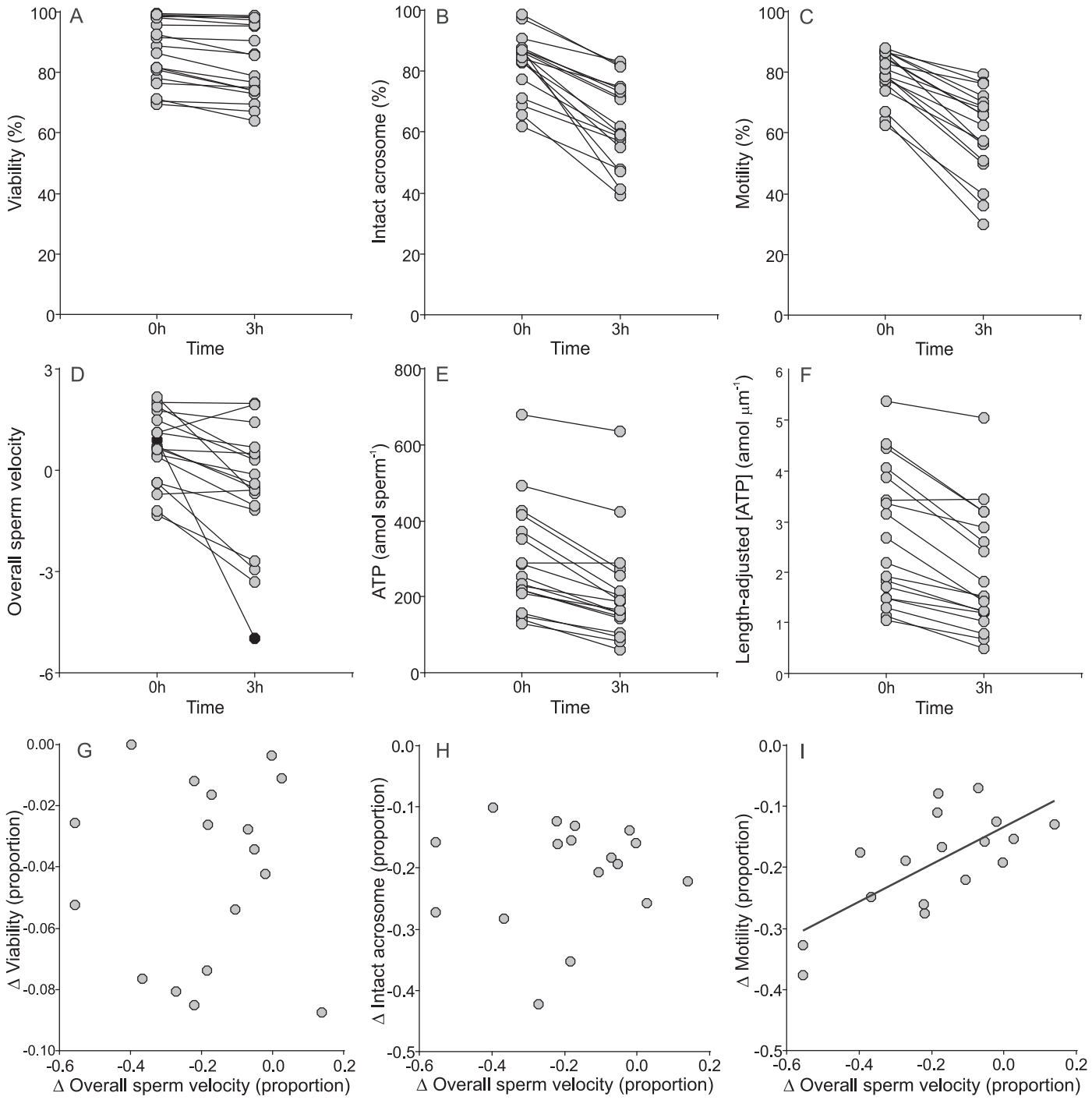


FIG. 1. Variation in sperm parameters of 18 muroid rodent species after 3 h of incubation in mT-H. **A**–**F**) Sperm parameters measured immediately after collection (0 h) and after incubation for 3 h. Percentage of viable sperm (**A**). Percentage of sperm with intact acrosome (**B**). Percentage of motile sperm (**C**). Overall sperm velocity (OSV): score of the first principal component (PC) of an analysis including curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) (**D**). The black dot represents *M. minutoides*. ATP content per sperm (**E**). Length-adjusted ATP concentration: ratio between ATP content per sperm and total sperm length (**F**). **G**–**I**) Relationships between proportional differences over time, that is, Δ = (value at 3 h − value at 0 h)/(value at 0 h), between freshly collected sperm and sperm incubated for 3 h. Δ of OSV and Δ percentage of viable sperm (**G**). Δ of OSV and Δ percentage of sperm with intact acrosome (**H**). Δ of OSV and Δ percentage of motile sperm (**I**).

and Table 3), OSV (Fig. 3H and Table 3), ATP content per sperm (Table 3), and length-adjusted ATP concentration (Fig. 3K and Table 3) were positively and significantly related to relative testis size. Additionally, all three velocity parameters included in the OSV measure were strongly associated with relative testes mass (Supplemental Fig. S3, B, E, and H, and Supplemental Table S3).

To test the effect of sperm competition on the changes in sperm parameters after 3 h of incubation, we analyzed the possible association between relative testes size and proportional variation (Δ 0 h − 3 h) for each parameter. The variation in the percentage of viable sperm was not associated to relative testes mass (Table 3). Similarly, there was no relation between relative testes mass and variation in the percentage of sperm

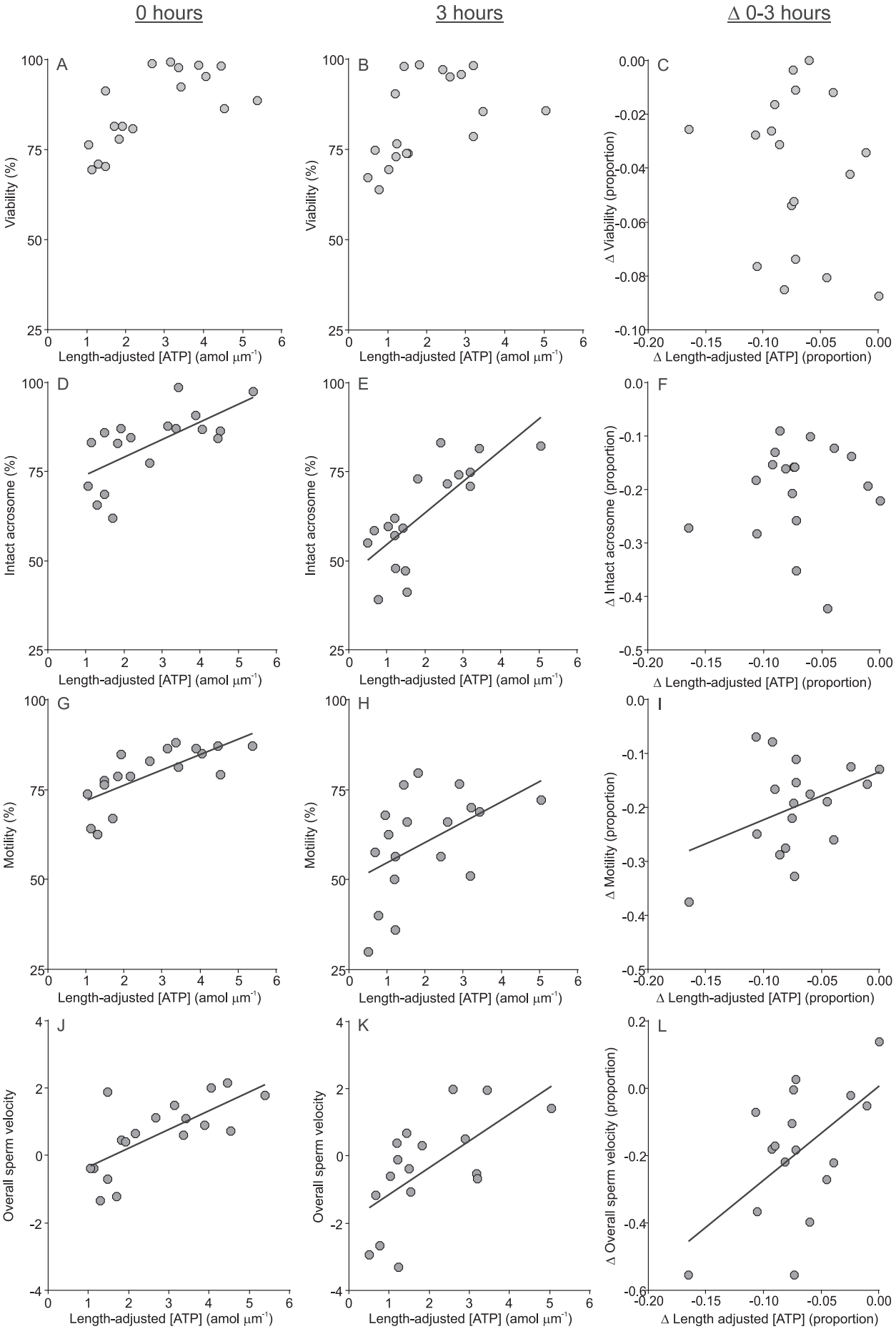


TABLE 1. Relations between sperm parameters measured at 0 h and after 3 h of incubation in Hepes-buffered modified Tyrode medium (mT-H) using phylogenetically controlled multiple regression analyses (PGLS).[a]

Dependent variable	Independent variable	Slope	R^2	t	P	λ[b]	r	CL(−)	CL(+)
Viability 3 h	Viability 0 h	1.0583	0.96	19.7756	**<0.0001**	$<0.001^{ns,*}$	0.9802	**1.7953**	**2.8074**
Intact acrosome 3 h	Intact acrosome 0 h	0.6284	0.56	4.5547	**0.0003**	$0.554^{ns,ns}$	0.7514	**0.4700**	**1.4822**
Motility 3 h	Motility 0 h	1.2751	0.71	6.1856	**<0.0001**	$0.855^{ns,ns}$	0.8397	**0.7142**	**1.7263**
ATP content per sperm 3 h	ATP content per sperm 0 h	1.1882	0.86	10.0826	**<0.0001**	$<0.001^{ns,*}$	0.9295	**1.1488**	**2.1609**
Length-adjusted [ATP] 3 h	Length-adjusted [ATP] 0 h	1.0922	0.87	10.5183	**<0.0001**	$<0.001^{ns,*}$	0.9347	**1.1882**	**2.2004**
Overall sperm velocity 3 h	Overall sperm velocity 0 h	0.9002	0.24	2.2222	**0.0410**	$0.127^{ns,ns}$	0.4856	**0.0243**	**1.0364**
Overall sperm velocity 3 h[c]	Overall sperm velocity 0 h[c]	0.9680	0.46	3.6058	**0.0026**	$0.128^{ns,*}$	0.6814	**0.3079**	**1.3556**
Δ 0 h − 3 h viability	Δ 0 h − 3 h overall sperm velocity	−0.0323	0.04	−0.7941	0.4395	$<0.001^{ns,*}$	−0.2009	−0.7275	0.3202
Δ 0 h − 3 h intact acrosome	Δ 0 h − 3 h overall sperm velocity	−0.0859	0.05	−0.9031	0.3808	$0.415^{ns,ns}$	−0.2271	−0.7549	0.2927
Δ 0 h − 3 h motility	Δ 0 h − 3 h overall sperm velocity	0.2559	0.46	3.5605	**0.0028**	$0.857^{ns,ns}$	0.6768	**0.2993**	**1.3470**

[a] Effect size r calculated from the t values and the noncentral 95% confidence limits (CLs) for the z-transformed value of r are presented. Confidence intervals excluding 0 indicate statistically significant relationships. P-values and CL that indicate statistical significance are shown in bold. Overall sperm velocity (OSV) represents the first component of a principal components analysis that included curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), and average path velocity (VAP, μm/sec).
[b] Superscripts following the λ value indicate significance levels (n.s. $P > 0.05$; * $P < 0.05$) in likelihood ratio tests against models with $\lambda = 0$ (first position) and $\lambda = 1$ (second position).
[c] *M. minutoides* excluded from the analysis.

with intact acrosome (Fig. 3C and Table 3), although there were relations between relative testes mass and acrosome integrity at both 0 and 3 h. In contrast, the proportional variation after incubation in percentage of motile sperm (Fig. 3F and Table 3) and OSV (Fig. 3I and Table 3), and its three constituent variables (Supplemental Fig. S3, C, F, and I, and Supplemental Table S3), was positively related to relative testes mass because declines in these sperm parameters was almost nonexistent as relative testes mass increased. Finally, the variation in sperm ATP content (Table 3) and length-adjusted ATP concentration (Fig. 3L and Table 3) over time was positively related to relative testes mass. Thus, species with higher relative testes mass showed a proportionally lower decrease in several sperm parameters after 3 h of incubation.

DISCUSSION

The results of the present study revealed a high degree of interspecific variability in the sperm traits measured soon after sperm collection. We also observed that most sperm parameters tended to have lower values after a 3 h incubation period. In addition, our study revealed that species with high sperm competition levels exhibit higher levels of sperm motility and faster sperm swimming velocity when sperm cells are incubated under conditions that support sperm survival. Furthermore, maintenance of such levels of sperm performance was due to the ability of sperm to sustain high concentrations of intracellular ATP over time.

Sperm acrosomal integrity, motility, OSV, and ATP content showed a mean decrease of between 20% and 30% of their initial value after spermatozoa were incubated for 3 h in vitro. An exception to this pattern was sperm viability, which revealed a much lower average decrease (2%). Importantly, the magnitude of decrease of each parameter was not the same among the 18 species included in this study. The values of all the variables in freshly collected sperm were strongly correlated with those of sperm incubated for 3 h. This indicated that spermatozoa of species that had high values for sperm traits immediately after collection maintained these high values after the 3 h incubation.

We found that ATP concentrations predicted sperm acrosome integrity, motility, and swimming velocity, whereas no relationship was found with sperm viability; these associations remained significant after 3 h of incubation. In addition, the proportional decrease in sperm motility and swimming velocity (but not in acrosome integrity) between 0 and 3 h was positively related to the proportional decrease in sperm ATP concentration. Thus, the sperm of species that were able to better maintain their ATP supply over the incubation period, showed a higher postincubation sperm velocity and motility. Positive associations between intracellular ATP content and sperm motility and velocity have been reported before in intraspecific and interspecific studies in mammals [25, 26, 28, 45, 75, 76] and fish (reviewed in [44]). However, the present study is the first to analyze the time-related variation of these associations in a comparative data frame of closely related species with ample variation of sperm performance. The absence of relation between the decreases in ATP content and acrosomal integrity is somewhat surprising, taking in account that (a) the two variables were highly correlated at 0 and 3 h and (b) both the stability, exocitosis, and internal pH of the acrosomal vesicle are regulated by ATP-dependent ion (Na^+/K^+ and Ca^{2+}) transporters in mammalian sperm [35–42]. However, because sperm motility accounts for about 70% of total ATP consumption in these cells [23], it is possible that the traits related to sperm motility are more sensitive to a decrease in intracellular ATP

FIG. 2. Relationships between length-adjusted ATP concentration and sperm parameters in 18 muroid rodent species. Parameters measured in freshly collected sperm at 0 h (**A**, **D**, **G**, **J**). Parameters measured after 3 h of incubation in mT-H (**B**, **E**, **H**, **K**). Proportional differences over time, that is, Δ = (value at 3 h – value at 0 h)/(value at 0 h) (**C**, **F**, **I**, **L**). Percentage of viable sperm (**A**, **B**, **C**). Percentage of sperm with intact acrosome (**D**, **E**, **F**). Percentage of motile sperm (**G**, **H**, **I**). Overall sperm velocity (OSV): score of the first principal component (PC) of an analysis including curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) (**J**, **K**, **L**).

TABLE 2. Relations between sperm ATP content, viability, acrosome integrity, and velocity using phylogenetically controlled regression analyses (PGLS).[a]

Time	Dependent variable	Independent variable	Slope	R^2	t	P	λ[b]	r	CL(−)	CL(+)
0 h	Viability	Length-adjusted [ATP]	0.5466	0.18	1.8859	0.0776	$0.999^{*,ns}$	0.4265	−0.0505	0.9616
	Intact acrosome	Length-adjusted [ATP]	0.8517	0.42	3.3981	**0.0037**	$<0.001^{ns,*}$	0.6474	**0.2648**	**1.2769**
	Motility	Length-adjusted [ATP]	0.5902	0.58	4.6992	**0.0002**	$<0.001^{ns,*}$	0.7615	**0.4937**	**1.5058**
	Overall sperm velocity	Length-adjusted [ATP]	5.9444	0.43	3.5007	**0.0030**	$<0.001^{ns,*}$	0.6586	**0.2842**	**1.2964**
3 h	Viability	Length-adjusted [ATP]	0.2279	0.03	0.6768	0.5082	$0.999^{*,ns}$	0.1668	−0.3377	0.6745
	Intact acrosome	Length-adjusted [ATP]	0.9115	0.69	5.9578	**<0.0001**	$<0.001^{ns,*}$	0.8302	**0.6828**	**1.6950**
	Motility	Length-adjusted [ATP]	0.6730	0.29	2.5499	**0.0214**	$0.814^{ns,ns}$	0.5375	**0.0946**	**1.1068**
	Overall sperm velocity	Length-adjusted [ATP]	6.9501	0.38	3.0310	**0.0084**	$<0.001^{ns,*}$	0.6163	**0.1952**	**1.2429**
Δ 0 h − 3 h	Viability	Length-adjusted [ATP]	−0.1792	0.05	−0.9206	0.3710	$<0.001^{ns,*}$	−0.2243	−0.7342	0.2779
	Intact acrosome	Length-adjusted [ATP]	−0.3890	0.04	−0.8073	0.4313	$0.570^{ns,ns}$	−0.1978	−0.7065	0.3056
	Motility	Length-adjusted [ATP]	1.0275	0.26	2.3726	**0.0305**	$0.928^{*,ns}$	0.5102	**0.0569**	**1.0690**
	Overall sperm velocity	Length-adjusted [ATP]	2.8960	0.33	2.7251	**0.0157**	$0.548^{ns,ns}$	0.5754	**0.1318**	**1.1795**

[a] Effect size r calculated from the t values and the noncentral 95% confidence limits (CLs) for the z-transformed value of r are presented. Confidence intervals excluding 0 indicate statistically significant relationships. P-values and CL that indicate statistical significance are shown in bold. 0 h: freshly collected sperm; 3 h: sperm incubated for 3 h in Hepes-buffered modified Tyrode medium (mT-H); Δ 0 h − 3 h: over time proportional differences between freshly collected sperm and sperm incubated for 3 h in mT-H. Overall sperm velocity (OSV) represents the first component of a principal components analysis that included curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), and average path velocity (VAP, μm/sec).
[b] Superscripts following the λ value indicate significance levels (n.s. $P > 0.05$; $^*P < 0.05$) in likelihood ratio tests against models with $\lambda = 0$ (first position) and $\lambda = 1$ (second position).

TABLE 3. Relations between relative testes mass and sperm traits using phylogenetically controlled multiple regression analyses (PGLS).[a]

Time	Dependent variable	Independent variable	Slope	R^2	t	P	λ[b]	r	CL(−)	CL(+)
0 h	Viability	Body mass	−0.2937	0.37	−2.9400	**0.0101**	$0.999^{*,ns}$	−0.6046	**−1.2065**	**−0.1943**
		Testes mass	0.1451		1.6722	0.1152		0.3964	−0.0867	0.9254
	Intact acrosome	Body mass	−0.4819	0.51	−3.8328	**0.0016**	$0.747^{ns,ns}$	−0.7034	**−1.3801**	**−0.3679**
		Testes mass	0.3519		3.3828	**0.0041**		0.6578	**0.2829**	**1.2951**
	Motility	Body mass	−0.3173	0.77	−6.7785	**<0.0001**	$0.323^{ns,*}$	−0.8683	**−1.8321**	**−0.8199**
		Testes mass	0.2277		6.2873	**<0.0001**		0.8514	**0.7552**	**1.7674**
	Overall sperm velocity	Body mass	−1.4914	0.24	−1.7220	0.1056	$0.867^{ns,ns}$	−0.4063	−0.9372	0.0749
		Testes mass	1.5841		2.1668	**0.0468**		0.4882	**0.0277**	**1.0398**
	ATP content per sperm	Body mass	−0.2102	0.31	−1.6345	0.1230	$0.999^{*,ns}$	−0.3888	−0.9165	0.0957
		Testes mass	0.2898		2.5946	**0.0203**		0.5566	**0.1218**	**1.1339**
	Length-adjusted [ATP]	Body mass	−0.2934	0.64	−4.9900	**0.0002**	$0.999^{*,ns}$	−0.7900	**−1.5774**	**−0.5653**
		Testes mass	0.1206		2.3615	**0.0322**		0.5206	**0.0711**	**1.0832**
3 h	Viability	Body mass	−0.2796	0.25	−2.2281	**0.0416**	$0.999^{*,ns}$	−0.4987	**−1.0536**	**−0.0415**
		Testes mass	0.1362		1.2495	0.2306		0.3070	−0.1888	0.8233
	Intact acrosome	Body mass	−0.3623	0.40	−3.1466	**0.0067**	$0.665^{ns,ns}$	−0.6306	**−1.2484**	**−0.2363**
		Testes mass	0.1909		2.0281	0.0607		0.4639	−0.0038	1.0083
	Motility	Body mass	−0.4500	0.79	−5.7180	**<0.0001**	$<0.001^{ns,*}$	−0.8280	**−1.6877**	**−0.6755**
		Testes mass	0.4162		7.6107	**<0.0001**		0.8912	**0.9218**	**1.9340**
	Overall sperm velocity	Body mass	−1.4914	0.24	−1.7220	0.1056	$<0.001^{ns,*}$	−0.4181	−0.9692	0.0785
		Testes mass	1.5841		2.1668	**0.0468**		0.5011	**0.0270**	**1.0747**
	ATP content per sperm	Body mass	−0.2960	0.43	−1.9414	0.0712	$0.999^{*,ns}$	−0.4481	−0.9884	0.0237
		Testes mass	0.4453		3.3624	**0.0043**		0.6556	**0.2789**	**1.2911**
	Length-adjusted [ATP]	Body mass	−0.3206	0.61	−4.8032	**0.0002**	$0.999^{*,ns}$	−0.7785	**−1.5475**	**−0.5354**
		Testes mass	0.1954		3.3706	**0.0042**		0.6565	**0.2805**	**1.2927**
Δ 0 h – 3 h	Viability	Body mass	−0.0214	0.02	−0.5730	0.5751	$<0.001^{ns,*}$	−0.1464	−0.6535	0.3587
		Testes mass	0.0094		0.3624	0.7221		0.0932	−0.4126	0.5995
	Intact acrosome	Body mass	0.0324	0.12	0.3927	0.7001	$0.678^{ns,ns}$	0.1009	−0.4048	0.6073
		Testes mass	−0.0821		−1.2166	0.2426		−0.2997	−0.8152	0.1969
	Motility	Body mass	−0.1791	0.57	−2.7300	**0.0155**	$<0.001^{ns,*}$	−0.5761	**−1.1627**	**−0.1506**
		Testes mass	0.2006		4.4010	**0.0005**		0.7507	**0.4685**	**1.4806**
	Overall sperm velocity	Body mass	−0.1907	0.48	−0.9708	0.3481	$<0.001^{ns,*}$	−0.2511	−0.7805	0.2672
		Testes mass	1.5841		3.0997	**0.0078**		0.6380	**0.2309**	**1.2785**
	ATP content per sperm	Body mass	−0.0436	0.25	−1.1664	0.2617	$0.894^{ns,ns}$	−0.2884	−0.8029	0.2093
		Testes mass	0.0685		2.1612	**0.0473**		0.4873	**0.0264**	**1.0386**
	Length-adjusted [ATP]	Body mass	−0.0435	0.25	−1.1648	0.2623	$0.894^{ns,ns}$	−0.2880	−0.8025	0.2097
		Testes mass	0.0685		2.1611	**0.0473**		0.4873	**0.0264**	**1.0385**

[a] Effect size r calculated from the t values and the noncentral 95% confidence limits (CLs) for the z-transformed value of r are presented. Confidence intervals excluding 0 indicate statistically significant relationships. P-values and CL that indicate statistical significance are shown in bold. 0 h: freshly collected sperm; 3 h: sperm incubated for 3 h in Hepes-buffered modified Tyrode medium (mT-H); Δ 0 h − 3 h: over time proportional differences between freshly collected sperm and sperm incubated for 3 h in mT-H. Overall sperm velocity (OSV) represents the first component of a principal components analysis that included curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), and average path velocity (VAP, μm/sec).
[b] Superscripts following the λ value indicate significance levels (n.s. $P > 0.05$; $^*P < 0.05$) in likelihood ratio tests against models with $\lambda = 0$ (first position) and $\lambda = 1$ (second position).

content. In that case, sperm acrosomal (and other membranes) integrity would be affected only under severe ATP depletion.

When we tested the influence of sperm competition on sperm traits, our results revealed that species with higher sperm competition levels showed higher sperm motility, a higher proportion of sperm with intact acrosomes, faster sperm swimming velocities, and higher sperm ATP concentrations in sperm freshly collected from epididymis (i.e., without incubation), which is in agreement with previously published results [28, 29]. Notably, for sperm traits examined after 3 h of incubation, the positive associations remained significant. Moreover, the motility, swimming velocity, and ATP content of sperm from species with higher sperm competition levels showed a lower decrease over time in sperm trait values than those from species with low competition levels.

A notable exception to this pattern is the unusual decline of sperm velocity in *M. minutoides* after 3 h of incubation. In this case, the pronounced decrease in sperm velocity does not seem to be related to low sperm competition levels because the relative testes mass of *M. minutoides* places this species in an intermediate range of sperm competition level (testes mass represent ~1.9% of body mass). Moreover, all the remaining sperm traits for this species (viability, acrosomal integrity, motility percentage, and length-adjusted ATP content) are around the intermediate range of the distribution even after incubation and conform to the trend observed for the rest of the species (values at 0 h predict values at 3 h). Additionally, the decline in velocity seems not to be associated with a decrease in ATP content or proportion of motile cells in *M. minutoides*. One peculiarity of this species is that it is the smallest within the dataset (and one of the smaller extant mammals species), with a mean adult weight of 4–6 g [77], a feature that could account for the relatively fast decline of this species' sperm velocity. Because the size of the female reproductive tract tends to be related to body mass in mammals [78], the sperm of *M. minutoides* could be adapted to provide a shorter burst of high speed to cover a relatively shorter distance in comparison to larger muroid species.

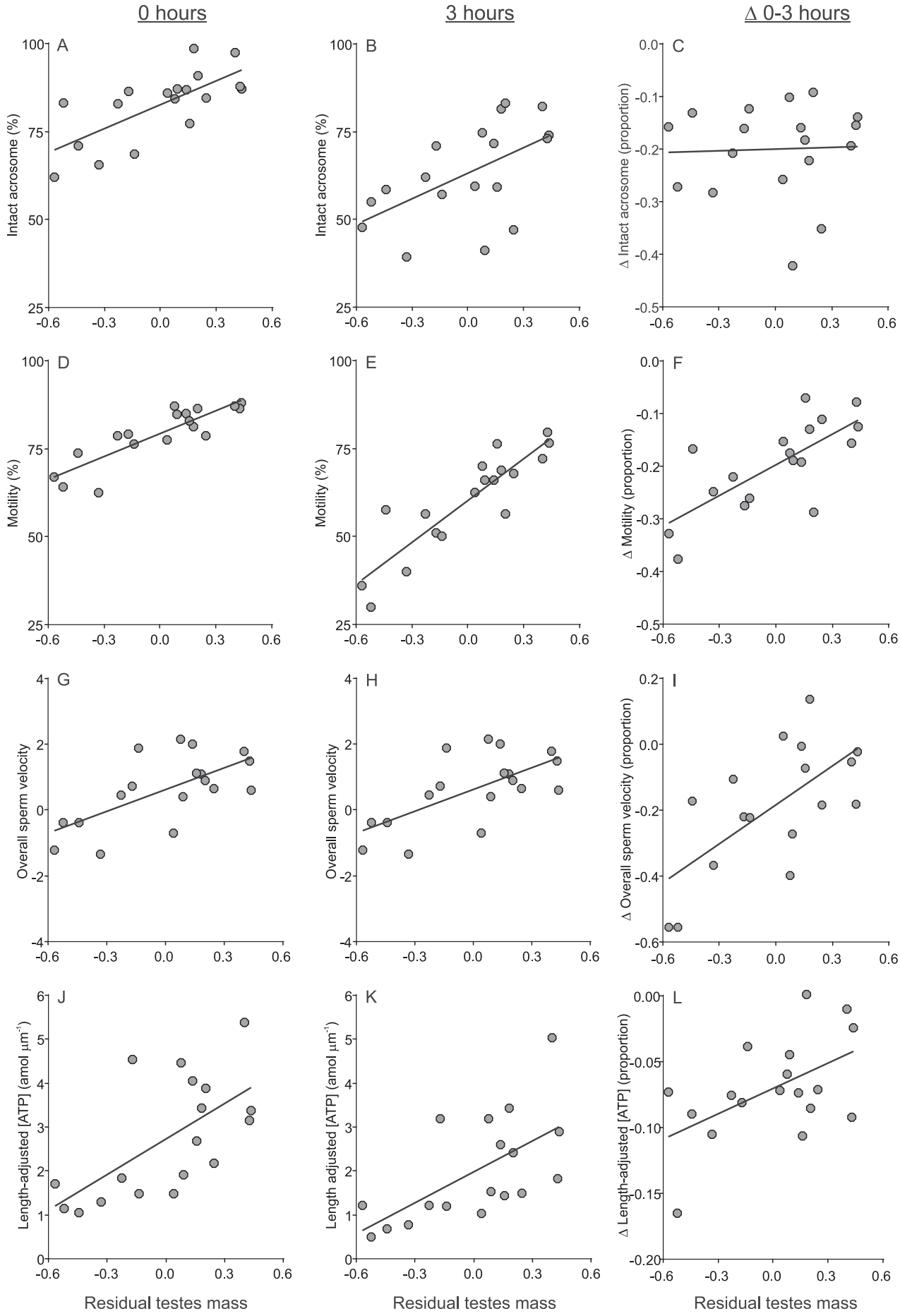
There are fundamental differences among taxa regarding the sources used by spermatozoa for ATP production. In externally fertilizing species that spawn in close proximity, fertilization occurs within a very short period of time (although there may be some exceptions [79]). In these species, the total duration of flagellar activity usually ranges from seconds to tens of minutes, and the energy necessary for flagellar beating may be only supplied by intracellular ATP stores present at the time of spawning and via rapid ATP synthesis from endogenous substrates [44]. In this metabolic scenario, with a very short and intense burst of energy output, the initial ATP load would probably be the only energetic variable with a significant impact on sperm competitiveness.

In contrast, sperm from internally fertilizing species, such as mammals, may use endogenous substrates as well as a variety of exogenous substrates present in seminal plasma or in the female reproductive tract to synthesize ATP [18–20, 45]. In muroid rodents, fertilization may take place a few hours after copulation [13, 50–53], a period during which sperm need to remain viable and capable of sustaining motility in order to actively negotiate barriers in the female tract (i.e., cervix and utero-tubal junction), undergo capacitation, acquire hyperactivation, gain fertilizing ability, and swim along the oviduct toward the site of fertilization [10, 11]. In the species of this group in which sperm physiology has been more thoroughly studied, mature sperm stored in the cauda epididymis prior to activation have similar ATP levels than freshly activated sperm [80, 81]. However, when extracted and activated in the absence of external metabolic substrates, sperm initial ATP reserves are rapidly consumed [25, 26], and sperm become immotile [25, 26] and unable to remain hyperactivate [25].

A recent study in passerine birds [82] showed that, while levels of intracellular ATP were related to midpiece length, higher energy content did not translate into faster-swimming sperm and was not related to sperm competition level. The differences in the relationship between sperm competition and sperm ATP content observed between passerine birds and muroid rodents may be explained by the differences between avian and mammalian fertilization mechanisms. In birds, sperm enter sperm-storage tubules, where they remain motile (often for many days) in order to avoid displacement by the fluid current generated by epithelial cells [83]. Rowe et al. [82] hypothesize that because sperm storage duration prior to fertilization may vary among species the optimum rate of available ATP usage would vary too. In this way, initial swimming velocity would not be determined by the amount of intracellular ATP available at the moment of ejaculation because its rate of usage would reflect the duration of species-specific sperm storage periods [82]. In the absence of exogenous substrates, an increase in the duration of sperm motility would increase the competitiveness of an ejaculate. This hypothesis has been supported by results showing a negative relationship between sperm swimming speed and duration of female sperm storage in passerine birds [84]. However, according to previous studies, the epithelial cells of the storage tubules would secrete oxidizable lipidic components and glycolysable sugars into the lumen of the tubules (reviewed in [85]), suggesting that bird sperm could have an assortment of metabolic substrates at their disposal along the storage period.

Altogether, the differences regarding the timing of the fertilization process along with the results of the present study, suggest that muroid rodents would face an intermediate scenario between the frantic, short-timed rush of external fertilizers and the long-time survival process of passerine birds. In rodents, the scenario would be more akin to a race divided in stages where sperm that could sustain a fast swimming speed for the duration of the active movement phases would have the advantage. Thus, sperm competition would promote not only an increase of sperm ATP content at the time of sperm transfer to the female reproductive tract, but also a sustained ATP synthesis over time. It should be borne in mind that the temporal constraints imposed by female reproductive physiology have been demonstrated to influence sperm physiology in mammals. Thus, a positive association between sperm fertile lifespan and the interval between the onset of oestrus and ovulation has been found in mammals [78]. Regrettably, information about the actual interval of time that elapses between copulation and fertilization in many muroid rodents is scarce and is only described in a handful of species [13, 50–54]. Therefore, while a rather clear pattern emerges from our results, the timing of fertilization under species-specific physiological conditions is likely to constitute a fundamental factor regarding the functional implications of sperm metabolic features.

When fertilization takes place under the conditions that are common in external fertilizers (limited energetic resources, short competitive time frame), a trade-off between sperm velocity and motility duration (i.e., sperm longevity) would arise. Because sperm would be constrained by their finite amount of endogenous substrate reserves, highly competitive scenarios would favor faster swimming sperm, whereas sperm-limited scenarios would promote longer motility duration [5, 7, 44]. However, among internal fertilizers, the limitation of



energetic resources depends on the composition of the fluids of the female reproductive tract [19, 46, 47]. Thus, in conditions where substrates are abundant and sperm have evolved flexible and adaptable metabolic processes to face the changing relative concentrations of exogenous metabolic substrates in the different regions of the female tract (as in the case of muroid rodents) [12, 20, 31], the trade-off between sperm velocity and motility duration might not exist. An intraspecific study in the house mouse [86] showed a significant positive relationship between sperm velocity and sperm motility decrease, supporting the idea of a trade-off between these two traits. In contrast, our results indicate that the species that experienced a lower over-time decrease in sperm velocity also presented a lower decrease in percentage of motile sperm and that the magnitude of the reduction in both traits was associated with the variation in sperm ATP concentration. Moreover, the decrease in the values of these three sperm parameters (motility, velocity, and ATP content) was predicted by sperm competition level, suggesting that species with high levels of sperm competition are able to sustain a high proportion of fast moving sperm over time.

It would be intuitive to think that the above-mentioned metabolic feature could be the result of a higher substrate oxidation capacity, in which case, the species with higher sperm respiratory or glycolytic rates would be able to synthesize ATP at a faster rate to meet the cellular energy demands and thus allow cells to be propelled at faster velocities and for longer time. Nonetheless, the high substrate turnover rates could not be solely responsible of the ATP content diversity. Numerous studies have identified the presence of a variety of signaling pathways operated by sperm-specific kinases that contribute to the control of sperm motility [87–92]. Moreover, some of these pathways include enzymes that are sensitive to variations in intracellular ATP concentrations [93] and have specific functions in the modulation of ATP production [92, 94, 95]. Thus, it is highly probable that the species-specific sperm motility phenotype is a result of the complex interplay between the total energetic input capabilities of the cell, represented by its substrate turnover rate, and the regulatory pathways that control this process and its translation to flagellar motility. In this scenario, the sequence, expression, and posttransductional regulation of the enzymes belonging to these regulatory pathways would constitute likely targets of selection by sperm competition. In conclusion, sperm competition favors spermatozoa that generate and sustain more energy (ATP) over time, preventing a high decrease in ATP concentration, which allows sperm cells to maintain higher levels of sperm performance.

ACKNOWLEDGMENT

We are grateful to François Bonhomme and Annie Orth (Institut des Sciences de l'Evolution, CNRS-Université Montpellier 2, France) for facilitating the purchase of animals. We also thank Juan Antonio Rielo for managing the animal facilities and Esperanza Navarro for animal care at the Museo Nacional de Ciencias Naturales (CSIC), Madrid.

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FIG. 3. Relationships between residual testes mass and sperm parameters in 18 muroid rodent species. Parameters measured in freshly collected sperm (0 h) (**A**, **D**, **G**, **J**). Parameters measured after 3 h of incubation in mT-H (**B**, **E**, **H**, **K**). Proportional differences over time, that is, Δ = (value at 3 h – value at 0 h)/(value at 0 h) (**C**, **F**, **I**, **L**). Percentage of sperm with intact acrosome (**A**, **B**, **C**).Percentage of motile sperm (**D**, **E**, **F**). Overall sperm velocity (OSV): score of the first principal component (PC) of an analysis including curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) (**G**, **H**, **I**). Length-adjusted ATP concentration: ratio between ATP content per sperm and total sperm length (**J**, **K**, **L**).

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BOR Papers in Press. Published as DOI: 10.1095/biolreprod.114.127621.

Performance of rodent spermatozoa over time is enhanced by increased ATP concentrations. The role of sperm competition[1]

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Supplemental Data

Files in this data supplement:

1. Supplemental Table S1. Body mass, testes mass and sperm parameters at 0 and 3 hours of incubation for 18 species of muroid rodents. Values for sperm parameters are for 0 and 3 h of incubation in mT-H medium.

2. Supplemental Table S2. Loadings and correlation of sperm traits with principal components of sperm velocity in muroid rodents.

3. Supplemental Figure S1. Phylogenetic tree for the species analyzed in this study.

4. Supplemental Figure S2. Relationship between overall sperm velocity in freshly collected sperm and after 3 h of incubation in mT-H medium in 18 rodent species.

5. Supplemental Figure S3. Relationships between relative testes mass and sperm velocity parameters after 0 and 3 h of incubation in mT-H medium, and proportional differences over time (between 0 and 3 h) in sperm velocity parameters.

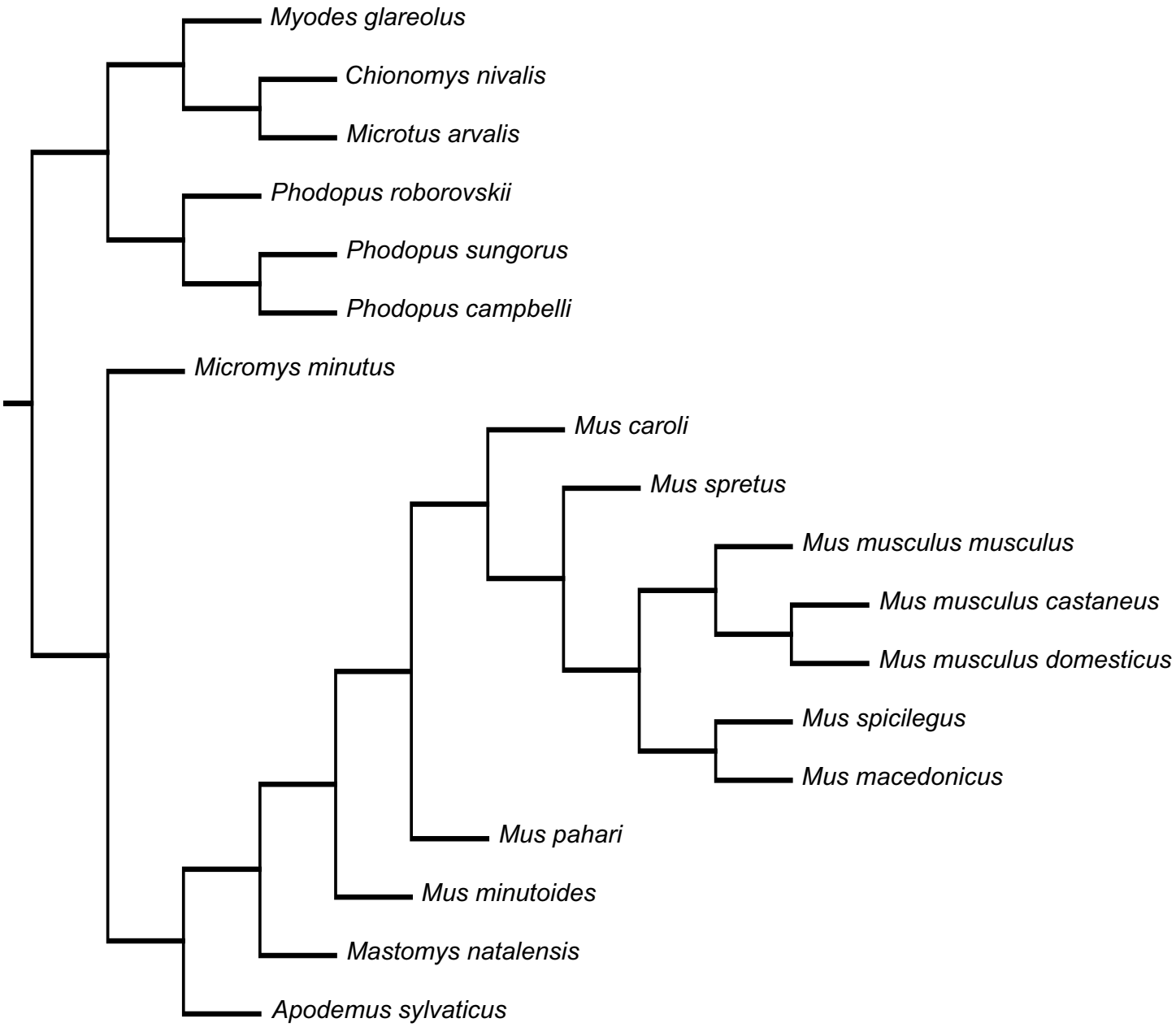
6. Supplemental Table S3. Relationships between relative testes mass and sperm velocity parameters after 0 and 3 h of incubation in mT-H medium, and proportional differences over time (between 0 and 3 h) in sperm velocity parameters.

Supplemental Table S1. Body mass, testes mass and sperm parameters of 18 species of muroid rodents. Values for spem parameters are for 0 and 3h of incubation in mT-H medium. N: number of individuals per species. BM: body mass (g). TM: testes mass (g). NSPZ: total sperm numbers (x 10^6) in both cauda epididymis. TSL: total sperm lengthl (μm). MOT: percentage of motile sperm (%). NAR: percentage of sperm with intact acrosome (%). VIA: percentage of viable sperm (%). ATP/SPZ: ATP content per sperm (amol $sperm^{-1}$). VCL: curvilinear velocity (μm s^{-1}). VSL: straight-line velocity (μm s^{-1}). VAP: average path velocity (μm s^{-1}).

Species	n	BM	TM	NSPZ	TSL	Time	MOT	NAR	VIA	ATP/SPZ	VCL	VSL	VAP
Myodes glareolus	4	29.27	0.626	239.3	83.90	0h	81.3	98.5	92.5	287.97	113.26	91.53	103.79
						3h	68.8	81.5	85.5	288.60	134.64	103.43	120.11
Chionomys nivalis	5	50.33	1.028	295.6	105.20	0h	85.0	86.8	95.4	426.76	131.91	110.38	119.77
						3h	66.0	71.6	95.2	273.15	133.63	108.67	117.99
Microtus arvalis	6	44.26	0.441	134.5	91.70	0h	79.2	86.3	86.3	416.24	108.05	85.87	94.60
						3h	51.0	70.8	78.7	255.05	93.84	63.82	73.69
Phodopus roborovskii	6	26.09	0.992	193.7	146.43	0h	88.0	87.1	97.8	492.57	149.69	59.24	84.03
						3h	76.5	74.1	95.7	423.56	138.58	61.93	83.15
Phodopus sungorus	7	46.70	0.995	174.4	131.83	0h	82.9	77.3	98.9	352.41	154.86	68.96	92.50
						3h	76.4	59.2	97.9	188.51	146.94	62.76	84.82
Phodopus campbelli	5	48.52	1.928	317.8	118.06	0h	86.5	87.7	99.3	372.15	162.79	73.81	100.78
						3h	79.5	73.1	98.5	215.39	154.48	50.37	79.36
Micromys minutus	5	8.09	0.119	34.4	64.66	0h	87.0	84.2	98.2	286.76	135.15	110.68	125.71
						3h	70.0	74.8	98.2	204.62	85.01	65.71	74.36
Mus caroli	7	18.07	0.144	14.7	118.31	0h	78.6	82.9	78.0	215.83	116.50	70.27	89.17
						3h	56.4	62.0	73.0	143.86	106.17	63.91	79.45
Mus spretus	18	18.17	0.300	64.7	108.41	0h	84.7	87.2	81.5	209.15	107.74	76.22	88.90
						3h	65.9	41.1	73.9	164.56	105.36	48.12	62.00
Mus musculus musculus	18	21.85	0.139	22.2	121.21	0h	62.5	65.5	71.0	158.02	87.61	51.46	62.51
						3h	40.0	39.2	63.8	92.66	75.51	36.80	48.48
Mus musculus castaneus	6	18.82	0.076	11.8	122.70	0h	64.2	83.2	69.5	139.27	98.73	64.46	75.11
						3h	30.0	55.0	67.2	61.74	78.52	32.03	45.33
Mus musculus domesticus	4	22.05	0.109	22.0	123.75	0h	73.8	71.0	76.3	129.75	97.54	62.36	77.96
						3h	57.5	58.5	74.8	83.73	93.88	50.77	63.99
Mus spicilegus	13	18.10	0.428	133.7	99.43	0h	78.6	84.6	80.8	217.25	111.60	80.51	92.80
						3h	68.0	47.1	73.9	147.75	108.35	58.71	72.38
Mus macedonicus	6	20.10	0.298	44.3	100.08	0h	77.5	86.0	70.3	148.63	106.02	50.40	71.39
						3h	62.5	59.5	69.3	103.80	98.65	59.10	72.09
Mus pahari	10	33.15	0.128	7.7	136.73	0h	66.9	62.0	81.4	233.15	96.35	48.82	62.79
						3h	36.0	47.8	76.6	156.36	76.25	28.09	43.03
Mus minutoides	10	5.54	0.105	40.6	65.17	0h	86.5	90.8	98.5	252.98	109.33	87.92	100.20
						3h	56.5	83.1	97.2	157.63	46.36	26.90	32.95
Mastomys natalensis	8	81.17	0.922	107.4	154.28	0h	76.3	68.6	91.4	229.23	129.09	109.07	117.05
						3h	50.0	57.0	90.5	185.57	108.66	76.96	85.98
Apodemus sylvaticus	5	30.46	1.085	120.5	126.10	0h	87.0	97.4	88.6	679.73	124.40	108.40	115.39
						3h	72.0	82.2	85.8	635.83	119.99	97.95	108.64

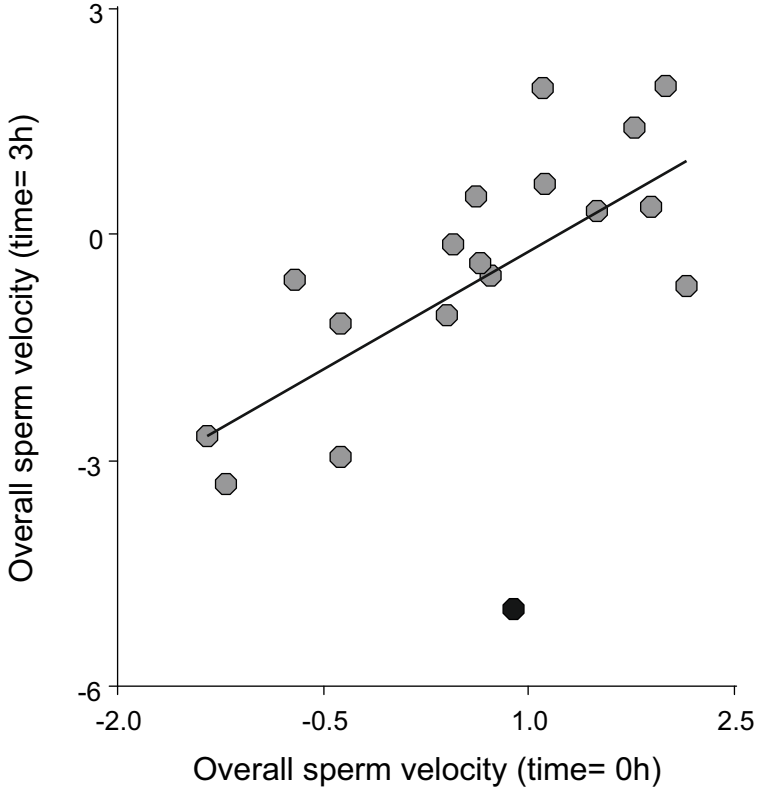
Supplemental Table S2. Loadings and correlation of sperm traits with principal components of sperm velocity in muroid rodent species. Values presented are Pearson's correlation coefficients. Significant correlation coefficients ($p < 0.05$) are shown in bold. PC1: principal component 1. PC2: principal component 2. Variable values were Log_{10} transformed prior to analysis.

Variables	Factor loadings		Factor correlation	
	PC1	PC2	PC1	PC2
Curvilinear velocity	0.5394	0.8124	**0.8779**	**0.4787**
Straight-line velocity	0.2809	-0.5495	**0.9454**	-0.3238
Average path velocity	0.6096	-0.1952	**0.9922**	-0.1150

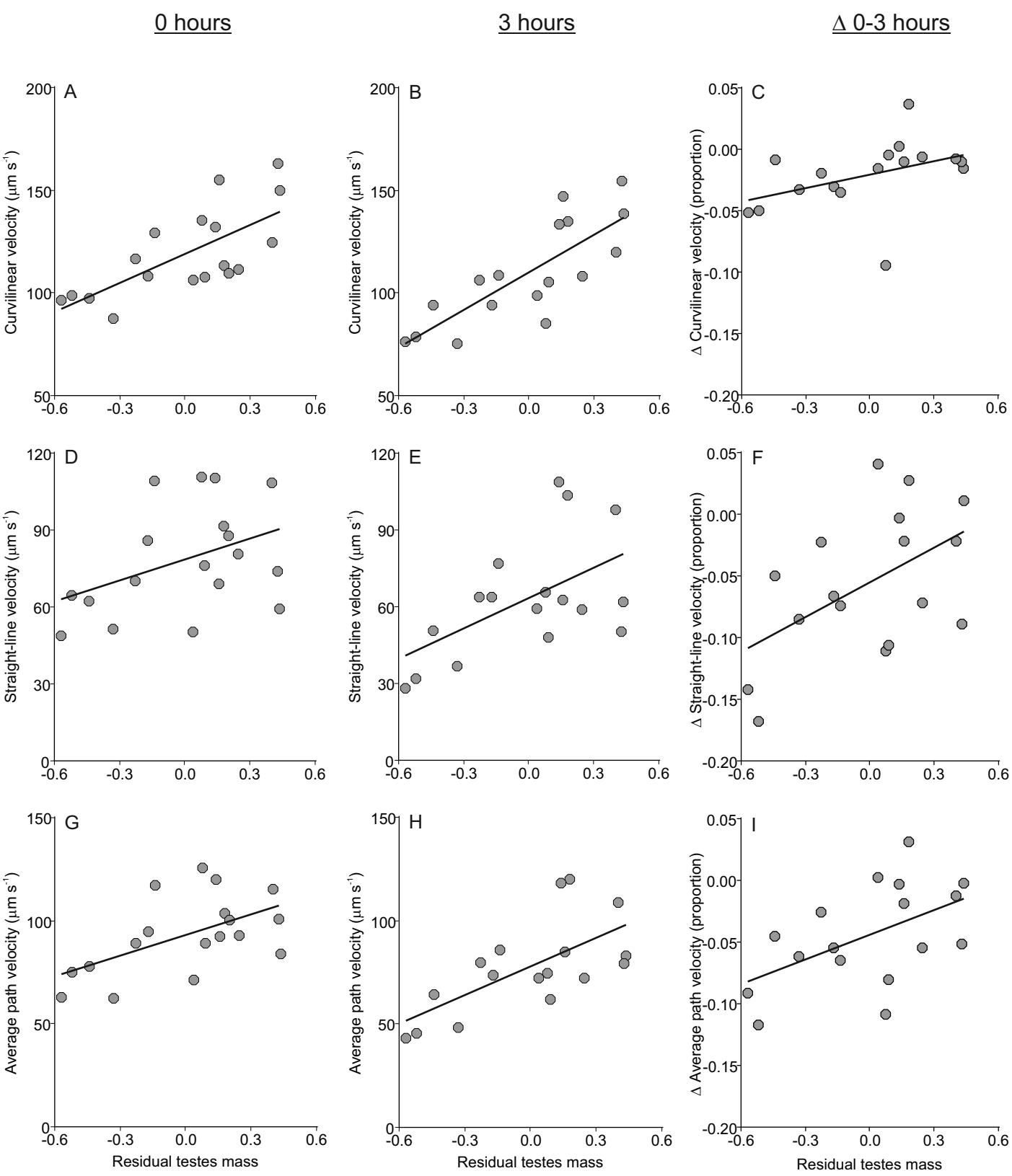


Supplemental Figure S1. Phylogenetic tree for the species analyzed in this study. Relationships were inferred from Fabre et al., *BMC Evolutionary Biology* 12: 88 [73] and Macholán et al., *Evolution of the House Mouse*, Cambridge University Press, Cambridge [74].

BOR Papers in Press. Published as DOI: 10.1095/biolreprod.114.127621.



Supplemental Figure S2. Relationship between overall sperm velocity assessed in freshly collected sperm (0 h) and after 3 h of incubation in mT-H medium in 18 rodent species. Overall sperm velocity is presented as the score of the first principal component (PC) of an analysis including curvilinear velocity, straight-line velocity, and average-path velocity. The black circle represents *Mus minutoides*, an outlier in the relationship.



Supplemental Figure S3. Relationships between relative testes mass and sperm velocity parameters after 0 and 3 h of incubation in mT-H medium, and proportional differences over time (between 0 and 3 h) in sperm velocity parameters.

Supplemental Table S3. Relations between relative testes mass and sperm velocity parameters after 0 and 3 hours of incubation in mT-H medium, and proportional differences over time (between 0 and 3h) in sperm velocity parameters. Phylogenetically controlled multiple regression analyses (PGLS). Superscripts following the λ value indicate significance levels (n.s. $p > 0.05$; *$p < 0.05$) in likelihood ratio tests against models with $\lambda = 0$ (first position) and $\lambda = 1$ (second position). Effect size r calculated from the t values and the non-central 95% confidence limits (CLs) for the z-transformed value of r are presented. Confidence intervals excluding 0 indicate statistically significant relationships. P-values and CL that indicate statistical significance are shown in bold. 0h: freshly collected sperm, 3h: sperm incubated for 3 hours in mt-H medium, Δ 0h - 3h: over-time proportional differences between freshly collected sperm and sperm incubated for 3h in mT-H medium.

Time	Dependent variable	Independent variable	Slope	R^2	t	P	λ	r	CL(-)	CL(+)
0h	Curvilinear velocity	Body mass	-0.0639	0.27	-1.3477	0.1978	$0.999^{*,ns}$	-0.3286	-0.8474	0.1648
		Testes mass	0.0960		2.3323	**0.0340**		0.5159	**0.0646**	**1.0768**
	Straight-line velocity	Body mass	-0.1597	0.17	-1.4568	0.1658	$0.963^{*,ns}$	-0.3521	-0.8739	0.1383
		Testes mass	0.1598		1.6935	0.1110		0.4006	-0.0817	0.9305
	Average path velocity	Body mass	-0.1533	0.26	-1.9709	0.0675	$0.999^{*,ns}$	-0.4535	-0.9952	0.0169
		Testes mass	0.1474		2.1827	**0.0454**		0.4910	**0.0313**	**1.0434**
3h	Curvilinear velocity	Body mass	-0.0950	0.82	-1.5607	0.1409	$<0.001^{ns,*}$	-0.3850	-0.9297	0.1180
		Testes mass	0.2464		6.4781	**<0.0001**		0.8659	**0.7928**	**1.8404**
	Straight-line velocity	Body mass	-0.2407	0.32	-1.4140	0.1792	$0.766^{ns,ns}$	-0.3535	-0.8933	0.1544
		Testes mass	0.3019		2.5392	**0.0236**		0.5615	**0.1112**	**1.1589**
	Average path velocity	Body mass	-0.1807	0.44	-1.5460	0.1444	$0.374^{ns,ns}$	-0.3819	-0.9261	0.1216
		Testes mass	0.2579		3.1947	**0.0065**		0.6493	**0.2503**	**1.2980**
Δ 0h - 3 h	Curvilinear velocity	Body mass	-0.0141	0.45	-0.4042	0.6922	$0.999^{*,ns}$	-0.1074	-0.6317	0.4160
		Testes mass	0.0674		2.8673	**0.0124**		0.6083	**0.1823**	**1.2300**
	Straight-line velocity	Body mass	-0.0541	0.32	-0.8242	0.4237	$<0.001^{ns,*}$	-0.2151	-0.7424	0.3053
		Testes mass	0.0934		2.2758	**0.0391**		0.5197	**0.0520**	**1.0997**
	Average path velocity	Body mass	-0.0146	0.39	-0.3002	0.7684	$<0.001^{ns,*}$	-0.2767	-0.8079	0.2397
		Testes mass	0.0689		2.2737	**0.0393**		0.6395	**0.2335**	**1.2811**