Sperm number trumps sperm size in mammalian ejaculate evolution

Stefan Lüpold† and John L. Fitzpatrick‡

Computational and Evolutionary Biology, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK

1. Introduction

Female promiscuity can cause sperm from different males to compete to fertilize a female’s limited supply of eggs [1]. The resulting selection on ejaculates to maximize fertilization success is viewed as the leading factor driving the dramatic diversification of sperm size and shape, as well as ejaculate traits more broadly (reviewed in [2]). Sperm competition theory initially focused on sperm numbers, proposing that males transferring more sperm gained a numerical competitive advantage analogous to buying more raffle tickets [3–5]. Within finite resources allocated to sperm production, however, investing in greater sperm numbers can be achieved only by reducing sperm size. This trade-off between sperm size and number soon became integral to sperm competition models, including those explaining the evolution of numerous tiny sperm relative to the fewer and usually much larger ova [3,6], which is ultimately also an important contributor to the evolution of mating systems [7–9].

Although sperm competition was predicted to favour smaller but more numerous spermatozoa, there is accumulating evidence that longer sperm may also result in fitness benefits and thus be under directional selection, resulting in longer sperm in species experiencing relatively high levels of sperm competition (e.g. [10–16]). To address the inconsistent directions of sperm evolution in response to sperm competition among taxa (see [17–20] for reviews), two hypotheses were recently proposed. First, the ‘sperm dilution hypothesis’ states that although males under intense sperm competition should always invest more in gametes, the strength of selection on sperm size depends on the sperm density prior to fertilization [21,22]. As the limits of sperm production are approached at high levels of sperm competition (e.g. [23,24]), trade-offs between sperm size and number are expected to generate stronger selection on sperm size when dilution effects are low and sperm actively interact with one another [21,22], such as in small organisms where the high sperm density within a relatively small reproductive tract often results in sperm displacement [25–27]. However, trade-offs result in stronger selection on sperm number when dilution effects are high.
(i.e. in large organisms where sperm are diluted in the female’s reproductive tract and thus at relatively low densities [21,22]). Alternatively, the ‘metabolic constraint hypothesis’ argues that sperm size, and gamete investment generally, responds to selection more intensely in species with higher mass-specific and cellular metabolic rates, based on the assumption that in these species resource processing of fast-dividing cells like germ cells is more efficient, resulting in enough resources to increase sperm size and/or number when under selection [24,28,29]. Following this hypothesis, the metabolic constraints on evolving longer and/or more abundant sperm should be relatively low in small-bodied endothermic animals with high metabolic rates, but high in large-bodied species with relatively low metabolic rates [24,28]. If so, both sperm size and number would be predicted to covary strongly positively with the level of sperm competition in small-bodied species but to be independent of, or only weakly associated with, sperm competition in larger bodied species owing to the stronger constraint on their evolution. Consequently, whereas both hypotheses suggest that the strength of selection acting on sperm size is ultimately dependent on body size, their predictions regarding the effect of body size on gamete investment are fundamentally different.

Here, we aimed to resolve the debate between the sperm dilution and the metabolic constraint hypotheses and to assess their applicability in explaining the macroevolutionary variation in sperm size by assessing it jointly with variation in sperm number. Using Parker et al.’s [21] terminology, we examined the relationship between sperm competition and both total gamete investment (m*s; i.e. product of sperm size m* and sperm number s*) and relative investment in sperm size and number (m*/s*), respectively. Examining the response of these combined traits to sperm competition along the body-size spectrum targets the contrasting predictions between the two hypotheses to address our goal.

The sperm dilution hypothesis, under which m*s* is driven almost exclusively by variation in sperm number [21,22], predicts a positive relationship between m*s* and relative testes size (as a proxy of sperm competition [30,31]) for all body sizes, but this relationship should become stronger with increasing body size because sperm number is under intense selection by both sperm competition and a growing risk of sperm loss or dilution in the longer and more voluminous female reproductive tract of larger species [32]. By contrast, to support the metabolic constraint hypothesis, we predicted that the relationship between m*s* and sperm competition should be strong in small species due to their high propensity to increase both ejaculate traits in response to sperm competition and low metabolic constraints, but it should become weaker in larger species where lower metabolic rates and less efficient sperm production are thought to constrain their ability to increase sperm size and number [24,28].

For the relative investment in sperm size and number, expressed by the m*/s* ratio, the sperm dilution hypothesis predicts that selection acting primarily on sperm number (i.e. raffle-like sperm competition) should result in a negative relationship between m*/s* and sperm competition risk, and this relationship should gradually become more strongly negative with any incremental increase in body size. This is due to the intense selection on sperm number by both sperm competition and the risk of sperm loss, which accelerates the interspecific variance in sperm number, s*, lowers the m*/s* ratio at a higher rate with greater body size and thus strengthens its negative relationship with the measure of sperm competition [21]. Under the metabolic constraint hypothesis, however, we predicted that the strength of the relationship between m*/s* and sperm competition should not change systematically with body size. This is because of the assumption underlying this hypothesis that the high resource turnover and efficient sperm production allows small species to increase both sperm size and number in response to selection, and both traits are increasingly constrained in their response to selection as body size increases [24,28]. Thus, although selection may consistently favour one trait over the other, leading to a bias towards either m* or s* within any body-size category, the m*/s* ratio itself is not predicted to covary more tightly with sperm competition levels among large-bodied compared to smaller bodied species.

Using these contrasting predictions between the two hypotheses, we evaluated the evolution of sperm size and number in mammals and tested whether the observed macroevolutionary patterns supported the sperm dilution or the metabolic constraint hypothesis. The extraordinary variation in body size in this taxon, spanning six orders of magnitude in our study, makes mammals a powerful system to test whether the response of ejaculates to sperm competition changes with female body size, which we use as a proxy of the female reproductive tract, given that the different parts of the female reproductive tract strongly covary with body size [33,34] (also see §2b). Additionally, the mammalian phylogeny is well resolved (e.g. [35,36]) and their reproductive physiology and ejaculate traits are well characterized [14,37]. Finally, there has been a long-standing debate about the variation in sperm size in response to sperm competition among mammals, with conflicting results between studies depending on the combination of species examined [14,38–40]. This ambiguity in evolutionary responses to sperm competition may in part be due to unresolved impacts of body-size-dependent relationships between gamete investment and sperm competition levels.

Across the large sample of mammalian species examined, our results support the sperm dilution, not metabolic constraint hypothesis, and demonstrate that body size-mediated trade-offs between sperm size and number can explain the extreme diversification in sperm phenotypes.

2. Material and methods

(a) Data collection and study taxa

We compiled species-specific values of sperm length, total number of sperm in ejaculates, combined testes mass, and male and female body masses of 100 mammalian species from the literature (electronic supplementary material, table S1). We used only sperm counts from samples that were obtained by electroejaculation or as natural ejaculates (for details see [37]) and omitted any samples collected from the epididymes as these are less likely to reflect natural ejaculates. In some cases, the total sperm number was not provided but it could be calculated as the product of ejaculate volume and sperm concentration.

(b) Statistical analyses

We conducted all analyses using the statistical package R v. 3.2 (R Core Team 2015) and transformed all data by logarithmic transformations to meet the parametric requirements of the
Table 1. Phylogenetically controlled associations between different measures of gamete investment and relative testes mass across 100 mammalian species. (The partial correlation coefficients \( r \) are presented with their lower (LCL) and upper (UCL) non-central 95% confidence limits.)

<table>
<thead>
<tr>
<th>response</th>
<th>predictor</th>
<th>partial ( r ) (LCL, UCL)</th>
<th>( t )</th>
<th>( p )</th>
<th>( \lambda )</th>
</tr>
</thead>
<tbody>
<tr>
<td>total sperm number</td>
<td>testes mass</td>
<td>0.61 (0.47, 0.70)</td>
<td>7.52</td>
<td>&lt;0.0001</td>
<td>0.050.04 &lt;0.001</td>
</tr>
<tr>
<td></td>
<td>body mass</td>
<td>-0.08 (-0.27, 0.12)</td>
<td>-0.80</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>total sperm length</td>
<td>testes mass</td>
<td>0.04 (-0.15, 0.23)</td>
<td>0.42</td>
<td>0.68</td>
<td>0.900.003 &lt;0.17</td>
</tr>
<tr>
<td></td>
<td>body mass</td>
<td>-0.17 (-0.35, 0.02)</td>
<td>-1.74</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>total gamete investment ((m^<em>s^</em>))</td>
<td>testes mass</td>
<td>0.61 (0.48, 0.71)</td>
<td>7.62</td>
<td>&lt;0.0001</td>
<td>&lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td></td>
<td>body mass</td>
<td>-0.15 (-0.33, 0.06)</td>
<td>-1.44</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>relative investment ((m^<em>/s^</em>))</td>
<td>testes mass</td>
<td>-0.59 (-0.69, -0.44)</td>
<td>-7.11</td>
<td>&lt;0.0001</td>
<td>0.050.05 &lt;0.01</td>
</tr>
<tr>
<td></td>
<td>body mass</td>
<td>0.01 (-0.19, 0.20)</td>
<td>0.10</td>
<td>0.92</td>
<td></td>
</tr>
</tbody>
</table>

Statistical models. These transformations also helped to avoid the problematic use of ratios in phylogenetic models in the case of the ratio between sperm size and number, because ln(sperm size : sperm number) is equivalent to ln(sperm size) – ln(sperm number). To statistically account for shared ancestry among species, we conducted phylogenetic generalized least-squared (PGLS) regressions [41,42], based on the same molecular phylogenies as in previous large-scale studies on the traits and taxa used [14,37] (electronic supplementary material, figure S1). PGLS models estimate the phylogenetic scaling parameter \( \lambda \) to evaluate the phylogenetic relationship of the covariance in the residuals [41]. Using likelihood ratio tests, we established whether the models with the maximum-likelihood value of \( \lambda \) differed from models with values of \( \lambda \) set to 0 or 1, respectively. Values of \( \lambda \) close to 0 indicate phylogenetic independence and those close to 1 suggest a strong phylogenetic association of the traits [41]. Throughout this paper, we present \( \lambda \) values with their associated \( p \)-values of these likelihood ratio tests in superscripts (first against \( \lambda = 0 \), second against \( \lambda = 1 \)).

To examine the relationship between ejaculate traits and sperm competition levels, we conducted phylogenetic multiple regressions as described above, using combined testes mass as the predictor and male body size as a covariate to account for allometric effects [43,44]. Relative testes mass is a widely used proxy of sperm competition levels as it covaries positively with indices of multiple paternity in various taxa, including mammals [30]. To better illustrate shifts in \( m^*s^* \) or \( m^*/s^* \) in relation to female body mass (as a proxy of the size of the reproductive tract; see below), we used a sliding-window approach [45]. In brief, we fitted the above multiple PGLS models within a sliding window that was four units wide on the scale of ln(female body mass) and then slid the window along this scale from the smallest to the largest female body masses at increments of 1.5 units. For each such window, we calculated the partial correlation coefficient between combined testes mass and either \( m^*s^* \) or \( m^*/s^* \) and transformed it to \( Z_r \), using Fisher’s transformation, weighted by sample size [46]. We then plotted these \( Z_r \)-values against the mean female body mass calculated for each sliding window. Finally, to confirm the consistency and robustness of these analyses, we repeated them using different window sizes and starting points (electronic supplementary material, figure S2).

We used female body mass as a proxy of the size of the reproductive tract because not enough data were available to use the reproductive tract size directly. However, restricting Anderson et al.’s [34] data on oviduct length and female body mass to the taxa used in our study (i.e. excluding Marsupialia and Chiroptera) revealed a highly significant positive relationship between the two traits (\( r = 0.87, p < 0.0001 \); \( \lambda < 0.0001 \).) with an allometric slope between oviduct length and the body mass (cube-root-transformed for equal dimensionality) of 0.79 (95% confidence interval: 0.66–0.94). Whereas female body mass covaries with oviduct length and other components of the female reproductive tract (also see [33,34]), it is also highly correlated with the mass-specific metabolic rate [47], thereby providing a good proxy for the assumptions underlying both the sperm dilution and metabolic constraint hypotheses.

3. Results
First, we examined the relationship of both sperm length and sperm number with sperm competition risk, using body size-corrected testes mass as a proxy measure [17,30,31], in separate phylogenetic multiple regressions across all 100 species. These analyses revealed that sperm number, but not sperm length, increased significantly with relative testes mass (table 1). Additionally, as predicted theoretically [21], across all 100 mammalian species examined the association between relative testes mass and the total investment in the cellular component of ejaculates \((m^*s^*)\) was strongly positive (table 1 and figure 1a), whereas that with relative investments \((m^*/s^*)\) was strongly negative (table 1 and figure 1b). The same patterns were also apparent within each of the four most speciose taxa in our dataset (Artiodactyla, Carnivora and Primates, Rodentia), albeit not significantly so in the Rodentia, which was limited to only nine species (compared to \( N > 20 \) in the other three taxa; electronic supplementary material, table S2).

Next, we determined if the strength of the association between sperm competition risk and total and relative gamete investment varied across different body-size categories. The response to sperm competition can be expressed as the effect size, \( r \), of the relationship between measures of gamete investment (which dilution effects and metabolism could constrain) and relative testes mass as a proxy measure of sperm competition [17,30,31]. Large effect sizes indicate that gamete investment varies tightly with different degrees of selection, whereas small effect sizes would suggest a limited response to variation in sperm competition levels. However, as effect sizes are influenced strongly by sample size, we converted \( r \) values to Fisher’s sample-size-corrected \( Z_r \) (i.e. using weighted Fisher’s transformations of \( r \) [46]) to facilitate comparisons of responses of total and relative gamete investment in response to sperm competition risk across the broad range of mammalian body sizes.

Using standardized \( Z_r \) values of the relationships between either \( m^*s^* \) or \( m^*/s^* \) and relative testes mass, we
employed a sliding-window approach [45] to determine how the strength of these associations varied across different body-size categories, using female body mass as a proxy of female reproductive tract size (for details see §2b). Comparing the calculated $Z_r$-values between different body size categories allowed us to estimate size-dependent responses to selection in order to test specific predictions that can distinguish between the two major hypotheses (§1). These analyses revealed that the relationships between relative testes mass and either $m^*s^*$ or $m^*/s^*$ became stronger, albeit in opposing directions, with increases in female body mass (figure 2). These results highlight that the total ejaculate investment increases with sperm competition risk and body size, but that the relative investment in sperm number becomes stronger compared to that in sperm length as species increase in size, consistent with the predictions from the sperm dilution hypothesis [21,22]. Since the unequal distribution of species along the body-size spectrum rendered these analyses sensitive to changes in both the width of the sliding window and the position of the first window (which then determined the cut-offs for each of the following windows), we repeated these analyses with varying window sizes and starting points and found largely consistent patterns regardless of the parameter settings (electronic supplementary material, figure S2).

To test whether the size-dependent variation in the relationship of $m^*s^*$ or $m^*/s^*$ with relative testes size was driven solely by sperm number, given that sperm length was not associated with size-controlled testes mass across all 100 species (table 1), we also applied the sliding-window approach to both ejaculate traits separately. We found sperm number to covary positively with relative testes mass within each body-size category (all $p \leq 0.01$), but more tightly so among larger bodied species (electronic supplementary material, figure S3a). The corresponding associations with sperm length were statistically significant only within the second-smallest size category ($p = 0.02$), and a negative trend between the weighted $Z_r$ values and mean female body mass was apparent (electronic supplementary material, figure S3b). We confirmed this trend across the much larger dataset of sperm length in Tourmente et al.’s [14] study ($n = 226$ eutherian mammal species; electronic supplementary material, figure S4). These separate results combined suggest that the size-dependent shifts in $m^*s^*$ or $m^*/s^*$ reported above are not the result of increasing variation in sperm

**Figure 1.** Partial regression plots showing the relationships across 100 mammalian species of (a) the total gametic investment (product of sperm length and number) and (b) the relative gametic investment (sperm length/sperm number) with testes mass after removing variation in body size from both variables.

**Figure 2.** Sliding-window analysis expressing, for each window, In(mean female body mass) and the weighted $Z_r$-value as the sample size-controlled effect size of a relationship between relative testes mass and (a) the total ejaculate investment ($m^*s^*$), or (b) the relative investment between sperm size and number ($m^*/s^*$). These $Z_r$-values reflect the strength of the examined relationship within each window (the further away from zero, the stronger the relationship). For each window, we restricted the dataset to a range of body sizes spanning four units along the $x$-axis and moved the window along this axis at intervals of 1.5 units. The first window had its midpoint at 4.3, the last at 11.8. Numbers above points indicate the number of species falling into each window. All $p$-values of the relationships underlying each data point were $p < 0.001$ except for the one labelled with 11 in (a) ($p = 0.015$) and the one labelled with 16 in (b) ($p = 0.056$).
number alone, with the effect of sperm competition on sperm length being invariably weak across all body size categories. Rather, the strength of the relationship between sperm length and relative testes mass tended to decline gradually with increasing body size while it became stronger for sperm number, as predicted by the sperm dilution hypothesis [21,22].

4. Discussion

Across our sample of eutherian mammals, we found greater total investment in the gametic fraction of ejaculates in response to increasing sperm competition risk. However, increases in sperm number in response to sperm competition were stronger than increases in sperm size. Furthermore, the strength of these effects increased with female body size, and thus likely with the size of the female reproductive tract [33,34]. These results offer robust support for the sperm dilution hypothesis [21,22,32], suggesting that in large species the marginal benefits of transferring greater sperm numbers outweigh those of transferring longer sperm to a greater extent than in smaller species.

In their comparison of passerine birds and fruit flies, Immler et al. [22] illustrated that high sperm density within the female reproductive tract of the small-bodied flies increases the benefits of sperm size due to direct competition for storage and sperm displacing one another [25–27], whereas selection is stronger on sperm number than on sperm size in the birds, in which the mechanism of sperm competition is more raffle-like [22,48] (also see [49] for a similar pattern in mammals). Our meta-analysis corroborates and expands these findings by showing continuous size-dependent variation in the importance of sperm number over that of sperm size. With increasing mammalian body size, incremental increases in sperm competition risk were tightly associated with a shift towards investing more in sperm number rather than sperm length. Thus, among mammals, selection is stronger on sperm number in large-bodied compared to small-bodied species. Interestingly, it is among these smaller species where more complex sperm morphology is observed, including apical hooks or other processes protruding from the sperm heads in various murid rodents, for example, which are thought to facilitate sperm cooperation and sperm transport [50,51]. It is thus plausible that the smaller body size increases the density of sperm relative to the female reproductive tract and lowers the risk of sperm loss, thereby creating an environment where variation in sperm morphology (or sperm quality in general) can explain a relatively greater proportion of overall ejaculate competitiveness. Consistently, several sperm-quality traits that covaried positively with the level of sperm competition also decreased with body size, whereas sperm number tended to increase, albeit not significantly so, in a previous study across a broad range of mammalian species [37]. Additionally, in our separate analyses of sperm length and number, the association with the level of sperm competition across different body-size categories decreased for sperm length but increased for sperm number, indicating that selection on sperm length is likely to become weaker as that on sperm number increases. These findings corroborate the general trends for the total and relative gametic investment (above) and highlight the increasing importance of sperm number in response to counteract the risk of sperm dilution or loss as body size increases.

Our results do not dismiss the importance of sperm morphology or sperm quality, even for relatively large mammals where sperm quality has been shown to influence male fertility (e.g. Iberian red deer Cervus elaphus hispanicus [52]). However, sperm-quality effects are likely to be more subtle and manifested mainly after controlling for sperm numbers (also see [52]). Superior quality may help sperm traverse the challenging environment of the female reproductive tract faster and thus in greater numbers [53,54], and it may be critical once sperm compete for fertilization near the ovum. Yet, sperm dilution and sperm loss are likely to be chance effects, thereby raising the importance of sperm number where greater distance has to be covered. For example, in humans, a mere 0.004% (approx. 250) of all motile sperm inseminated make it past the uterus [55], and it seems likely that the efficiency of sperm transport would vary with the size of the female reproductive tract.

In contrast to the sperm dilution hypothesis, our joint examination of sperm size and number variation did not support the metabolic constraint hypothesis. It seems plausible that the lack of a link between sperm size and sperm competition in large species, which was attributed to a metabolic constraint [28,29], may simply be the result of relaxed selection on sperm size due to the comparatively much greater advantage of increasing sperm number. Effects of the metabolic rate and of body size per se are inherently difficult to separate. In fact, the mass-specific metabolic rate (basal metabolic rate divided by body mass), as used in the tests of the metabolic constraint hypothesis [24,28,29], does not remove the effect of body size from the metabolic rate (e.g. [56]). Across mammals, approximately 90% of the mass-specific metabolic rate is explained by body size variation alone [47], so its relationship with sperm length largely reflects an inverted association of sperm length with body mass. Consequently, it is possible that the metabolic rate and the intensity of selection on sperm size independently vary with body size.

In conclusion, we document size-dependent and gradual shifts in ejaculate allocation, with large species exhibiting stronger selection on sperm number than on sperm size compared to smaller species. This gradual change in the relative investment in ejaculate traits supports the sperm dilution hypothesis, but is not consistent with the metabolic constraint hypothesis, thereby resolving the debate between these two competing explanations of how body size affects patterns of sperm evolution. Additionally, Parker [57] recently proposed an extensive theoretical framework explaining how changes in the selective environment, associated with stepwise transitions from simple sperm-broadcasting life forms to complex and mobile organisms with internal fertilization, have shaped the evolutionary dynamics of, and selection on, gamete investment throughout animal history. In this context, our findings add an important piece to Parker’s [57] predictions and have broad implications for our understanding of ejaculate evolution by showing how variation in body size affects the selective environment in internal fertilizers and thus the trajectory of sperm size evolution.
References


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