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No evidence of tradeoffs in the evolution of sperm numbers and sperm size in mammals

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Short title: Sperm numbers and size in mammals

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Abstract

Postcopulatory sexual selection, in the form sperm competition, has influenced the evolution of several male reproductive traits. However, theory predicts that sperm competition would lead to tradeoffs between numbers and size of spermatozoa because increased costs per cell would result in a reduction of sperm number if both traits share the same energetic budget.

Theoretical models have proposed that, in large animals, increased sperm size would have minimal fitness advantage compared with increased sperm numbers. Thus, sperm numbers would evolve more rapidly than sperm size under sperm competition pressure. We tested in mammals whether sperm competition maximizes sperm numbers and size, and whether there is a tradeoff between these traits. Our results showed that sperm competition maximizes sperm numbers in eutherian and metatherian mammals. There was no evidence of a tradeoff between sperm numbers and sperm size in any of the two mammalian clades since we did not observe any significant relationship between sperm numbers and sperm size once the effect of sperm competition was taken into account. Maximization of both numbers and size in mammals may occur because each trait is crucial at different stages in sperm's life; e.g., size-determined sperm velocity is a key determinant of fertilization success. In addition, numbers and size may also be influenced by diverse energetic budgets required at different stages of sperm formation.

Keywords: sexual selection, sperm competition, metabolic rate, energetic constraints, sperm size, sperm numbers
Introduction

One of the key predictions of sperm competition theory (Parker, 1970) is that males belonging to species with high levels of sperm competition should produce more sperm in order to outcompete their rivals in their quest to fertilize ova. If it is assumed that sperm competition resembles a raffle, in which sperm are equivalent to fertilizing opportunity increments, more sperm would increase the chances of fertilization (Parker, 1970; 1993). In line with this "raffle hypothesis", high levels of sperm competition associate with increases in testes mass relative to body size (Harcourt et al., 1981; Gage, 1994; Birkhead & Møller, 1998; Simmons, 2001; Birkhead et al., 2009; Soulsbury, 2010) and with high relative sperm numbers (Birkhead & Møller, 1998; Parker & Pizzari, 2010; Gomez Montoto et al., 2011a) in many taxa.

Since the production of a vast amount of sperm would be highly expensive in terms of energy, and larger sperm may increase the energetic cost per cell, a "direct" energetic tradeoff would exist between sperm numbers and sperm size (Parker, 1982). This line of reasoning assumed that total energetic budget for sperm production equaled the number of sperm produced multiplied by the energetic cost of producing each sperm. Consequently, in order to produce more sperm there should be a reduction in the cost of producing each individual cell (i.e., via a reduction in sperm size).

Nevertheless, sperm size could provide an adaptive advantage. If sperm competition resembles a race in which the first sperm to reach the ovum engages in fertilization, an increase in sperm size (e.g., due to a longer flagellum) could provide increased sperm velocity, thus affecting the outcome of sperm competition (Gomendio & Roldan, 1991). This scenario adds a temporal component to the raffle hypothesis. Evidence supporting positive relationships between sperm competition levels and sperm size (Gage, 1994; Briskie et al., 1997; Tourmente et al., 2009; Tourmente et al., 2011b; a), sperm size and sperm velocity...
(Gomendio & Roldan, 2008; Fitzpatrick et al., 2009; Lüpold et al., 2009; Gomez Montoto et al., 2011b; Tourmente et al., 2011b), and sperm competition and sperm velocity (Fitzpatrick et al., 2009; Kleven et al., 2009; Gomez Montoto et al., 2011b; Tourmente et al., 2011b) has been found in many taxa, including mammals. Therefore, since faster sperm increase fertilization success in competitive scenarios (Birkhead et al., 1999; Gage et al., 2004; Gasparini et al., 2010), males in species with high levels of sperm competition should maximize both sperm numbers and sperm size to increase their fitness.

Subsequent theoretical models developed the concept of an "indirect tradeoff" by placing the emphasis on total reproductive budget rather than just on sperm production (Parker, 1993). This tradeoff definition was expanded to incorporate a new partition of the total reproductive energetic budget between mate acquisition and total ejaculate investment (the latter encompassing sperm numbers and sperm size) (Parker, 1993; Parker et al., 2010). Nevertheless, this model is still based on the idea of a direct tradeoff between sperm numbers and sperm size within the ejaculate investment component, for a given value of sperm competition risk. In addition, two sperm competition mechanisms have been defined on the basis of the volume scale differences between the ejaculate and the female reproductive tract. On the one hand, there is a raffle mechanism in which female reproductive tract volume and ejaculate volume scales differ considerably and there is no space constraint for fertilization (Parker, 1990). This mechanism describes conditions for externally fertilizing species and many vertebrates with internal fertilization, especially those with large bodies. On the other hand, a displacement mechanism, in which the volumes of the ejaculate and the female sperm storage organs are similar and, thus, successive ejaculates will volumetrically displace the preceding ones (Parker & Simmons, 1991). This mechanism describes the conditions prevalent in insects, in which females have sperm stores that can hold a small volume of ejaculate.
An early theoretical model on sperm size proposed that the effect of sperm size on sperm competition is mainly mediated by sperm density (Parker, 1993). This model predicted that, if the effect of sperm density in the female tract on sperm competitiveness is negligible, there would be a single optimal size for sperm, independently of the sperm competition level.

In a later paper (Parker et al., 2010), the probable effects of sperm density on sperm competitiveness were investigated in detail. Since sperm numbers ejaculated (and hence the density of competing sperm) often change with sperm competition level, this study (Parker et al., 2010) examined how the balance between sperm size and number is expected to change with sperm competition. Applying the indirect tradeoff hypothesis to sperm competition mechanisms, theoretical studies proposed that (a) a tradeoff between sperm numbers and sperm size would occur; (b) fitness advantages related to sperm size would be mediated by "sperm density" (i.e., sperm numbers in relation to the space available for sperm competition); and (c) in the raffle models, an increase in sperm numbers would generally yield greater fitness gain than an increase in sperm size since sperm density is considered to be negligible in this mechanism due to the scaling difference between ejaculate volume and female reproductive tract volume (Parker et al., 2010). Thus, in "raffle" models, the effect of sperm size on sperm competitiveness is assumed as a weak force "reduced mainly to effects on sperm motility and survival" (Immler et al., 2011). A comparative study presented empirical support for these predictions by comparing the relationships between sperm numbers, sperm size and sperm competition risk in two taxa with different sperm competition mechanisms (birds: raffle; flies: displacement) (Immler et al., 2011).

In mammals, sperm competition is associated with increases in both sperm numbers (Møller, 1989; Gomendio et al., 1998; Gomez Montoto et al., 2011a; Lüpold, 2013), and sperm size (Tourmente et al., 2011b; a). However, the existence of a positive association between both sperm size and number with the risk of sperm competition does not allow any
predictions about the existence of a tradeoff between these two traits, since the investment in one of them might be higher in relation to the investment in the other (Parker et al., 2010). Mammals and birds share a number of similarities regarding the reproductive traits which were taken in account by the models cited above. Firstly, volume of the female reproductive tract is considerably higher than the volume of the ejaculate (Brillard & Bakst, 1990; Suarez & Pacey, 2006). Secondly, sperm swimming velocity is a main factor in fertility (Birkhead et al., 1999; Malo et al., 2005), is positively related to sperm size (Lüpold et al., 2009; Gomez Montoto et al., 2011b; Tourmente et al., 2011b) and is positively selected by sperm competition (Kleven et al., 2009; Gomez Montoto et al., 2011b; Tourmente et al., 2011b; a; Tourmente et al., 2013). Thirdly, immotile sperm are not transported towards the egg despite passive sperm transport to the site of fertilization (Froman & Kirby, 2005; Suarez & Pacey, 2006). Therefore, according to current theoretical models, the existence of a tradeoff between sperm numbers and size in mammals would be anticipated.

In this study, we analyzed the relationships between sperm numbers and sperm size in mammals by comparing two sister mammalian clades, Eutheria and Metatheria. We chose to compare these two groups because they present clear differences in sperm numbers, sperm size, and reproductive physiology, which could strongly influence responses of sperm phenotype to sperm competition. In general terms, Metatheria (i.e., marsupials) have lower relative testes mass (Tourmente et al., 2011a), lower sperm numbers in relation to body size (Taggart et al., 1998; Taggart et al., 2003), longer sperm (Tourmente et al., 2011a), and a higher efficiency of sperm transport, with an extremely high proportion of ejaculated sperm reaching the fertilization site in relation to Eutheria (i.e., the so-called placental mammals) (Taggart et al., 1998; Taggart et al., 2003). Furthermore, in Metatheria sperm storage in the female reproductive tract takes place during relatively long time periods (up to two weeks) in comparison to Eutheria (with the exception of Chiroptera) (Bedford et al., 1984).
We predicted that a tradeoff between sperm numbers and sperm size exists among mammals but that, based on their reproductive physiology, there may be differences between Eutheria and Metatheria with regards to the relative increase in sperm size and numbers in response to sperm competition.

Materials and Methods

Sperm numbers, sperm size, and relative testes mass

Data on body mass (g), testes mass (g), total sperm length (µm), and number of sperm in caudae epididymides (x 10^6 sperm) were obtained from the literature for 64 species (21 families) of Eutheria and 14 species (7 families) of Metatheria (Table S1 for data and references). Only species for which all these measures were available were included. Data collection was restricted to studies on adult, healthy, reproductively mature individuals. In cases of experimental studies or clinical trials, only the values of control groups were used as data sources. Whenever possible, data were collected from studies in which the higher number of variables analyzed were present. In those cases in which different values for the same variable and species were available from different studies, averages were used to obtain a representative measure. For total sperm length, only species where this parameter was measured from digital images were incorporated into the dataset.

The measure of sperm number used in this study was the number of sperm in epididymal reserves (cauda epididymides), since it is a more robust parameter of sperm production than the number of sperm in the ejaculate, although both traits are strongly correlated (delBarco-Trillo et al., 2013). We are aware that the number of sperm in epididymal reserves may not always be extremely accurate due to insufficient flushing of the epididymis, seasonal or age-related variation (Johnson & Thompson, 1983), and perceived risk of sperm competition during spermatogenesis (Ramm & Stockley, 2009). However, the
number of sperm in the ejaculate is a less direct measure of sperm production and may be affected by many factors, namely number of copulations (Pierce et al., 1990; Pitnick & Markow, 1994; Ambriz et al., 2002), number of potential partners (Sheldon, 2000), sperm depletion in successive ejaculates (Preston et al., 2001), differential sperm allocation through perceived sperm competition risk (Dewsbury, 1982; Wedell et al., 2002; delBarco-Trillo, 2011; Kelly & Jennions, 2011), or the effect of electrostimulation (a common procedure in large mammals) in comparison to normal copulation (Mattner & Voglmayr, 1962; Aulerich et al., 1972; Schneiders et al., 2004).

**Data analysis**

To test the influence of sperm competition we used relative testes size as predictor of number of sperm in caudae epididymides (dependent variable). Since testes size relative to body mass is a reliable indicator of investment in sperm production, this trait is considered to be a very good proxy of sperm competition levels (Gage, 1994; Birkhead & Møller, 1998).

Experimental selection assays in insects have demonstrated that lines subjected to high sperm competition environments evolve larger testes (Hosken & Ward, 2001), while lines subjected to enforced monogamy evolve reduced testes size and sperm production (Pitnick et al., 2001).

Moreover, a recent comparative study (Soulsbury, 2010) found that levels of multiple paternity correlate well with relative testes size in mammals. We performed multiple regression analyses introducing the following predictors in the model: body mass, testes mass, a quadratic term for testes mass, and the interaction between body mass and testes mass (Tomkins & Simmons, 2002; Immler et al., 2011).

When testing the effect of sperm size on sperm numbers, we used two sets of predictor variables in order to control for the effect of body size (body mass and total sperm length as predictors), and sperm competition (body mass, testes mass and total sperm length as
predictors) on sperm numbers. All variables were log₁₀-transformed to meet parametric assumptions.

All regressions were performed using phylogenetic generalized least-squares analyses (PGLS) (Freckleton et al., 2002) since species trait values may be similar as a result of phylogenetic association rather than selective evolution (Felsenstein, 1985; Harvey & Pagel, 1991). 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 non-central confidence limits (CLs) for the z-transformed value of r (Nakagawa & Cuthill, 2007). The CLs value [values?] indicate that the effect size is statistically significant if 0 is not contained within the interval (Smithson, 2002).

All statistical analyses were performed using the CAPER v0.5 (Orme et al., 2012) package for R (v3.0.1; R Foundation for Statistical Computing 2013). P values were considered statistically significant at α < 0.05. Residual testes mass was calculated for each group as the residual of a log-log linear regression of testes mass on body mass (Eutheria: p<0.0001, R²=0.87; Metatheria: p<0.0001, R²=0.89) and used exclusively to illustrate the results. The phylogenetic reconstruction used in the PGLS analyses is included in the Supplementary Information (Fig. S1). This reconstruction was based on a mammalian supertree (Bininda-Emonds et al., 2007) for the determination of the phylogenetic position of the higher groups (orders and families). Group-specific phylogenies were used to resolve the
within group relationships for Artiodactyla (Prothero & Foss, 2007; Agnarsson & May-Collado, 2008), Carnivora (Flynn et al., 2005), Soricomorpha (Dubey et al., 2007), Lagomorpha (Robinson & Matthee, 2005), Rodentia (Pages et al., 2010; Fabre et al., 2012), and Metatheria (Meredith et al., 2008; 2009).

Results

Data on sperm numbers, sperm size, body mass and testes mass are presented in Table S1. Eutherian mammals had a mean of $8.6 \times 10^9$ spermatozoa in their sperm reserves (i.e., sperm in caudae epididymides), ranging from $2.6 \times 10^5$ spermatozoa in the Damaraland mole rat (*Fukomys damarensis*) to $1.4 \times 10^{11}$ spermatozoa in the sheep (*Ovis aries*) (a range of 6 orders of magnitude; Table S1). On the other hand, metatherian mammals showed a mean value of $4.6 \times 10^8$ sperm in cauda epididymides, which ranged from $1.2 \times 10^6$ spermatozoa in the fat-tailed dunnart (*Sminthopsis crassicaudata*) to $4.8 \times 10^9$ spermatozoa in the tammar wallaby (*Macropus eugenii*) (a range of 2 orders of magnitude; Table S1).

In both Eutheria and Metatheria, sperm number exhibited a strong negative allometric association with body mass (Eutheria: $\log_{10}$ sperm numbers $= 6.52 + 0.67 \times \log_{10}$ body mass, $F_{1,62} = 90.95, p < 0.0001, R^2 = 0.59$; Metatheria: $\log_{10}$ sperm numbers $= 5.11 + 0.81 \times \log_{10}$ body mass, $F_{1,12} = 38.72, p < 0.0001, R^2 = 0.76$). However, the log-log equation for Metatheria presents a higher slope and a lower intercept, which means that small metatherians would have lower sperm numbers than their eutherian counterparts of the same body mass. To show this, we calculated the "linearized mean body mass" (the antilogarithm of the mean $\log_{10}$-transformed body mass) for each group (Eutheria= 2851.02g, Metatheria= 2299.85g) and used the previously stated equations to predict the sperm numbers. Comparison of predicted sperm numbers between the two clades revealed that Metatheria had roughly one order of magnitude less sperm ($6.66 \times 10^7$) than their Eutheria counterpart ($7.02 \times 10^8$).
Our analyses regarding the effect of sperm competition on sperm numbers revealed that in both Eutheria and Metatheria, there is a positive association between relative testes size and sperm numbers in caudae epididymides (Table 1). Neither the quadratic term for testes mass, nor the interaction between body mass and testes mass were significantly related to sperm numbers or sperm size in any of the two clades (Table S2). We next examined possible relationships between sperm size and sperm numbers. Since sperm size is negatively associated with body size in both Eutheria (Gomendio et al., 2011) and Metatheria (Tourmente et al., 2011a), as a consequence of a decrease in mass-specific metabolic rate in large-bodied animals, we included body size as a controlling variable in our analyses. We found that, after controlling for body size, sperm numbers in reserves and sperm size were positively associated in Eutheria (Table 1, Fig. 1A) but showed no significant relationship in Metatheria (Table 1, Fig. 1B).

According to recent theoretical models, the influence of sperm competition risk on both sperm numbers and sperm size should be taken into account to identify a possible tradeoff between these traits (Parker et al., 2010). Thus, in order to accurately predict the possible effect of one sperm trait on the other, the effect of relative testes size was included in the model. When relative testes size was controlled for, no significant association between sperm numbers and sperm length was observed in either Eutheria or Metatheria (Table 1, Fig. 1C, D).

Further assessments of the relationships between sperm numbers and sperm size were performed by calculating two additional parameters (Parker, 1990; Parker & Simmons, 1991; Parker et al., 2010; Immler et al., 2011): (a) total sperm investment (i.e., sperm size * sperm numbers), which represents an approximation of the total energy a male spends in sperm production, and (b) relative investment in sperm size (i.e., sperm size / sperm numbers), which represents an approximation of the proportion between investment in sperm numbers
and investment in sperm size. We found that total sperm investment was positively related to relative testes size in both Eutheria and Metatheria but that there were differences between clades when comparing increase rates (Eutheria, slope = 4.2121, $F_{1,61} = 91.54$, $p < 0.0001$; Metatheria, slope = 2.9936, $F_{1,11} = 11.68$, $p = 0.0057$; Table 2, Fig. 2A, B). On the other hand, relative investment in sperm size showed a significant negative relationship with relative testes size in Eutheria (slope = -0.0489, $F_{1,61} = 71.80$, $p < 0.0001$; Table 2, Fig. 2C) but not in Metatheria (slope = -0.0398, $F_{1,11} = 1.58$, $p = 0.2352$, Table 2, Fig. 2D). Overall, these results suggest that, in Eutheria, sperm numbers increase faster than sperm size in response to sperm competition.

**Discussion**

The results of this study showed that sperm competition maximizes sperm numbers in eutherian and metatherian mammals. Previous work on both clades also revealed a positive relationship between sperm competition and sperm size (Tourmente et al., 2011b; a). Both an increase in sperm numbers and sperm size would promote a positive association between total sperm investment (sperm size * sperm numbers) and sperm competition levels (Parker, 1990; Parker & Simmons, 1991; Parker et al., 2010; Immler et al., 2011), which is supported for both Eutheria and Metatheria in our analyses.

Theoretical models (Parker, 1990; Parker et al., 2010) predict that, in large vertebrates, the vast scale difference between the size of the sperm cell and that of the female reproductive tract favors a "raffle" sperm competition mechanism, and that competitive advantage may be gained mainly through increases in sperm numbers, which would tradeoff against sperm size. Our results regarding the relationship between sperm competition and relative investment in sperm size (sperm size/sperm numbers) in Eutheria suggest that, in this group, sperm numbers increase more rapidly than sperm size in response to sperm competition. At first glance, these
trends appear to be consistent with those described for passerine birds (Immler et al., 2011) and seem to adjust to theoretical models (Parker et al., 2010). However, we did not find any significant relationship between sperm numbers and sperm size in any of the two mammalian clades once the effect of sperm competition was controlled for. That is, an increase in sperm size is not associated to a decrease in sperm numbers at a given level of sperm competition. In addition, studies in birds showed an “inverted U-shaped” relationship between sperm competition and sperm size, which led to the conclusion that little or no increase in sperm size at high sperm competition risks occurred as a result of a tradeoff between sperm numbers and sperm size (Parker et al., 2010; Immler et al., 2011). In contrast, sperm size was not significantly associated with the quadratic term of testes mass, nor with the interaction term between body mass and testes mass in any of the two clades analyzed. Thus, there is no evidence of a deceleration in the slope of increase of sperm size in relation to sperm competition in mammals. Moreover, extremes cases such as Cricetulus griseus (total sperm length: 258 µm) and Tarsipes rostratus (total sperm length: 337 µm) show the longest sperm, respectively, in Eutheria and Metatheria, along with extremely high relative testes size (Gomendio et al., 2011; Tourmente et al., 2011b; a).

Raffle models predict that sperm numbers would have a higher rate of increment than sperm size in response to sperm competition because of the differences in the relative competitive advantages provided by each trait in a tradeoff scenario. Since our results found no evidence of such tradeoff, we propose that a similar relationship between rates of increase of these two traits could exist in mammals because their morpho-physiological reproductive characteristics do not conform to certain assumptions of the general models (Parker et al., 2010). These reproductive characteristics of mammals are discussed below.

Sperm numbers and size may be regulated by different energetic budgets
Sperm numbers and size are determined at different stages of spermatogenesis and are related to separate, independent processes of sperm formation. Sperm numbers may depend on testes architecture, kinetics of spermatogenesis, cell proliferation (i.e., the number of cell divisions of spermatogonia) and apoptosis (i.e., cell loss) resulting in a net number of cells after meiosis. These traits show considerable variations in mammals (Hess & Franca, 2008) and have been shown to covary with sperm competition levels (delBarco-Trillo et al., 2013).

Sperm size, on the other hand, depends exclusively on the post-meiotic differentiation process, when spermatids give rise to sperm cells. This process also presents a high degree of variability among mammals (Hess & Franca, 2008), although the possible associations between sperm competition and the duration of sperm differentiation remain to be established.

Differential constraints on sperm size and numbers

At least in Eutheria, longer sperm take longer to produce, and sperm competition selects for shorter sperm production times (Ramm & Stockley, 2010; delBarco-Trillo et al., 2013). This suggests that to produce increasingly longer sperm in a competitive timeframe, an enhanced mass-specific metabolic rate is required. In both Eutheria and Metatheria, sperm size correlates negatively with body size, which is a consequence of differences in mass-specific metabolic rates (low in large species and high in small species) (Gomendio et al., 2011; Tourmente et al., 2011a). Thus, in large mammalian species the increase on sperm size in relation to high levels of sperm competition appears to be constrained by the influence of mass-specific metabolic rate (Gomendio et al., 2011; Tourmente et al., 2011a). On the other hand, an increase in sperm numbers can be attained simply by increasing the quantity of sperm-producing units (i.e., increasing testes size). Absolute body mass and testes mass are positively correlated with sperm numbers in sperm reserves in both Eutheria and Metatheria, meaning that larger bodied species have larger testes and hence more sperm. Moreover, a
recent study found that sperm numbers in ejaculate, while increasing with relative testes size, appear not to be associated with metabolism (Lüpold, 2013). In addition, it should be pointed out that sperm size may be limited by factors other than energetic budget because size could relate to hydrodynamic, biomechanical (Ishijima, 2012), and biochemical (e.g., energy production, and ATP diffusion) (Takei et al., 2014) constraints, beyond which motility would not be efficient. Therefore, if sperm numbers and sperm size have a diverse set of energetic constraints, a tradeoff between these two sperm traits is not required to explain a more pronounced increase in sperm numbers associated to sperm competition.

Variable scaling of the female reproductive tract

The models described assume that the female reproductive tract is a uniform space, which increases proportionally to the size of the female. In mammals, the female reproductive tract generally comprises three major organs: the vagina, the uterus, and the oviducts, which contain both the sperm reservoir (at the lower isthmus) and the site of fertilization (ampulla) (Suarez, 2008). The size of the vagina and uterus exhibit considerable variation across mammalian species and are related to adult body mass, individual offspring size, and total litter mass (Austin & Short, 1985). However, the volume of the oviduct represents a small fraction of the total size of the tract. The oviduct volume represents about 0.2% of the total volume of the reproductive tract (estimated from (Peters & Ball, 1987; Kunhbar et al., 2003)) and relates to body mass with an extremely low exponent (0.2809, (Gomendio & Roldan, 1993; Anderson et al., 2006)). As an example, a cow has an oviduct 10-fold longer than a mouse, while having an approximately 13,000-fold body mass (Gomendio & Roldan, 1993). In this scenario, the effect of interspecific body mass variation on the scaling proportion between ejaculate size and the volume of the fertilization space would be very different according to the region of the female reproductive tract in which the sperm is located.
(extremely high in the uterus and vagina; much lower in the oviduct, the actual fertilization space in mammals).

Sperm numbers at the site of fertilization and polyspermy

Parker et al.’s raffle model assumes that the ejaculated sperm mass is affected only by passive sperm loss in its passage through the female reproductive tract, and that the amount of sperm lost is proportional to its size. In birds, this sperm loss is considerable since only 1-2% of the ejaculated sperm reaches the oviductal sperm storage tubules (Brillard & Bakst, 1990; Birkhead, 1992). In mammals, the sperm mass is rapidly transported (within minutes) from sperm deposition sites (uterus or vagina depending on the species) to the oviduct by muscular contractions. During this process, the sperm mass also sustains passive sperm loss, with a reduction of sperm numbers of approximately one order of magnitude (Suarez & Pacey, 2006). However, at least in the Eutheria, this reduction in sperm numbers is relatively low when compared to the reduction (5 to 6 orders of magnitude) that takes place when spermatozoa swim through the junction between the uterus and the oviduct (utero-tubal junction) (Hunter, 1993; Suarez & Pacey, 2006; Coy et al., 2012). As a result, only a few thousands of sperm are trapped and stored in the oviductal reservoir (Suarez, 2008; Coy et al., 2012). It is noteworthy that, while the number of ejaculated sperm in mammals presents considerable variation (a range of 5 orders of magnitude), and is related to body size [40], similar sperm numbers are found in the oviductal reservoir in many species with different body sizes (Harper, 1982). Following storage in the oviductal sperm reservoir, and after completing the process of capacitation, sperm are released in discrete small-numbered populations, which swim along the oviduct towards the site of fertilization (the oviductal ampulla) (Suarez, 2008; Hunter, 2012). A final step in the reduction of sperm numbers relates to the loss of cells that are not able to reach the ampulla, a process in which both thermotaxis
(Eisenbach & Giojalas, 2006) and chemotaxis (Guidobaldi et al., 2012) are involved. Thus, as a result of these complex physiological steps, the sperm:egg ratios at the time of fertilization are close to unity (Hunter, 1996; Burkitt et al., 2012; Coy et al., 2012; Hunter, 2012). The most likely explanation for such dynamics of sperm storage and release in mammals is the need to prevent polyspermy (Coy & Aviles, 2010; Coy et al., 2012; Hunter, 2012), which generally results in the death of the embryo (Snook et al., 2011). On the contrary, polyspermy appears to be the rule in birds, and multiple sperm penetration in the egg’s germinal disk (up to 60 in some cases (Perry, 1987)) does not lead to any developmental problem for the embryo (Snook et al., 2011). Moreover, multiple sperm penetration in birds appears to be an adaptation to assure fertility, since there is a positive correlation between the number of sperm entering the ova and the ovum size (Birkhead et al., 1994).

Taken together, differences in reproductive physiology suggest that, contrary to what happens in birds, in mammals sperm velocity (which is related to sperm size) would play a more essential role than sperm number in the last stage of the sperm journey to the site of fertilization. Thus, this final step in sperm transport would resemble a short race, with only a few participants, rather than a raffle.

**Differences between Eutheria and Metatheria**

In Eutheria sperm numbers tend to increase in a more pronounced way than sperm size under increasing levels of sperm competition. This pattern was not observed in Metatheria. A possible explanation for this difference may lay in fundamental differences that exist between Eutheria and Metatheria with regards to their relative investments in sperm numbers and sperm size. Metatheria have, on average, longer and fewer spermatozoa than their Eutheria counterparts (mean total sperm length, Metatheria = 163µm; Eutheria = 91µm) (Tourmente et al., 2011b; a). Several species of Metatheria appear to have evolved extremely efficient
mechanisms to transport sperm to the fertilization site (Taggart et al., 2003), measures of sperm protection (such as the ability to store sperm in specialized crypts of the oviductal epithelium) (Taggart et al., 1998), and the formation of sperm pairs (which appear to enhance velocity and/or protect the acrosomes) (Bedford et al., 1984). Consequently, the proportion of ejaculated sperm reaching the oviduct is up to four orders of magnitude higher than in some Eutheria (Taggart et al., 2003). Additionally, Metatheria have approximately 40% lower testes mass in relation to their body mass than Eutheria (Tourmente et al., 2011a). These particular physiological characteristics (more efficient transport, high sperm survival rate, and sperm storage in the female reproductive tract) may have resulted in a lower selective pressure on the production of high sperm numbers in Metatheria when compared to Eutheria, which may explain the comparatively lower relative testes mass and sperm numbers in the former.

Conclusions

While we found some relationships that matched the prediction of the raffle model of sperm competition (Parker et al., 2010) regarding the relationships between sperm numbers and size in mammals, we did not find evidence of a tradeoff between numbers and size when controlling for sperm competition levels. These relationships between sperm traits may be explained, in the absence of a tradeoff, as the product of two main features of mammalian reproductive physiology: (1) The number and size of spermatozoa are regulated by different molecular and cellular mechanisms underlying sperm proliferation and differentiation, which are subjected to different energetic budgets and constraints. (2) The extraordinary variation in the scaling between the volumes of the ejaculate and the female reproductive tract, and the extreme reduction in sperm numbers at the fertilization site make increasing sperm numbers and sperm size (through its influence on sperm velocity) adaptive advantages in different
stages of fertilization. In this way, increased sperm numbers would be more important at the
levels of the vagina and uterus, where sperm are likely to be diluted in a large environment,
while increased sperm size would be key in the oviduct, where there are only a few sperm
competing to reach the oocyte. These findings demand for revised or more detailed
hypotheses in order to elucidate the relationship between sperm traits in a sperm competition
context. Increased knowledge of the general and reproductive physiology of different taxa
should be taken into account to perform general predictions regarding the evolutionary effects
of sperm competition on sperm phenotype. In particular, physiological variables such as
mass-specific metabolic rate, timing, duration and efficiency of spermatogenesis in general
(and their different stages), accurate measures of the relative scaling of female reproductive
tract and sperm:egg ratio at the site of fertilization, and effects of sperm design and
physiology on sperm performance will be of foremost significance when predicting the
coevolution of sperm numbers and sperm size.

Acknowledgements
We are very grateful to Geoff A. Parker and Leigh W. Simmons for critically reading the
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Cierva" fellowship (JCI-2011-10381) both from the Spanish Ministry of Economy and
Competitiveness.

References


Table 1. Relation between sperm numbers, relative testes size and total sperm size in mammals. Phylogenetically controlled multiple regression analyses (PGLS). Superscripts following the $\lambda$ 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. All variables were log$_{10}$-transformed. n: number of species.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Dependent variable</th>
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<th>Slope</th>
<th>$R^2$</th>
<th>$p$</th>
<th>$t$</th>
<th>$\lambda$</th>
<th>$r$</th>
<th>CL(-)</th>
<th>CL(+)</th>
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<tr>
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Table 2. Relation between sperm investment and relative testes size in mammals. Phylogenetically controlled multiple regression analyses (PGLS). Superscripts following the $\lambda$ 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. All variables were log$_{10}$-transformed. n: number of species.

<table>
<thead>
<tr>
<th>Clade</th>
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<th>$R^2$</th>
<th>$p$</th>
<th>$t$</th>
<th>$\lambda$</th>
<th>$r$</th>
<th>CL(-)</th>
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<td>-0.3541</td>
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FIGURE LEGENDS

**Figure 1.** Relationships between sperm numbers and sperm size in mammals. Figure points are partial residuals estimated from multiple regression analysis using sperm numbers as dependent variable. A, B: body mass and total sperm length used as predictors. C, D: body mass, testes mass, and total sperm length used as predictors. All variables were log_{10}-transformed. A, C: Eutheria. B, D: Metatheria.

**Figure 2.** Relationships between sperm investment and relative testes size in mammals. Figure points are partial residuals estimated from multiple regression analysis using total sperm investment (sperm size * sperm numbers) (A, B), or relative investment in sperm size (sperm size / numbers) (C, D) as dependent variable, and body mass and testes mass as predictors. All variables were log_{10}-transformed. A, C: Eutheria. B, D: Metatheria.
Figure 1
No evidence of tradeoffs in the evolution of sperm numbers and sperm size in mammals

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¹Reproductive Ecology and Biology Group, Museo Nacional de Ciencias Naturales (CSIC), 28006-Madrid, Spain

Supporting Information

Table S1. Sperm size, sperm numbers, body mass, and testes mass in 78 species of mammals.

Table S2. Alternative models for the relation between sperm numbers, sperm size and relative testes size in mammals.

Figure S1. Phylogenetic reconstruction for the 78 mammal species utilized in the PGLS analysis.
Table S1. Sperm size, sperm numbers, body mass, and testes mass in 78 species of mammals. Abbreviations: TSL: total sperm length (μm). SN: sperm numbers in caudae epididymides (x 10^6). BM: body mass (g). TM: testes mass (g).

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<tr>
<th>Species</th>
<th>Order</th>
<th>Family</th>
<th>TSL</th>
<th>SN</th>
<th>BM</th>
<th>TM</th>
<th>Ref. TSL</th>
<th>Ref. SN</th>
<th>Ref. BM &amp; TM</th>
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<td>Artiodactyla</td>
<td>Bovidae</td>
<td>53.53</td>
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<td>(22, 23)</td>
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<td>(27, 28)</td>
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References


54. Banzato TP: Avaliação de parâmetros reprodutivos e análise histológica do testículo de ratos expostos ao cromio in utero e durante a lactação. Universidade Federal de São Carlos, Ciências Biológicas; 2010.


Table S2. Alternative models for the relation between sperm numbers, sperm size and relative testes size in mammals. 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 λ = 0 (first position) and λ = 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. All variables were log10-transformed. n: number of species. Testes mass*2: quadratic term of testes mass. Body mass * Testes mass: interaction between body mass and testes mass.

| Clade      | Dependent variable | Independent variable | Slope  | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ    | R²   | p    | t    | λ    | p  | r     | λ
Figure S1. Phylogenetic reconstruction for the 78 mammal species utilized in the PGLS analysis.