Postprint del artículo publicado en: Journal of Evolutionary Biology 27(1): 55-62 (2014)

Effects of metabolic rate and sperm competition on the fatty-acid composition of mammalian sperm

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Running title: Evolution of Mammalian Sperm Membrane

ABSTRACT

The sperm membrane is a key structure affecting sperm function and thus reproductive success. Spermatozoa are highly specialized and differentiated cells that undergo a long series of processes in the male and female reproductive tracts until they reach the site of fertilization. During this transit, the sperm membrane is prone to damage such as lipid peroxidation. The characteristics and performance of the sperm membrane are strongly determined by the fatty-acid composition of membrane phospholipids. Polyunsaturated fatty-acids (PUFAs) are the most prone to lipid peroxidation. Lipid peroxidation and other types of oxidative damage increase with higher metabolism and with higher levels of sperm competition due to the increased ATP production to fuel higher sperm velocities. Consequently, we hypothesized that, in order to avoid oxidative damage, and the ensuing impairment of sperm function, sperm cells exhibit a negative relationship between PUFA content and mass-specific metabolic rate (MSMR). We also hypothesized that higher sperm competition leads to a reduction in the proportion of sperm PUFAs. We performed a comparative study in mammals and found that high MSMR and high levels of sperm competition both promote a decrease in the proportion of PUFAs that are more prone to lipid peroxidation. The negative relationship between MSMR and these PUFAs in sperm cells is surprising, because a positive relationship is found in all other cell types so far investigated. Our results support the idea that the effects of MSMR and sperm competition on sperm function can operate at very different levels.

Keywords: sperm membrane; sperm competition; mass-specific metabolic rate; polyunsaturated fatty acids; lipid peroxidation

INTRODUCTION

The efficient functionality of spermatozoa determines the reproductive success of males (Yanagimachi, 1994). Sperm function differs greatly among species and it is specially affected by mass-specific metabolic rate (MSMR) (Lüpold, 2013) and sperm competition (Gómez Montoto *et al.*, 2011; Lüpold, 2013); sperm competition occurs when females mate with more than one male and the sperm of those males compete to fertilize the female's ova (Parker, 1970; Birkhead & Møller, 1998). For example, comparative studies on mammals report that an increase in both MSMR and sperm competition levels favours an increase in sperm swimming velocity (Gómez Montoto *et al.*, 2011; Lüpold, 2013).

One of the sperm features that most directly affects sperm function is the cellular membrane, which is involved not only in sperm motility and viability, but also in the processes that precede and enable the fusion of the spermatozoon with the oocyte (Eddy & O'Brien, 1994; Florman & Ducibella, 2006). The membrane bilayer is mainly constituted by phospholipids and their fatty acids. The proportion of different types of fatty acids can influence many aspects of membrane function (Hulbert & Else, 1999). A key difference among these different types of fatty acids is their level of unsaturation, which is determined by the number of double bonds within the molecule (Wathes *et al.*, 2007). Saturated fatty acids (SFAs), monounsatured fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) have zero, one, or more than one double bond, respectively. The most polyunsaturated PUFA is docosahexaenoic acid (DHA), with six double bonds.

Lipid peroxidation occurs when lipids react with reactive oxygen species (ROS) and can have several negative effects on sperm function, including loss of motility, structural damage to the sperm membrane, and inability to undergo capacitation and fuse with the oocyte (de Lamirande & Gagnon, 1992; White, 1993; Aitken & Bennetts, 2006; Costantini *et al.*, 2010). SFAs and MUFAs are not susceptible to lipid peroxidation, but PUFAs are

(Hulbert *et al.*, 2007), and the greater the degree of polyunsaturation of fatty acids, the more susceptible they are to lipid peroxidation, with DHA being the PUFA most prone to lipid peroxidation (Hulbert *et al.*, 2007). Sperm cells have high PUFA content (Mann & Lutwak-Mann, 1981; White, 1993) and are thus more vulnerable to oxidative damage (including lipid peroxidation) than other cell types (Aitken, 1997; Sikka, 2001). Such high PUFA content in sperm cells confers fluidity to the sperm membrane and it also seems to be important in the regulation of lipid metabolism and cell movement (Stubbs & Smith, 1984; Gliozzi, *et al.*, 2009). Given that smaller sized species have higher MSMR and thus a higher production of ROS, there should be a reduction in membrane unsaturation in the spermatozoa of these species as a defensive mechanism to minimize the oxidative damage produced by external ROS.

In addition to MSMR, sperm competition is another evolutionary force that could affect the fatty-acid composition of sperm cells. In species that experience high levels of sperm competition there will be a reproductive advantage by improving sperm function, notably sperm velocity (Gómez Montoto *et al.*, 2011). One way to obtain faster swimming sperm may be to increase the production of ATP; however, this will result in an upregulation of metabolic activity and thus a higher production of ROS. A strategy to counteract the negative effects of internally produced ROS would be to reduce the proportion of fatty acids that are easily peroxidized (such as DHA).

The level of sperm competition across species is unrelated to body size and thus MSMR and sperm competition may have independent effects on the fatty-acid composition of sperm membranes. Consequently, the goal of our study was to study for the first time the different effects that metabolic rate and sperm competition may have on the fatty-acid composition of sperm membranes. To accomplish this goal we gathered and analysed information on the proportion of sperm phospholipids and fatty acids in mammalian species.

We considered separately the proportion of n-6 and n-3 PUFAs (where 3 and 6 refer to the first double bond from the terminal CH_3 of the carbon chain), given that n-3 PUFAs are more prone to peroxidation than n-6 PUFAs (Hulbert *et al.*, 2007). We predicted that the proportion of n-3 PUFAs (but not necessarily the proportion of n-6 PUFAs) will decrease in species with higher MSMR and higher levels of sperm competition. Similarly, we predicted that the proportion of DHA (i.e., the PUFA most prone to peroxidation) will show a negative relationship with MSMR and levels of sperm competition.

Our prediction that higher MSMR will lead to sperm cells with lower proportions of n-3 PUFAs and DHA is, however, opposite to what we know to be the case in all other tissues so far investigated, in which higher metabolism is coupled with higher levels of polyunsaturation in bilayer membranes (Hulbert & Else, 2000). Indeed, the "membrane pacemaker theory of metabolism" makes a direct connection between the MSMR of a species and its level of membrane polyunsaturation (Hulbert & Else, 1999; Hulbert, 2005). This theory proposes that higher levels of membrane polyunsaturation cause membrane proteins to have a higher molecular activity, which results in higher metabolic rates in those cells and thus in the whole organism (Hulbert, 2005). Consequently, this theory predicts a positive relationship between MSMR and the level of membrane polyunsaturation; given that there is a negative correlation between body size and MSMR, this theory also predicts a negative relationship between body size and the level of membrane polyunsaturation. A series of studies have supported the membrane pacemaker theory of metabolism in birds (Hulbert *et al.*, 2002a) and mammals (Hulbert *et al.*, 2002b), and in many different tissues, including cardiac muscle, skeletal muscle, liver, and kidney (Hulbert *et al.*, 2002b).

We also considered the proportion of the main types of phospholipids (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) in relation to MSMR and sperm competition. Given that in other tissues the distribution of membrane phospholipid

117 classes do not vary with body size, with phosphatidylcholine and phosphatidylethanolamine
118 being the main phospholipid classes regardless of body size (Nealon *et al.*, 2008), we
119 predicted no relationship between MSMR (or sperm competition) and phospholipid
120 proportions in mammalian sperm.

121 Finally, we investigated two potential compensatory mechanisms to counterbalance
122 any reduction in the level of polyunsaturation. First, we considered the
123 cholesterol:phospholipid ratio. Cholesterol is an important structural component in cell
124 membranes, where it contributes to an impermeable and cohesive membrane (White, 1993).
125 We predicted that in species with higher levels of sperm competition there will be a lower
126 proportion of cholesterol for two reasons: (a) higher levels of cholesterol are associated with
127 longer duration of capacitation (Davis, 1981), and a reduction in the time for capacitation is a
128 competitive feature (Gomendio *et al.*, 2006); (b) if sperm competition selects for sperm with
129 lower levels of polyunsaturation (which would reduce membrane fluidity) to decrease lipid
130 peroxidation, there may be a concomitant decrease in the proportion of cholesterol (which
131 would increase membrane fluidity) to maintain similar levels of membrane fluidity. Second,
132 we investigated the desmosterol:cholesterol ratio in mammalian sperm. Desmosterol is an
133 intermediate compound in the synthesis of cholesterol (Lin *et al.*, 1993; Zalata *et al.*, 2010).
134 In mammals, desmosterol is mostly restricted to sperm cells and testes (Connor *et al.*, 1998).
135 Desmosterol has two double bonds while cholesterol has only one double bond, which may
136 result in desmosterol providing more fluidity to the membrane (Lin *et al.*, 1993; Connor *et*
137 *al.*, 1998). Consequently, we predicted that the desmosterol:cholesterol ratio may increase
138 with higher MSMR and higher levels of sperm competition to counterbalance a possible
139 decrease in the proportion of PUFAs to reduce the incidence of lipid peroxidation.

140 We found that high MSMR and high levels of sperm competition both promote a
141 decrease in the proportion of PUFAs that are more prone to lipid peroxidation. These results,

compared to those of previous studies, indicate that the fatty-acid composition of membranes in sperm cells differs from that found in all other cell types.

MATERIALS AND METHODS

We collected data on the composition of phospholipids, fatty acids, and sterols (cholesterol and desmosterol) in the sperm of 21 mammalian species (see Table S1 in Additional file 1). For all these 21 species we also collected data on body mass (g) and testes mass (g), whereas data on mass-specific metabolic rate (ml O_2 / h x g) was found for a subset of 16 species (Table S1). Only data for the three main classes of phospholipids (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) were available for a sufficient number of species (n = 13; Table S1). For two of these phospholipids (phosphatidylcholine and phosphatidylethanolamine), we compiled data from the literature on their fatty-acid composition for 9 species (Tables S2 and S3), and studied the relationship of these data with relative testes mass (we did not have MSMR data for all these 9 species, so we did not perform analyses on the effect of MSMR).

We calculated five variables regarding the total fatty-acid content in sperm: percentage of saturated fatty acids (% SFA), percentage of polyunsaturated fatty acids (% PUFA), percentage of n-6 polyunsaturated fatty acids (% n6), percentage of n-3 polyunsaturated fatty acids (% n3), and percentage of docosahexaenoic acid (% DHA). We also compiled or calculated two ratios: cholesterol:phospholipid and desmosterol:cholesterol. For any of these variables, when more than one value was reported for the same species, we calculated an average value weighted by sample size. Data on MSMR, body mass, testes mass, and ratios were $\log_{10}$-transformed. All the other variables, being percentage data, were arcsine-transformed (calculating arcsine of the square root of the variable).

We tested the influence of metabolic rate on the composition of the sperm membrane, using regression analyses in which each variable of interest was a dependent variable and using mass-specific metabolic rate as the predictor. Each dependent variable was analysed separately. We also tested the influence of sperm competition on those same dependent variables (each dependent variable being analysed separately), using body mass and testes mass as the predictors. This provided a measure of the relationship between each dependent variable and relative testes mass. A higher relative testes mass has been found to strongly associate with the percentage of multiple paternity and, thus, relative testes mass can be used as a proxy of sperm competition levels (Ramm *et al.*, 2005; Firman & Simmons, 2008; Soulsbury, 2010). Given that body mass and testes mass are related to each other (i.e., they are non-orthogonal), a sequential (Type I) sum of squares was used, adding the two predictors to the models in the following order: body mass, testes mass. For all these analyses we conducted phylogenetic generalized least squares (PGLS) models in R 2.13.0 (R Core Team, 2012) using a code written by R. Freckleton. The PGLS estimates a phylogenetic scaling parameter lambda (λ), which is then incorporated in the models to control for phylogenetic effects. If λ values are close to 0, the variable in question is likely to have evolved independently of phylogeny, whereas values close to 1 indicate strong phylogenetic association of the variables. The phylogenetic reconstruction used in the PGLS analyses in included in the Additional file 1. For the graphical representation of the data (Fig. 1), and only in this case, relative testes size was calculated using Kenagy and Trombulak´s mammalian-specific regression equation: relative testes size = testes mass / 0.035 × body mass$^{0.72}$ (Kenagy & Trombulak, 1986).

RESULTS

Values compiled from the literature for the different variables are summarized in the Datasets in the Additional file 1 (Tables S1-S3). There were large differences across species in many variables. For example, the percentage of n-3 PUFAs ranged from 0% in the rat to 68% in the African elephant.

MSMR was not significantly correlated with the proportion of SFAs, the proportion of total or n-6 PUFAs, the cholesterol:phospholipid ratio, the proportion of phosphatidylcholine, the proportion of phosphatidylethanolamine, or the proportion of sphingomyelin (PGLS: $P > 0.05$ for all analyses; see Table 1). On the other hand, species with high MSMR had lower proportions of n-3 PUFAs (PGLS: $F_{1,12} = 4.84$, $P = 0.048$; fig. 1a) and DHA (PGLS: $F_{1,14} = 8.07$, $P = 0.01$), and higher amounts of desmosterol relative to cholesterol (PGLS: $F_{1,9} = 9.19$, $P = 0.01$; Table 1).

Relative testes size was not significantly correlated with the proportion of SFAs, the proportion of total or n-6 PUFAs, the proportion of DHA, the cholesterol:phospholipid ratio, the desmosterol:cholesteriol ratio, the proportion of phosphatidylcholine, or the proportion of phosphatidylethanolamine ($P > 0.05$ for all analyses; see Table 1). However, an increase in relative testes size was associated with a reduction in the proportion of n-3 PUFAs (PGLS: $F_{1,15} = 4.82$, $P = 0.04$; fig. 1b) and with a reduction in the proportion of sphingomyelin (PGLS: $F_{1,10} = 18.75$, $P = 0.001$; see Table 1 and Table S4).

The proportion of SFAs, PUFAs, and DHA in phosphatidylcholine or phosphatidylethanolamine were not related to body mass or relative testes mass ($P > 0.05$ for all analyses; see Tables S5 and S6 in Additional file 1).

There was no significant relationship between MSMR and relative testes mass (PGLS: $F_{1,13} = 1.62$, $P = 0.23$; see Table S7 in Additional file 1), which indicates that MSMR and sperm competition may have independent effects on the proportion of n-3 PUFAs.

DISCUSSION

We found that the proportion of n-3 PUFAs, which are the membrane fatty acids most prone to lipid peroxidation, decrease in the sperm of mammalian species with high MSMR and high levels of sperm competition. These results, which support our initial predictions, are strikingly different from those found in other mammalian tissues. As mammal and bird species decrease in size (and thus increase their MSMR), the cellular membranes in several organs become progressively more polyunsaturated (Hulbert *et al.*, 2002a; Hulbert *et al.*, 2002b). Interestingly, in mammals, the proportion of total PUFAs is not affected by body mass in all tissues investigated, whereas the proportion of n-3 PUFAs correlate negatively with body mass in heart, skeletal muscle, liver and kidney (Hulbert *et al.*, 2002b). In sperm cells, the proportion of total PUFAs is also unrelated to body mass, whereas the proportion of n-3 PUFAs correlate positively with body mass. Therefore, sperm cells represent an exception to the membrane pacemaker theory of metabolism, which postulates a positive association between MSMR and membrane polyunsaturation.

One of the main predictions of the membrane pacemaker theory of metabolism is that species with high MSMR have membranes that are predominantly polyunsaturated and with high DHA content, whereas those species with low MSMR have less polyunsaturated membranes, with a low DHA content (Hulbert, 2005). This prediction of the membrane pacemaker theory has been so well supported for several tissues in mammals and birds (Käkelä & Hyvärinen, 1995; Hulbert *et al.*, 2002a; Hulbert *et al.*, 2002b), that it seemed to be an overarching explanation for all organs and cell types. Here we show that sperm cells are, however, a striking exception. Our results suggest that the unusual fatty-acid composition of sperm cells is due to the need to counterbalance the negative effects of lipid peroxidation in order to maintain effective levels of sperm function. On the one hand, MSMR leads to higher

metabolic rates in all tissues with a consequent increase in the production of external ROS. On the other hand, sperm competition promotes a higher production of ATP to fuel faster swimming speeds (Tourmente *et al.*, 2013), which is in turn likely to increase the production of internal ROS. To minimize the negative effects of ROS on sperm function, species with high MSMR and/or high levels of sperm competition have evolved sperm membranes that are less prone to lipid peroxidation. This seems to have been accomplished not only by reducing the proportion of PUFAs in the membrane, but also by increasing the proportion of plasmalogens in sperm cells. Plasmalogens are a type of glycerophospholipid that has antioxidant properties and are found in high levels in the sperm cells of several mammalian groups (Fuchs *et al.*, 2007; Fuchs *et al.*, 2009). An increase in the proportion of plasmalogens, together with the antioxidants contained in the seminal plasma (Koziorowska-Gilun *et al.*, 2011), would also reduce the susceptibility of sperm cells to lipid peroxidation. Unfortunately, data for a sufficient number of species on the proportion of plasmalogens in sperm cells are not yet available to determine how the proportion of plasmalogens may be affected by MSMR and/or different levels of sperm competition.

The proportion of DHA in mammalian sperm varies across species much more than in any other tissues. While the proportion of DHA across species in heart, skeletal muscle, liver, kidney, and brain ranges approximately between 1% and 12% (Hulbert *et al.*, 2002b), DHA in sperm ranges from very low percentages in rat (0%) and rabbit (1%) to 68% in the African elephant. Such higher values of DHA have also been reported in the muscle mitochondria of cold-water fish (Guderley *et al.*, 1997). Although the proportion of DHA did not relate significantly with sperm competition, it showed a negatively relationship with MSMR. Again, this result is opposite to results in other tissues, where DHA is negatively correlated with body size (and thus positively correlated with MSMR) in heart, skeletal muscle, liver and kidney. The membrane pacemaker theory of metabolism states that high proportions of

DHA in most tissues of small-sized species can explain their high MSMR. Our results in sperm cells suggest that the high MSMR of such small-sized species may have in turn forced a reduction in the proportion of DHA in sperm to minimize the negative effects of lipid peroxidation. One question that still remains unanswered is why the proportion of DHA is so high in sperm of some species in the first place.

The proportion of SFA in sperm was unrelated to MSMR, which is similar to what occurs in other mammalian tissues (Hulbert *et al.*, 2002b). The proportion of SFA was also unrelated to sperm competition. Therefore, the only fatty-acids that seem to be affected by sperm competition and MSMR are those that increase the risk of lipid peroxidation, i.e. n-3 PUFAs, and DHA in particular.

In the majority of studies from which we compiled data for our analyses (see Table S1), no distinction was made between phospholipids from the head and from the tail of sperm cells. In rhesus monkey, 99% of sperm DHA was located in the tail (Connor *et al.*, 1998). It is thus possible that the decrease in the proportion of polyunsaturation observed in small-bodied species and species with high levels of sperm competition may be restricted to the sperm tails. Furthermore, given that the sperm head contains the nuclear DNA, a maximal protection of this DNA may be attained by having a high proportion of saturated fatty acids in the membrane of the sperm head. In the rhesus monkey, the proportion of desmosterol in relation to cholesterol is also higher in sperm tails than in sperm heads (Connor *et al.*, 1998). In the same way that the six double bonds of DHA contribute to increase membrane fluidity, the two double bonds in desmosterol can confer more membrane fluidity than the single double bond in cholesterol (Connor *et al.*, 1998). Given that our results showed that the desmosterol:cholesterol ratio was positively associated with MSMR, we argue that the decrease in n-3 PUFAs in species with high MSMR (which can reduce the risk of lipid peroxidation but will also reduce membrane fluidity), can be counterbalanced with a higher

proportion of desmosterol, so that membrane fluidity can be maintained while reducing the incidence of lipid peroxidation. Unfortunately, no data are yet available to test this hypothesis.

The cholesterol:phospholipid ratio was not related to MSMR or sperm competition. The proportion of the main phospholipid classes (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) were also not related to MSMR, which is also the case for other tissues such as kidney and brain (Nealon *et al.*, 2008). However, the relative proportions reported for kidney and brain were partly different from the ones we found for sperm. In kidney, brain, and sperm, phosphatidylcholine is the main class of phospholipid, but while it represents around 70% of phospholipids in kidney and brain, it only represents an average of 44% (range: 28 – 65%) in sperm. The second main phospholipid (phosphatidylethanolamine) represents around 20% of phospholipids in kidney, brain, and sperm. However, the third class of phospholipid in sperm is sphingomyelin, which represents a much lower percentage in kidney and brain. For example, sphingomyelin was not detected in the kidney or brain of mice, but it represented 22% of phospholipids in mouse sperm (Alvarez *et al.*, 1987; Rejraji *et al.*, 2006). Interestingly, we found a negative relationship between the proportion of sphingomyelin and the level of sperm competition across species. Sphingomyelin in rats is one of the lipid classes that decrease the most during the acrosome reaction (Zanetti *et al.*, 2010), which suggests that a reduction in the proportion of sphingomyelin can result into a more stable membrane and thus a decrease in the proportion of sperm undergoing spontaneous acrosome reaction. It must also be noted that sphingomyelin in the sperm head is composed mostly by PUFAs (Oresti *et al.*, 2011), so a general reduction of PUFAs in relation to sperm competition levels could also be related to the significant reduction in the proportion of sphingomyelin.

CONCLUSIONS

Despite the importance that the cellular membrane has for the function of sperm cells, we have little understanding on how different evolutionary forces shape its composition. Our main finding that high MSMR and high levels of sperm competition both promote a decrease in the proportion of PUFAs that are more prone to lipid peroxidation emphasizes the importance of reducing the exposure of DNA, proteins and lipids to oxidative stress. The atypical composition of the sperm membrane in mammals (compared to somatic cells from other tissues examined to date) can be understood in a general framework in which high levels of both MSMR and sperm competition lead to the overall enhancement of sperm function.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for their comments. This work was supported by a Ramón y Cajal fellowship (RYC-2011-07943) to J.d.-T. and grants from the Spanish Ministry of Economy and Competitiveness (CGL2011-26341 to E.R.S.R. and CGL2012-37423 to J.d.-T.). The authors declare that they have no competing interests.

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455 **Table 1**. Effects of metabolic rate and sperm competition on fatty-acid composition of sperm

Dependent variable	Predictor	Estimate	*F*	e(MS,df)	*P* value	λ	*r*	CI	n
% SFA	MSMR	0.05	0.12	0.007,14	0.73	$0.999^{*, n.s.}$	0.09	-0.45 to 0.64	16
	RTS	0.03	0.15	0.004,18	0.7	$0.70^{n.s., n.s.}$	0.09	-0.37 to 0.55	21
% PUFA	MSMR	-0.10	0.36	0.009,14	0.56	$0.95^{*, n.s.}$	0.16	-0.38 to 0.70	16
	RTS	-0.06	0.51	0.004,18	0.48	$0.23^{n.s., *}$	0.17	-0.29 to 0.63	21
% n-6	MSMR	0.24	4.16	0.006,12	0.06	$<0.01^{n.s., n.s.}$	0.51	-0.03 to 1.15	14
	RTS	0.01	0.004	0.007,15	0.95	$<0.01^{n.s., n.s.}$	0.02	-0.49 to 0.52	18
% n-3	MSMR	-0.43	4.84	0.013,12	**0.048**	$0.64^{n.s., n.s.}$	0.54	**0.01 to 1.19**	14
	RTS	-0.31	4.82	0.009,15	**0.04**	$<0.01^{n.s., *}$	0.49	**0.03 to 1.05**	18
% DHA	MSMR	-0.51	8.07	0.02,14	**0.01**	$0.16^{n.s., *}$	0.61	**0.16 to 1.24**	16
	RTS	-0.18	1.57	0.012,18	0.23	$<0.01^{n.s., *}$	0.28	-0.17 to 0.75	21
CHO:PL	MSMR	0.07	0.01	0.23,10	0.94	$0.999^{*, n.s.}$	0.03	-0.63 to 0.68	12
	RTS	0.55	1.65	0.13,12	0.22	$0.999^{*, n.s.}$	0.35	-0.20 to 0.93	15
DES:CHO	MSMR	1.57	9.19	0.1,9	**0.01**	$<0.01^{n.s., *}$	0.71	**0.20 to 1.58**	11
	RTS	-0.09	0.03	0.13,11	0.87	$0.47^{n.s., n.s.}$	0.05	-0.54 to 0.64	14
% PC	MSMR	0.05	0.15	0.005,9	0.71	$<0.01^{n.s., n.s.}$	0.13	-0.61 to 0.87	11
	RTS	0.05	0.15	0.005,10	0.71	$<0.01^{n.s., *}$	0.12	-0.50 to 0.74	13
% PE	MSMR	-0.01	0.02	0.002,9	0.89	$<0.01^{n.s., *}$	0.05	-0.69 to 0.79	11
	RTS	0.11	2.24	0.002,10	0.17	$<0.01^{n.s., *}$	0.43	-0.16 to 1.08	13
% SM	MSMR	-0.14	2.32	0.003,9	0.16	$<0.01^{n.s., *}$	0.45	-0.25 to 1.23	11
	RTS	-0.29	18.75	0.003,10	**0.001**	$0.999^{n.s., n.s.}$	0.81	**0.50 to 1.74**	13

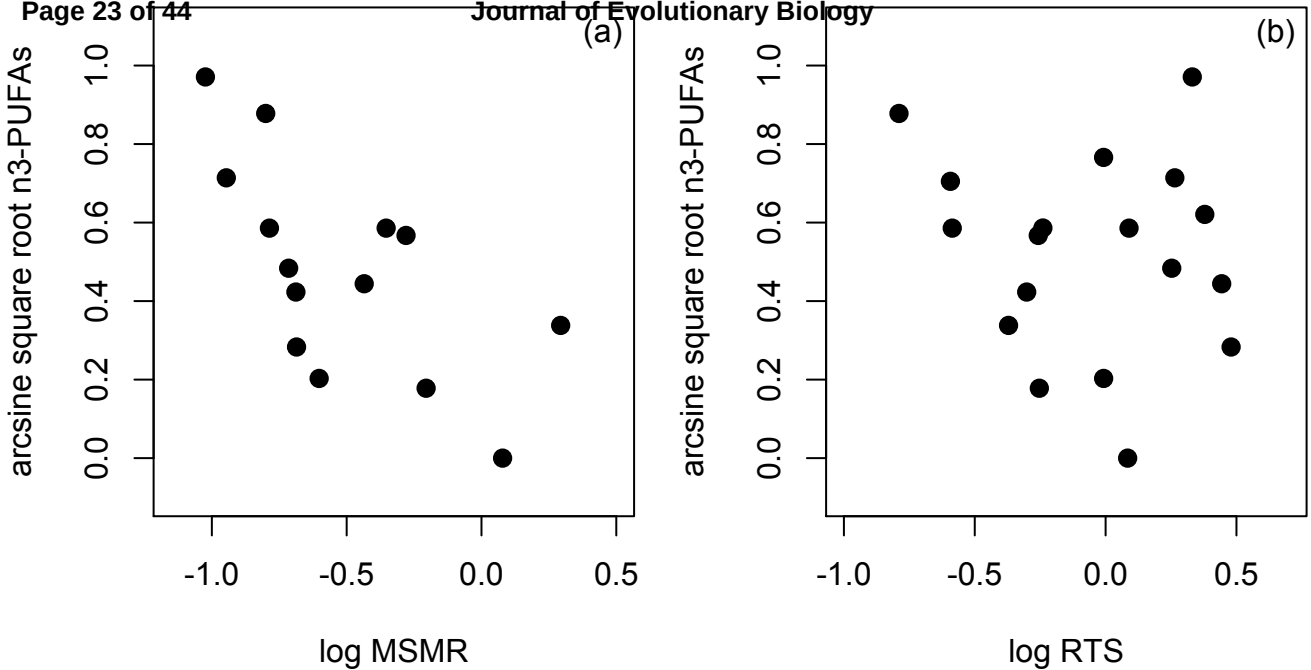
456 Phylogenetically controlled multiple regression analyses revealing the effects of mass-
457 specific metabolic rate (MSMR) and relative testes mass (RTS) on phospholipid, sterol, and
458 fatty-acid composition of sperm. In the RTS analyses, we report the values for the second
459 predictor (testes mass) after controlling for the effect of the first predictor (body mass; see

Additional file 1 for the values of body mass). Proportion data were arcsine-transformed (using arcsine root square) and ratio data were $\log_{10}$-transformed prior to analysis. The 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 superscript) and $\lambda = 1$ (second superscript). The effect size *r* was calculated from the *F* values; we also present the non-central 95% confidence interval (CI), an interval excluding 0 indicating statistically significant relationships. The *P* values and CI that indicate statistical significance are shown in bold. Abbreviations: e(MS,df), error MS and error degrees of freedom; % SFA, percentage of saturated fatty acids; % PUFA, percentage of polyunsaturated fatty acids; % n6, percentage of n-6 polyunsaturated fatty acids; % n3, percentage of n-3 polyunsaturated fatty acids; % DHA, percentage of docosahexaenoic acid (22:6 n-3); CHO:PL, ratio between cholesterol and phospholipid; DES:CHO, ratio between desmosterol and cholesterol; % PC, percentage phosphatidylcholine out of the total of phospholipids; % PE, percentage of phosphatidylethanolamine out of the total of phospholipids; % SM, percentage of sphingomyelin out of the total of phospholipids.

477 FIGURE LEGENDS

478

479 Figure 1. Proportion of n-3 polyunsaturated fatty acids in relation to mass-specific metabolic
480 rate and relative testes size. (a) Relation between mass-specific metabolic rate
481 (MSMR) and the content of n-3 PUFAs in the sperm membrane. (b) Relation between
482 relative testes size (RTS, sensu Kenagy & Trombulak 1986) and the content of n-3
483 PUFAs in the sperm membrane. These relations do not include the phylogenetic
484 corrections included in the statistical models.


(a)
arcsine square root n3-PUFAs
0.0
0.2
0.4
0.6
0.8
1.0
-1.0
-0.5
0.0
0.5
log MSMR
(b)
arcsine square root n3-PUFAs
0.0
0.2
0.4
0.6
0.8
1.0
-1.0
-0.5
0.0
0.5
log RTS

SUPPORTING INFORMATION

DATASETS

Table S1. Data on body mass, testes mass, mass-specific metabolic rate and sperm lipid variables

Table S2. Data on body mass, testes mass, mass-specific metabolic rate and fatty-acid composition of sperm phosphatidylcholine

Table S3. Data on body mass, testes mass, mass-specific metabolic rate and fatty-acid composition of sperm phosphatidylethanolamine

PHYLOGENETIC INFORMATION

Phylogeny S1. Phylogenetic reconstruction for the 21 mammalian species utilized in the PGLS analyses.

SUPPLEMENTARY REFERENCES

References S1. References cited in supplementary material.

SUPPLEMENTARY TABLES

Table S4. Effect of sperm competition on fatty-acid composition of sperm.

Table S5. Effect of sperm competition on the fatty-acid composition of sperm phosphatidylcholine.

Table S6. Effect of sperm competition on the fatty-acid composition of sperm phosphatidylethanolamine.

Table S7. Relationship between mass-specific metabolic rate and sperm competition.

Table S1. Data on body mass, testes mass, mass-specific metabolic rate and sperm lipid variables

Species	BM	TM	MSMR	%SFA	%PUFA	%n6	%n3	%DHA	CHO:PL	DES:CHO	%PC	%PE	%SM	Refs
Bos taurus	680385	681	0.16	45.33	47.08	11.23	30.57	59.05	0.25	0.13	53.8	24.25	10.81	[1-9]
Bubalus bubalis	680000	652		47.8	38.4			20.2			30.4	10.8	11.3	[4,10]
Capra hircus	25420	156.8	0.21	60.94	26.78	11.5	7.8	3.02	0.46	0.01	38.9	26.02	17.86	[11-13]
Ovis aries	57172.73	222.99		33.73	59.67	4.27	33.84	51.59	0.52	0.03	55.87	14.55	17.28	[8,14-21]
Cervus elaphus	104600	141.4		33.79	54.29	6.23	48.06	47.22						[22]
Sus scrofa	39700	128.2	0.19	36.2	53.46	33.12	21.63	21	0.43	0.28	43.54	26.65	17.11	[3,7,8,23-34]
Equus caballus	468000	416	0.25	29.54	64.4	60.21	4.06	1.89	0.29	0.05	64.6	12.5	11.9	[7,35-37]
Vulpes vulpes	5069	9	0.53	37.98	52.68	23.81	28.87	28.87	0.02	2.91				[38]
Alopex lagopus	4800	4.06	0.44	45.53	41.8	11.23	30.57	30.57	0.01	1.96				[38]
Canis familiaris	21620	27.66	0.36	41.4	43.4			3.9			27.5	20.1	18.3	[39]
Mesocricetus auratus	108	3.17	1.79	39	53.5			18.6	0.21	6.38	53	30.2	0.7	[40]
Rattus norvegicus	379.63	3.06	1.2	55.82	60.5	40.6	0.00	0.00	0.33	0.32	29.27	29.18	5.66	[14,41,42]
Mus musculus	21.13	0.13	1.97	46.3	41.2	28.5	11	11	0.29		56.47	14.3	22.34	[43,44]
Oryctolagus cuniculus	2888	6.06	0.62	46.45	44.45	41.54	3.14	1.12	0.89		46.59	13.19	13.88	[8,43,45-47]
Homo sapiens	63540	50.2	0.2	49.78	33.59	9.19	16.86	19.62	0.46	0.39	33.44	26.98	17.24	[8,48-67]
Macaca mulatta	10430	76	0.37	46.67	34.8	14.33	18.47	19.51		1.37	33	25	8.1	[68-70]
Loxodonta africana	4365500	4530	0.09	23.21	73.93	5.8	68.13	68.13						[71]
Elephas maximus	4545400	4000	0.11	35.02	52.24	9.36	42.88	42.88						[71]
Vombatus ursinus	40100	18.42		34.5	52.9	10.9	42	42	0.01	0.15				[72]
Phascolarctos cinereus	8150	3.72	0.16	12	80.2	21	59.2	59.2	0.00	0.09				[72]
Macropus giganteus	40720	42.02		18.1	61.5	30.9	30.6	30.6	0.01	0.04				[72]

Species are listed in the same order as they appear in the phylogenetic reconstruction (see Phylogeny S1). Abbreviations: BM, body mass; TM, testes mass; MSMR, mass-specific metabolic rate; % SFA, percentage of saturated fatty acids; % PUFA, percentage of polyunsaturated fatty acids; % n6, percentage of n-6 polyunsaturated fatty acids; % n3, percentage of n-3 polyunsaturated fatty acids; % DHA, percentage of docosahexaenoic acid (22:6 n-3); CHO:PL, ratio between cholesterol and phospholipid; DES:CHO, ratio between desmosterol and cholesterol; % PC, percentage phosphatidylcholine out of the total of phospholipids; % PE, percentage of phosphatidylethanolamine out of the total of phospholipids; % SM, percentage of sphingomyelin out of the total of phospholipids; Refs, references.

Table S2. Data on body mass, testes mass, mass-specific metabolic rate and fatty-acid composition of sperm phosphatidylcholine

Species	BM	TM	% SFA	% PUFA	% DHA	Refs
Bos taurus	680385	681	46.15	48.88	36.3	[5,7,9,73]
Bubalus bubalis	680000	652	45.2	38.4	23.5	[10]
Capra hircus	25420	156.8	73.39	10.8	1.8	[11,12]
Ovis aries	57172.73	222.99	19.16	76.61	36.03	[18,74]
Sus scrofa	39700	128.2	27.48	68.5	20.2	[7,32,75]
Equus caballus	468000	416	24.89	71.3	54.6	[7]
Mesocricetus auratus	108	3.17	46.78	49.49	21.2	[40]
Rattus norvegicus	379.63	3.06	37	44.1	0.00	[41]
Macaca mulatta	10430	76	42.2	35.4	21.6	[70]

Species are listed in the same order as they appear in the phylogenetic reconstruction (see Phylogeny S1). Abbreviations: BM, body mass; TM, testes mass; %SFA, percentage of saturated fatty acids; %PUFA, percentage of polyunsaturated fatty acids; %DHA, percentage of docosahexaenoic acid (22:6 n-3); Refs, references. Fatty-acid values differ from those in Table S1 because they are calculated using only information about the fatty-acid composition of phosphatidylcholine.

Table S3. Data on body mass, testes mass, mass-specific metabolic rate and fatty-acid composition of sperm phosphatidylethanolamine

Species	BM	TM	% SFA	% PUFA	% DHA	Refs
Bos taurus	680385	681	42.29	47.22	17.34	[5,7,9,73]
Bubalus bubalis	680000	652	55.2	30.1	22.6	[10]
Capra hircus	25420	156.8	73.89	11.57	2.6	[11,12]
Ovis aries	57172.73	222.99	44.4	44.25	22.21	[17,74]
Sus scrofa	39700	128.2	53.08	39.97	14.97	[7,24,32,75]
Equus caballus	468000	416	47.92	48.93	20.3	[7]
Mesocricetus auratus	108	3.17	39.59	58.2	24.9	[40]
Rattus norvegicus	379.63	3.06	35	57.3	0.00	[41]
Macaca mulatta	10430	76	39.4	48.8	30.4	[70]

Species are listed in the same order as they appear in the phylogenetic reconstruction (see Phylogeny S1). Abbreviations: BM, body mass; TM, testes mass; %SFA, percentage of saturated fatty acids; %PUFA, percentage of polyunsaturated fatty acids; %DHA, percentage of docosahexaenoic acid (22:6 n-3); Refs, references. Fatty-acid values differ from those in Table S1 because they are calculated using only information about the fatty-acid composition of phosphatidylethanolamine

Phylogeny S1. Phylogenetic reconstruction for the 21 mammalian species utilized in the PGLS analyses. For this phylogenetic reconstruction we used a previous reconstructed phylogeny [76], which we complemented with trees for the higher mammalian groups (orders and families) [77,78].

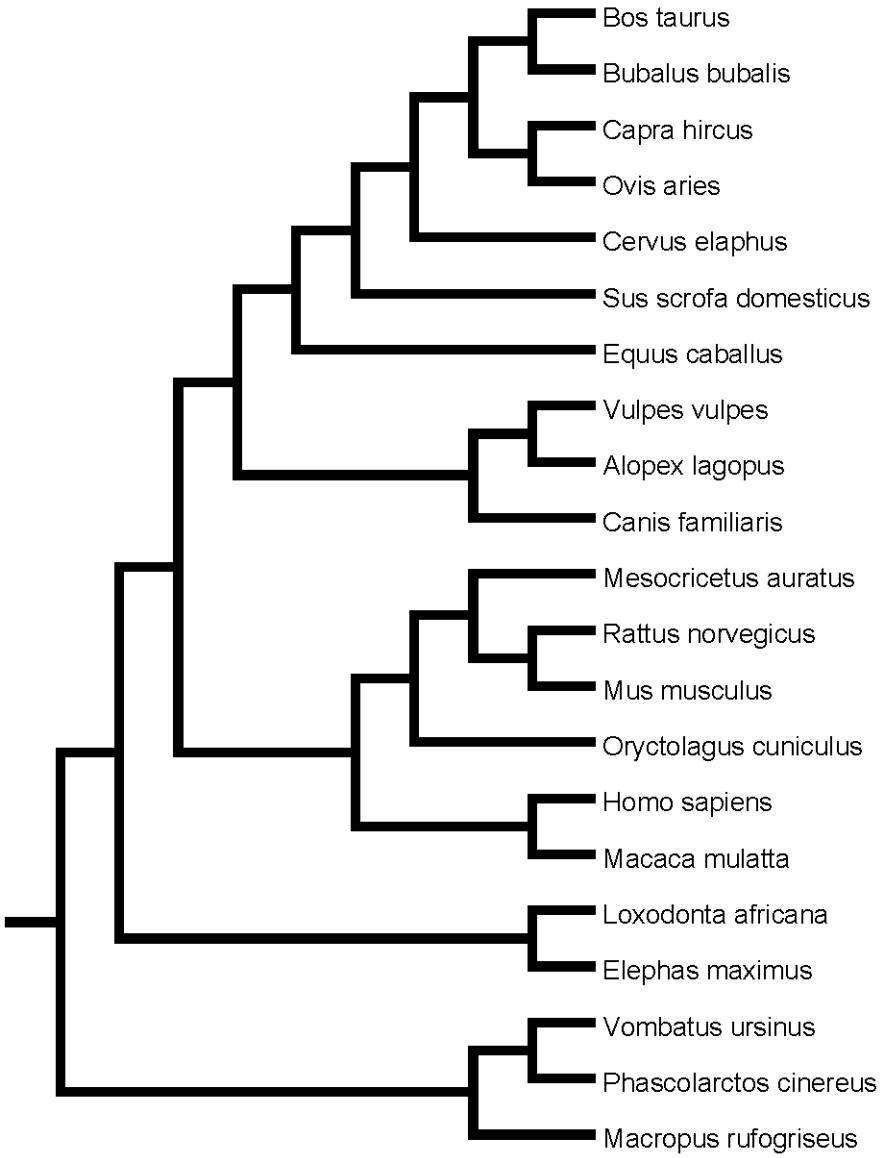


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Table S4. Effect of sperm competition on fatty-acid composition of sperm

Dependent variable	Predictor	Estimate	*F*	e(MS,df)	*P* value	λ	*r*	CI	n
% SFA	body mass	-0.03	0.12		0.73		0.08	-0.38 to 0.54	
	testes mass	0.03	0.15	0.004,18	0.7	$0.70^{n.s., n.s.}$	0.09	-0.37 to 0.55	21
% PUFA	body mass	0.07	0.56		0.47		0.17	-0.29 to 0.64	
	testes mass	-0.06	0.51	0.004,18	0.48	$0.23^{n.s., *}$	0.17	-0.29 to 0.63	21
% n-6	body mass	-0.06	2.98		0.11		0.41	-0.07 to 0.94	
	testes mass	0.01	0.004	0.007,15	0.95	$<0.01^{n.s., n.s.}$	0.02	-0.49 to 0.52	18
% n-3	body mass	0.36	7.42		**0.02**		0.58	**0.15 to 1.16**	
	testes mass	-0.31	4.82	0.009,15	**0.04**	$<0.01^{n.s., *}$	0.49	**0.03 to 1.05**	18
% DHA	body mass	0.24	5.68		**0.028**		0.49	**0.07 to 1.00**	
	testes mass	-0.18	1.57	0.012,18	0.23	$<0.01^{n.s., *}$	0.28	-0.17 to 0.75	21
CHO:PL	body mass	-0.24	1.01		0.34		0.28	-0.28 to 0.86	
	testes mass	0.55	1.65	0.13,12	0.22	$0.999^{*, n.s.}$	0.35	-0.20 to 0.93	15
DES:CHO	body mass	-0.38	4.97		**0.048**		0.56	**0.04 to 1.22**	
	testes mass	-0.09	0.03	0.13,11	0.87	$0.47^{n.s., n.s.}$	0.05	-0.54 to 0.64	14
% PC	body mass	-0.04	0.003		0.96		0.02	-0.60 to 0.64	
	testes mass	0.05	0.15	0.005,10	0.71	$<0.01^{n.s., *}$	0.12	-0.50 to 0.74	13
% PE	body mass	-0.10	0.77		0.40		0.27	-0.35 to 0.89	
	testes mass	0.11	2.24	0.002,10	0.17	$<0.01^{n.s., *}$	0.43	-0.16 to 1.08	13
% SM	body mass	0.17	0.21		0.65		0.14	-0.48 to 0.77	
	testes mass	-0.29	18.75	0.003,10	**0.001**	$0.999^{n.s., n.s.}$	0.81	**0.50 to 1.74**	13

Phylogenetically controlled multiple regression analyses revealing the effects of body mass and relative testes mass (testes mass) on phospholipid, sterol, and fatty-acid composition of sperm. The data for relative testes mass (testes mass) is also presented in Table 1 (with relative testes mass named RTS). Proportion data were arcsine-transformed (using arcsine root square) and ratio data were $\log_{10}$-transformed prior to analysis. The 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 superscript) and $\lambda = 1$ (second superscript). The effect size *r* was calculated from the *F* values; we also present the non-central 95% confidence interval (CI), an interval excluding

0 indicating statistically significant relationships. The *P* values and CI that indicate statistical significance are shown in bold. Abbreviations: e(MS,df), error MS and error degrees of freedom; % SFA, percentage of saturated fatty acids; % PUFA, percentage of polyunsaturated fatty acids; % n6, percentage of n-6 polyunsaturated fatty acids; % n3, percentage of n-3 polyunsaturated fatty acids; % DHA, percentage of docosahexaenoic acid (22:6 n-3); CHO:PL, ratio between cholesterol and phospholipid; DES:CHO, ratio between desmosterol and cholesterol; % PC, percentage phosphatidylcholine out of the total of phospholipids; % PE, percentage of phosphatidylethanolamine out of the total of phospholipids; % SM, percentage of sphingomyelin out of the total of phospholipids.

Table S5. Effect of sperm competition on the fatty-acid composition of sperm phosphatidylcholine

Dependent variable	Predictor	Estimate	*F*	e(MS,df)	*P* value	λ	*r*	CI	n
% SFA	body mass	-0.24	0.57		0.48		0.30	-0.50 to 1.11	
	testes mass	0.33	1.13	0.007,6	0.33	$<0.01^{n.s.,\ *}$	0.40	-0.38 to 1.22	9
% PUFA	body mass	0.31	0.63		0.46		0.31	-0.48 to 1.12	
	testes mass	-0.42	0.96	0.014,6	0.37	$<0.01^{n.s.,\ *}$	0.37	-0.41 to 1.19	9
% DHA	body mass	0.03	4.34		0.08		0.65	-0.03 to 1.57	
	testes mass	0.14	0.09	0.017,6	0.78	$<0.01^{n.s.,\ *}$	0.12	-0.68 to 0.92	9

Phylogenetically controlled multiple regression analyses revealing the effects of body mass and relative testes mass (testes mass) on fatty-acid composition of sperm phosphatidylcholine. Proportion data were arcsine-transformed (using arcsine root square) prior to analysis. The 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 superscript) and $\lambda = 1$ (second superscript). The effect size *r* was calculated from the *F* values; we also present the non-central 95% confidence interval (CI), an interval excluding 0 indicating statistically significant relationships. The *P* values and CI that indicate statistical significance are shown in bold. Abbreviations: e(MS,df), error MS and error degrees of freedom; % SFA, percentage of saturated fatty acids; % PUFA, percentage of polyunsaturated fatty acids; % DHA, percentage of docosahexaenoic acid (22:6 n-3).

Table S6. Effect of sperm competition on the fatty-acid composition of sperm phosphatidylethanolamine

Dependent variable	Predictor	Estimate	*F*	e(MS,df)	*P* value	λ	*r*	CI	n
% SFA	body mass	-0.10	1.80		0.23		0.48	-0.28 to 1.32	
	testes mass	0.21	1.08	0.003,6	0.34	$<0.01^{n.s.,\ *}$	0.39	-0.39 to 1.21	9
% PUFA	body mass	0.19	1.79		0.23		0.48	-0.28 to 1.32	
	testes mass	-0.36	1.98	0.005,6	0.21	$<0.01^{n.s.,\ *}$	0.50	-0.25 to 1.35	9
% DHA	body mass	-0.33	0.87		0.39		0.36	-0.43 to 1.17	
	testes mass	0.59	2.68	0.010,6	0.15	$<0.01^{n.s.,\ n.s.}$	0.56	-0.17 to 1.43	9

Phylogenetically controlled multiple regression analyses revealing the effects of body mass and relative testes mass (testes mass) on fatty-acid composition of sperm phosphatidylethanolamine. Proportion data were arcsine-transformed (using arcsine root square) prior to analysis. The 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 superscript) and $\lambda = 1$ (second superscript). The effect size *r* was calculated from the *F* values; we also present the non-central 95% confidence interval (CI), an interval excluding 0 indicating statistically significant relationships. The *P* values and CI that indicate statistical significance are shown in bold. Abbreviations: e(MS,df), error MS and error degrees of freedom; % SFA, percentage of saturated fatty acids; % PUFA, percentage of polyunsaturated fatty acids; % DHA, percentage of docosahexaenoic acid (22:6 n-3).

Table S7. Relationship between mass-specific metabolic rate and sperm competition

Dependent variable	Predictor	Estimate	*F*	e(MS,df)	*P* value	λ	*r*	CI	n
MSMR	body mass	-0.26	23.50		**0.0003**		0.80	**0.56 to 1.65**	
	testes mass	0.12	1.62	0.010,13	0.23	$0.999^{*, n.s.}$	0.33	-0.20 to 0.89	16

Phylogenetically controlled multiple regression analysis revealing the effect of body mass and relative testes mass (testes mass) on mass-specific metabolic rate (MSMR). All data were $\log_{10}$-transfomed prior to analysis. The 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 superscript) and $\lambda = 1$ (second superscript). The effect size *r* was calculated from the *F* values; we also present the non-central 95% confidence interval (CI), an interval excluding 0 indicating statistically significant relationships. The *P* values and CI that indicate statistical significance are shown in bold. Abbreviations: e(MS,df), error MS and error degrees of freedom; n: number of species in each analysis.