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

2   **mammalian sperm**

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14   Running title: Evolution of Mammalian Sperm Membrane

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18   ABSTRACT

19   The sperm membrane is a key structure affecting sperm function and thus reproductive

20   success. Spermatozoa are highly specialized and differentiated cells that undergo a long

21   series of processes in the male and female reproductive tracts until they reach the site of

22   fertilization. During this transit, the sperm membrane is prone to damage such as lipid

23   peroxidation. The characteristics and performance of the sperm membrane are strongly

24   determined by the fatty-acid composition of membrane phospholipids. Polyunsaturated fatty-

25   acids (PUFAs) are the most prone to lipid peroxidation. Lipid peroxidation and other types of

26   oxidative damage increase with higher metabolism and with higher levels of sperm

27   competition due to the increased ATP production to fuel higher sperm velocities.

28   Consequently, we hypothesized that, in order to avoid oxidative damage, and the ensuing

29   impairment of sperm function, sperm cells exhibit a negative relationship between PUFA

30   content and mass-specific metabolic rate (MSMR). We also hypothesized that higher sperm

31   competition leads to a reduction in the proportion of sperm PUFAs. We performed a

32   comparative study in mammals and found that high MSMR and high levels of sperm

33   competition both promote a decrease in the proportion of PUFAs that are more prone to lipid

34   peroxidation. The negative relationship between MSMR and these PUFAs in sperm cells is

35   surprising, because a positive relationship is found in all other cell types so far investigated.

36   Our results support the idea that the effects of MSMR and sperm competition on sperm

37   function can operate at very different levels.

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42   INTRODUCTION

43   The efficient functionality of spermatozoa determines the reproductive success of

44   males (Yanagimachi, 1994). Sperm function differs greatly among species and it is specially

45   affected by mass-specific metabolic rate (MSMR) (Lüpold, 2013) and sperm competition

46   (Gómez Montoto *et al.*, 2011; Lüpold, 2013); sperm competition occurs when females mate

47   with more than one male and the sperm of those males compete to fertilize the female's ova

48   (Parker, 1970; Birkhead & Møller, 1998). For example, comparative studies on mammals

49   report that an increase in both MSMR and sperm competition levels favours an increase in

50   sperm swimming velocity (Gómez Montoto *et al.*, 2011; Lüpold, 2013).

51   One of the sperm features that most directly affects sperm function is the cellular

52   membrane, which is involved not only in sperm motility and viability, but also in the

53   processes that precede and enable the fusion of the spermatozoon with the oocyte (Eddy &

54   O'Brien, 1994; Florman & Ducibella, 2006). The membrane bilayer is mainly constituted by

55   phospholipids and their fatty acids. The proportion of different types of fatty acids can

56   influence many aspects of membrane function (Hulbert & Else, 1999). A key difference

57   among these different types of fatty acids is their level of unsaturation, which is determined

58   by the number of double bonds within the molecule (Wathes *et al.*, 2007). Saturated fatty

59   acids (SFAs), monounsatured fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs)

60   have zero, one, or more than one double bond, respectively. The most polyunsaturated PUFA

61   is docosahexaenoic acid (DHA), with six double bonds.

62   Lipid peroxidation occurs when lipids react with reactive oxygen species (ROS) and

63   can have several negative effects on sperm function, including loss of motility, structural

64   damage to the sperm membrane, and inability to undergo capacitation and fuse with the

65   oocyte (de Lamirande & Gagnon, 1992; White, 1993; Aitken & Bennetts, 2006; Costantini *et*

66   *al.*, 2010). SFAs and MUFAs are not susceptible to lipid peroxidation, but PUFAs are

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67   (Hulbert *et al.*, 2007), and the greater the degree of polyunsaturation of fatty acids, the more

68   susceptible they are to lipid peroxidation, with DHA being the PUFA most prone to lipid

69   peroxidation (Hulbert *et al.*, 2007). Sperm cells have high PUFA content (Mann & Lutwak-

70   Mann, 1981; White, 1993) and are thus more vulnerable to oxidative damage (including lipid

71   peroxidation) than other cell types (Aitken, 1997; Sikka, 2001). Such high PUFA content in

72   sperm cells confers fluidity to the sperm membrane and it also seems to be important in the

73   regulation of lipid metabolism and cell movement (Stubbs & Smith, 1984; Gliozzi, *et al.*,

74   2009). Given that smaller sized species have higher MSMR and thus a higher production of

75   ROS, there should be a reduction in membrane unsaturation in the spermatozoa of these

76   species as a defensive mechanism to minimize the oxidative damage produced by external

77   ROS.

78   In addition to MSMR, sperm competition is another evolutionary force that could

79   affect the fatty-acid composition of sperm cells. In species that experience high levels of

80   sperm competition there will be a reproductive advantage by improving sperm function,

81   notably sperm velocity (Gómez Montoto *et al.*, 2011). One way to obtain faster swimming

82   sperm may be to increase the production of ATP; however, this will result in an upregulation

83   of metabolic activity and thus a higher production of ROS. A strategy to counteract the

84   negative effects of internally produced ROS would be to reduce the proportion of fatty acids

85   that are easily peroxidized (such as DHA).

86   The level of sperm competition across species is unrelated to body size and thus

87   MSMR and sperm competition may have independent effects on the fatty-acid composition

88   of sperm membranes. Consequently, the goal of our study was to study for the first time the

89   different effects that metabolic rate and sperm competition may have on the fatty-acid

90   composition of sperm membranes. To accomplish this goal we gathered and analysed

91   information on the proportion of sperm phospholipids and fatty acids in mammalian species.

92   We considered separately the proportion of n-6 and n-3 PUFAs (where 3 and 6 refer to the

93   first double bond from the terminal CH_3 of the carbon chain), given that n-3 PUFAs are more

94   prone to peroxidation than n-6 PUFAs (Hulbert *et al.*, 2007). We predicted that the

95   proportion of n-3 PUFAs (but not necessarily the proportion of n-6 PUFAs) will decrease in

96   species with higher MSMR and higher levels of sperm competition. Similarly, we predicted

97   that the proportion of DHA (i.e., the PUFA most prone to peroxidation) will show a negative

98   relationship with MSMR and levels of sperm competition.

99   Our prediction that higher MSMR will lead to sperm cells with lower proportions of

100   n-3 PUFAs and DHA is, however, opposite to what we know to be the case in all other

101   tissues so far investigated, in which higher metabolism is coupled with higher levels of

102   polyunsaturation in bilayer membranes (Hulbert & Else, 2000). Indeed, the "membrane

103   pacemaker theory of metabolism" makes a direct connection between the MSMR of a species

104   and its level of membrane polyunsaturation (Hulbert & Else, 1999; Hulbert, 2005). This

105   theory proposes that higher levels of membrane polyunsaturation cause membrane proteins to

106   have a higher molecular activity, which results in higher metabolic rates in those cells and

107   thus in the whole organism (Hulbert, 2005). Consequently, this theory predicts a positive

108   relationship between MSMR and the level of membrane polyunsaturation; given that there is

109   a negative correlation between body size and MSMR, this theory also predicts a negative

110   relationship between body size and the level of membrane polyunsaturation. A series of

111   studies have supported the membrane pacemaker theory of metabolism in birds (Hulbert *et*

112   *al.*, 2002a) and mammals (Hulbert *et al.*, 2002b), and in many different tissues, including

113   cardiac muscle, skeletal muscle, liver, and kidney (Hulbert *et al.*, 2002b).

114   We also considered the proportion of the main types of phospholipids

115   (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) in relation to MSMR

116   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,

142   compared to those of previous studies, indicate that the fatty-acid composition of membranes

143   in sperm cells differs from that found in all other cell types.

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146   MATERIALS AND METHODS

147   We collected data on the composition of phospholipids, fatty acids, and sterols

148   (cholesterol and desmosterol) in the sperm of 21 mammalian species (see Table S1 in

149   Additional file 1). For all these 21 species we also collected data on body mass (g) and testes

150   mass (g), whereas data on mass-specific metabolic rate (ml O_2 / h x g) was found for a subset

151   of 16 species (Table S1). Only data for the three main classes of phospholipids

152   (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) were available for a

153   sufficient number of species (n = 13; Table S1). For two of these phospholipids

154   (phosphatidylcholine and phosphatidylethanolamine), we compiled data from the literature on

155   their fatty-acid composition for 9 species (Tables S2 and S3), and studied the relationship of

156   these data with relative testes mass (we did not have MSMR data for all these 9 species, so

157   we did not perform analyses on the effect of MSMR).

158   We calculated five variables regarding the total fatty-acid content in sperm:

159   percentage of saturated fatty acids (% SFA), percentage of polyunsaturated fatty acids (%

160   PUFA), percentage of n-6 polyunsaturated fatty acids (% n6), percentage of n-3

161   polyunsaturated fatty acids (% n3), and percentage of docosahexaenoic acid (% DHA). We

162   also compiled or calculated two ratios: cholesterol:phospholipid and desmosterol:cholesterol.

163   For any of these variables, when more than one value was reported for the same species, we

164   calculated an average value weighted by sample size. Data on MSMR, body mass, testes

165   mass, and ratios were $\log_{10}$-transformed. All the other variables, being percentage data, were

166   arcsine-transformed (calculating arcsine of the square root of the variable).

167   We tested the influence of metabolic rate on the composition of the sperm membrane,

168   using regression analyses in which each variable of interest was a dependent variable and

169   using mass-specific metabolic rate as the predictor. Each dependent variable was analysed

170   separately. We also tested the influence of sperm competition on those same dependent

171   variables (each dependent variable being analysed separately), using body mass and testes

172   mass as the predictors. This provided a measure of the relationship between each dependent

173   variable and relative testes mass. A higher relative testes mass has been found to strongly

174   associate with the percentage of multiple paternity and, thus, relative testes mass can be used

175   as a proxy of sperm competition levels (Ramm *et al.*, 2005; Firman & Simmons, 2008;

176   Soulsbury, 2010). Given that body mass and testes mass are related to each other (i.e., they

177   are non-orthogonal), a sequential (Type I) sum of squares was used, adding the two predictors

178   to the models in the following order: body mass, testes mass. For all these analyses we

179   conducted phylogenetic generalized least squares (PGLS) models in R 2.13.0 (R Core Team,

180   2012) using a code written by R. Freckleton. The PGLS estimates a phylogenetic scaling

181   parameter lambda (λ), which is then incorporated in the models to control for phylogenetic

182   effects. If λ values are close to 0, the variable in question is likely to have evolved

183   independently of phylogeny, whereas values close to 1 indicate strong phylogenetic

184   association of the variables. The phylogenetic reconstruction used in the PGLS analyses in

185   included in the Additional file 1. For the graphical representation of the data (Fig. 1), and

186   only in this case, relative testes size was calculated using Kenagy and Trombulak´s

187   mammalian-specific regression equation: relative testes size = testes mass / 0.035 × body

188   mass$^{0.72}$ (Kenagy & Trombulak, 1986).

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191   RESULTS

192   Values compiled from the literature for the different variables are summarized in the

193   Datasets in the Additional file 1 (Tables S1-S3). There were large differences across species

194   in many variables. For example, the percentage of n-3 PUFAs ranged from 0% in the rat to

195   68% in the African elephant.

196   MSMR was not significantly correlated with the proportion of SFAs, the proportion

197   of total or n-6 PUFAs, the cholesterol:phospholipid ratio, the proportion of

198   phosphatidylcholine, the proportion of phosphatidylethanolamine, or the proportion of

199   sphingomyelin (PGLS: $P > 0.05$ for all analyses; see Table 1). On the other hand, species

200   with high MSMR had lower proportions of n-3 PUFAs (PGLS: $F_{1,12} = 4.84$, $P = 0.048$; fig.

201   1a) and DHA (PGLS: $F_{1,14} = 8.07$, $P = 0.01$), and higher amounts of desmosterol relative to

202   cholesterol (PGLS: $F_{1,9} = 9.19$, $P = 0.01$; Table 1).

203   Relative testes size was not significantly correlated with the proportion of SFAs, the

204   proportion of total or n-6 PUFAs, the proportion of DHA, the cholesterol:phospholipid ratio,

205   the desmosterol:cholesteriol ratio, the proportion of phosphatidylcholine, or the proportion of

206   phosphatidylethanolamine ($P > 0.05$ for all analyses; see Table 1). However, an increase in

207   relative testes size was associated with a reduction in the proportion of n-3 PUFAs (PGLS:

208    $F_{1,15} = 4.82$, $P = 0.04$; fig. 1b) and with a reduction in the proportion of sphingomyelin

209   (PGLS: $F_{1,10} = 18.75$, $P = 0.001$; see Table 1 and Table S4).

210   The proportion of SFAs, PUFAs, and DHA in phosphatidylcholine or

211   phosphatidylethanolamine were not related to body mass or relative testes mass ($P > 0.05$ for

212   all analyses; see Tables S5 and S6 in Additional file 1).

213   There was no significant relationship between MSMR and relative testes mass

214   (PGLS: $F_{1,13} = 1.62$, $P = 0.23$; see Table S7 in Additional file 1), which indicates that MSMR

215   and sperm competition may have independent effects on the proportion of n-3 PUFAs.

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218   DISCUSSION

219   We found that the proportion of n-3 PUFAs, which are the membrane fatty acids most

220   prone to lipid peroxidation, decrease in the sperm of mammalian species with high MSMR

221   and high levels of sperm competition. These results, which support our initial predictions, are

222   strikingly different from those found in other mammalian tissues. As mammal and bird

223   species decrease in size (and thus increase their MSMR), the cellular membranes in several

224   organs become progressively more polyunsaturated (Hulbert *et al.*, 2002a; Hulbert *et al.*,

225   2002b). Interestingly, in mammals, the proportion of total PUFAs is not affected by body

226   mass in all tissues investigated, whereas the proportion of n-3 PUFAs correlate negatively

227   with body mass in heart, skeletal muscle, liver and kidney (Hulbert *et al.*, 2002b). In sperm

228   cells, the proportion of total PUFAs is also unrelated to body mass, whereas the proportion of

229   n-3 PUFAs correlate positively with body mass. Therefore, sperm cells represent an

230   exception to the membrane pacemaker theory of metabolism, which postulates a positive

231   association between MSMR and membrane polyunsaturation.

232   One of the main predictions of the membrane pacemaker theory of metabolism is that

233   species with high MSMR have membranes that are predominantly polyunsaturated and with

234   high DHA content, whereas those species with low MSMR have less polyunsaturated

235   membranes, with a low DHA content (Hulbert, 2005). This prediction of the membrane

236   pacemaker theory has been so well supported for several tissues in mammals and birds

237   (Käkelä & Hyvärinen, 1995; Hulbert *et al.*, 2002a; Hulbert *et al.*, 2002b), that it seemed to be

238   an overarching explanation for all organs and cell types. Here we show that sperm cells are,

239   however, a striking exception. Our results suggest that the unusual fatty-acid composition of

240   sperm cells is due to the need to counterbalance the negative effects of lipid peroxidation in

241   order to maintain effective levels of sperm function. On the one hand, MSMR leads to higher

242   metabolic rates in all tissues with a consequent increase in the production of external ROS.

243   On the other hand, sperm competition promotes a higher production of ATP to fuel faster

244   swimming speeds (Tourmente *et al.*, 2013), which is in turn likely to increase the production

245   of internal ROS. To minimize the negative effects of ROS on sperm function, species with

246   high MSMR and/or high levels of sperm competition have evolved sperm membranes that are

247   less prone to lipid peroxidation. This seems to have been accomplished not only by reducing

248   the proportion of PUFAs in the membrane, but also by increasing the proportion of

249   plasmalogens in sperm cells. Plasmalogens are a type of glycerophospholipid that has

250   antioxidant properties and are found in high levels in the sperm cells of several mammalian

251   groups (Fuchs *et al.*, 2007; Fuchs *et al.*, 2009). An increase in the proportion of

252   plasmalogens, together with the antioxidants contained in the seminal plasma (Koziorowska-

253   Gilun *et al.*, 2011), would also reduce the susceptibility of sperm cells to lipid peroxidation.

254   Unfortunately, data for a sufficient number of species on the proportion of plasmalogens in

255   sperm cells are not yet available to determine how the proportion of plasmalogens may be

256   affected by MSMR and/or different levels of sperm competition.

257   The proportion of DHA in mammalian sperm varies across species much more than in

258   any other tissues. While the proportion of DHA across species in heart, skeletal muscle, liver,

259   kidney, and brain ranges approximately between 1% and 12% (Hulbert *et al.*, 2002b), DHA

260   in sperm ranges from very low percentages in rat (0%) and rabbit (1%) to 68% in the African

261   elephant. Such higher values of DHA have also been reported in the muscle mitochondria of

262   cold-water fish (Guderley *et al.*, 1997). Although the proportion of DHA did not relate

263   significantly with sperm competition, it showed a negatively relationship with MSMR.

264   Again, this result is opposite to results in other tissues, where DHA is negatively correlated

265   with body size (and thus positively correlated with MSMR) in heart, skeletal muscle, liver

266   and kidney. The membrane pacemaker theory of metabolism states that high proportions of

267   DHA in most tissues of small-sized species can explain their high MSMR. Our results in

268   sperm cells suggest that the high MSMR of such small-sized species may have in turn forced

269   a reduction in the proportion of DHA in sperm to minimize the negative effects of lipid

270   peroxidation. One question that still remains unanswered is why the proportion of DHA is so

271   high in sperm of some species in the first place.

272   The proportion of SFA in sperm was unrelated to MSMR, which is similar to what

273   occurs in other mammalian tissues (Hulbert *et al.*, 2002b). The proportion of SFA was also

274   unrelated to sperm competition. Therefore, the only fatty-acids that seem to be affected by

275   sperm competition and MSMR are those that increase the risk of lipid peroxidation, i.e. n-3

276   PUFAs, and DHA in particular.

277   In the majority of studies from which we compiled data for our analyses (see Table

278   S1), no distinction was made between phospholipids from the head and from the tail of sperm

279   cells. In rhesus monkey, 99% of sperm DHA was located in the tail (Connor *et al.*, 1998). It

280   is thus possible that the decrease in the proportion of polyunsaturation observed in small-

281   bodied species and species with high levels of sperm competition may be restricted to the

282   sperm tails. Furthermore, given that the sperm head contains the nuclear DNA, a maximal

283   protection of this DNA may be attained by having a high proportion of saturated fatty acids in

284   the membrane of the sperm head. In the rhesus monkey, the proportion of desmosterol in

285   relation to cholesterol is also higher in sperm tails than in sperm heads (Connor *et al.*, 1998).

286   In the same way that the six double bonds of DHA contribute to increase membrane fluidity,

287   the two double bonds in desmosterol can confer more membrane fluidity than the single

288   double bond in cholesterol (Connor *et al.*, 1998). Given that our results showed that the

289   desmosterol:cholesterol ratio was positively associated with MSMR, we argue that the

290   decrease in n-3 PUFAs in species with high MSMR (which can reduce the risk of lipid

291   peroxidation but will also reduce membrane fluidity), can be counterbalanced with a higher

292   proportion of desmosterol, so that membrane fluidity can be maintained while reducing the

293   incidence of lipid peroxidation. Unfortunately, no data are yet available to test this

294   hypothesis.

295   The cholesterol:phospholipid ratio was not related to MSMR or sperm competition.

296   The proportion of the main phospholipid classes (phosphatidylcholine,

297   phosphatidylethanolamine, and sphingomyelin) were also not related to MSMR, which is also

298   the case for other tissues such as kidney and brain (Nealon *et al.*, 2008). However, the

299   relative proportions reported for kidney and brain were partly different from the ones we

300   found for sperm. In kidney, brain, and sperm, phosphatidylcholine is the main class of

301   phospholipid, but while it represents around 70% of phospholipids in kidney and brain, it

302   only represents an average of 44% (range: 28 – 65%) in sperm. The second main

303   phospholipid (phosphatidylethanolamine) represents around 20% of phospholipids in kidney,

304   brain, and sperm. However, the third class of phospholipid in sperm is sphingomyelin, which

305   represents a much lower percentage in kidney and brain. For example, sphingomyelin was

306   not detected in the kidney or brain of mice, but it represented 22% of phospholipids in mouse

307   sperm (Alvarez *et al.*, 1987; Rejraji *et al.*, 2006). Interestingly, we found a negative

308   relationship between the proportion of sphingomyelin and the level of sperm competition

309   across species. Sphingomyelin in rats is one of the lipid classes that decrease the most during

310   the acrosome reaction (Zanetti *et al.*, 2010), which suggests that a reduction in the proportion

311   of sphingomyelin can result into a more stable membrane and thus a decrease in the

312   proportion of sperm undergoing spontaneous acrosome reaction. It must also be noted that

313   sphingomyelin in the sperm head is composed mostly by PUFAs (Oresti *et al.*, 2011), so a

314   general reduction of PUFAs in relation to sperm competition levels could also be related to

315   the significant reduction in the proportion of sphingomyelin.

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318   CONCLUSIONS

319   Despite the importance that the cellular membrane has for the function of sperm cells,

320   we have little understanding on how different evolutionary forces shape its composition. Our

321   main finding that high MSMR and high levels of sperm competition both promote a decrease

322   in the proportion of PUFAs that are more prone to lipid peroxidation emphasizes the

323   importance of reducing the exposure of DNA, proteins and lipids to oxidative stress. The

324   atypical composition of the sperm membrane in mammals (compared to somatic cells from

325   other tissues examined to date) can be understood in a general framework in which high

326   levels of both MSMR and sperm competition lead to the overall enhancement of sperm

327   function.

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454

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

460   Additional file 1 for the values of body mass). Proportion data were arcsine-transformed

461   (using arcsine root square) and ratio data were $\log_{10}$-transformed prior to analysis. The

462   superscripts following the λ value indicate significance levels (n.s., $p > 0.05$; *, $p < 0.05$) in

463   likelihood ratio tests against models with $\lambda = 0$ (first superscript) and $\lambda = 1$ (second

464   superscript). The effect size r was calculated from the F values; we also present the non-

465   central 95% confidence interval (CI), an interval excluding 0 indicating statistically

466   significant relationships. The P values and CI that indicate statistical significance are shown

467   in bold. Abbreviations: e(MS,df), error MS and error degrees of freedom; % SFA, percentage

468   of saturated fatty acids; % PUFA, percentage of polyunsaturated fatty acids; % n6,

469   percentage of n-6 polyunsaturated fatty acids; % n3, percentage of n-3 polyunsaturated fatty

470   acids; % DHA, percentage of docosahexaenoic acid (22:6 n-3); CHO:PL, ratio between

471   cholesterol and phospholipid; DES:CHO, ratio between desmosterol and cholesterol; % PC,

472   percentage phosphatidylcholine out of the total of phospholipids; % PE, percentage of

473   phosphatidylethanolamine out of the total of phospholipids; % SM, percentage of

474   sphingomyelin out of the total of phospholipids.

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476

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.
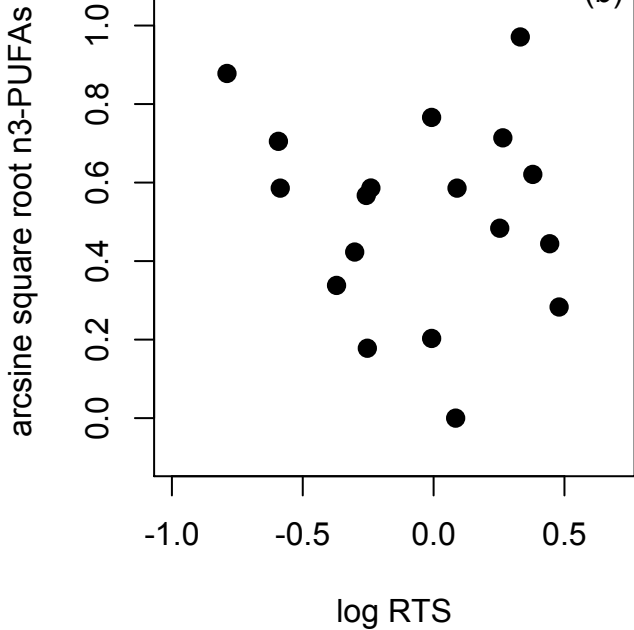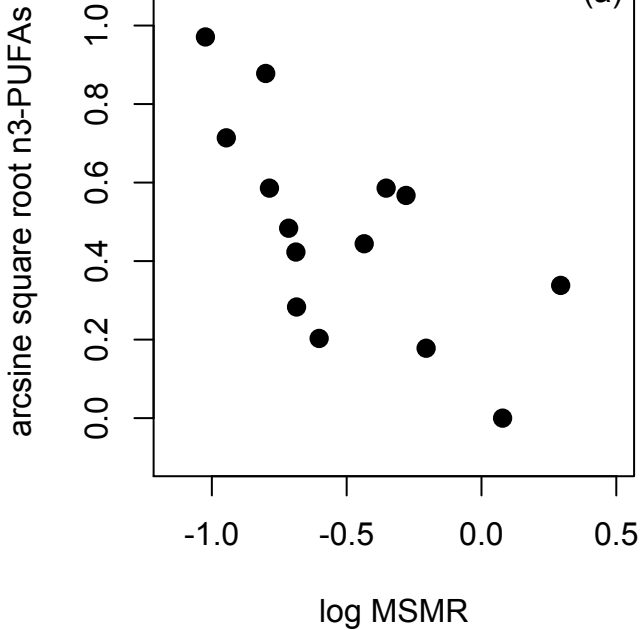
SUPPORTING INFORMATION

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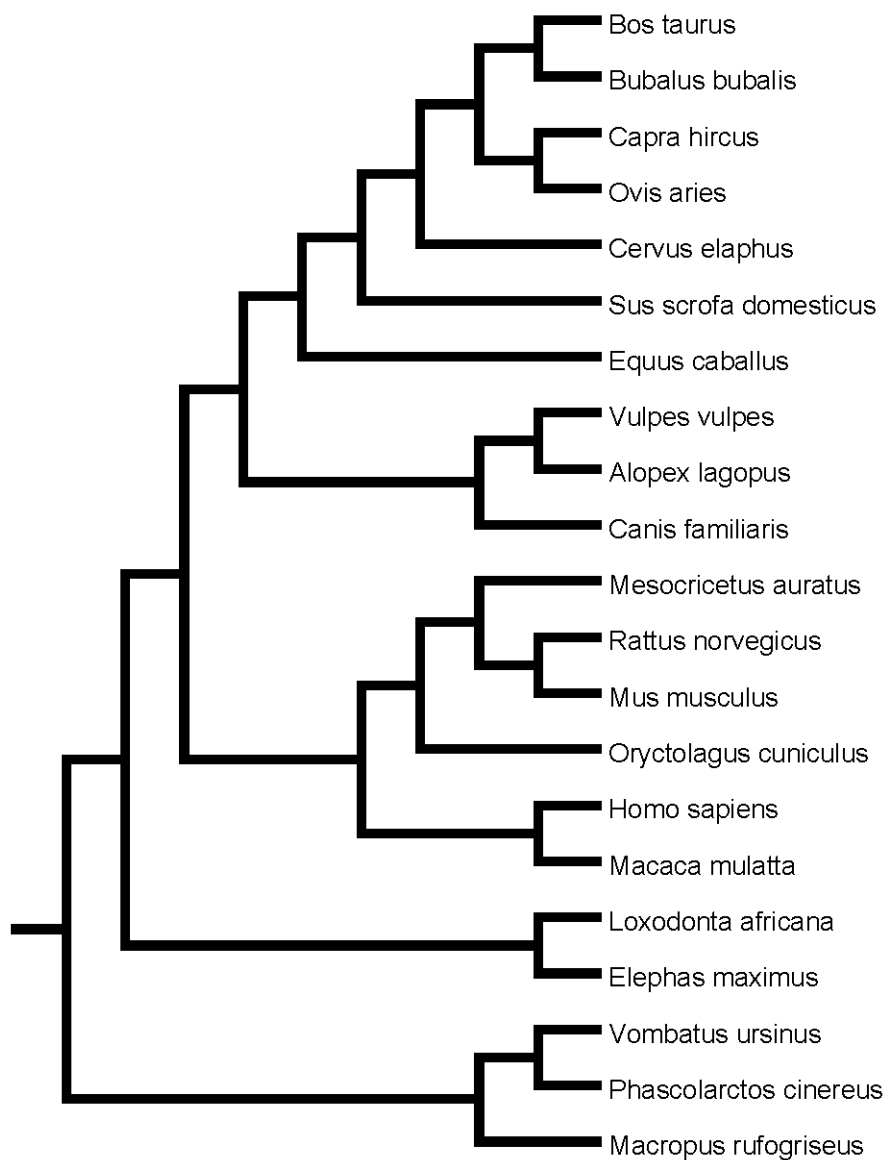
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Table S4. Effect of sperm competition on fatty-acid composition of sperm

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	$0.70^{\text{n.s., n.s.}}$	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	$0.23^{\text{n.s., *}}$	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	$<0.01^{\text{n.s., n.s.}}$	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**	$<0.01^{\text{n.s., *}}$	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	$<0.01^{\text{n.s., *}}$	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	$0.999^{\text{*, n.s.}}$	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	$0.47^{\text{n.s., n.s.}}$	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	$<0.01^{\text{n.s., *}}$	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	$<0.01^{\text{n.s., *}}$	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**	$0.999^{\text{n.s., n.s.}}$	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	$<0.01^{\text{n.s.,} *}$	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	$<0.01^{\text{n.s.,} *}$	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	$<0.01^{\text{n.s.,} *}$	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	$<0.01^{\text{n.s., }*}$	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	$<0.01^{\text{n.s., }*}$	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	$<0.01^{\text{n.s., n.s.}}$	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.