Morphology and Ultrastructure of the Amazon River Dolphin (*Inia geoffrensis*) Spermatozoa

RODRIGO S. AMARAL [1]*, VERA M. F. DA SILVA,[2] FABÍOLA X. VALDEZ DOMINGOS,[3] AND ANTHONY R. MARTIN[4]

[1]Federal Institute of Education, Science and Technology of the Amazonas (IFAM/CMZL), Manaus, Amazonas, Brazil

[2]Laboratory of Aquatic Mammals (LMA), National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil

[3]Biodiversity Department (CBio), National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil

[4]Centre for Remote Environments, University of Dundee, Dundee, UK

ABSTRACT

The spermatozoa from seven adult Amazon river dolphins (*Inia geoffrensis*, CETACEA: INIIDAE) were analyzed by light and electron microscopy. The spermatozoa showed an elongated ellipsoid shaped head and a long tail with a well distinguishable midpiece. The head spermatozoa have a smooth surface like other odontocetes examined, with the exception of the Delphinidae family. The mean dimensions of the spermatozoa were within the range already reported for other cetaceans. The spermatozoa midpiece, as in other cetaceans, showed a random pattern of mitochondria, different from that described for other mammals. Further studies of sperm morphology of a wider spectrum of cetacean families could help to better understand the reproductive biology of these animals and the intergeneric and intrageneric relationships among them, as well as, among other mammals. Anat Rec, 300:1519–1523, 2017.

Key words: Amazon river dolphin; cetacean; Inia geoffrensis; reproduction; sperm

The feasibility of morphological and ultrastructural analysis of spermatozoa in reproductive biology and phylogenetic studies has been demonstrated in several mammalian species, including cetaceans (Cummins and Woodall, 1985; Kita et al., 2001; Miller et al., 2002; Anderson et al., 2005; Meisner et al., 2005; Plön and Bernard, 2006; Tourmente et al., 2011). This tool has been used to add parameters for phylogenetic studies, to evaluate mating strategies, and to develop or improve the reproductive technologies.

Among cetaceans already evaluated, the spermatozoa morphology varies between whales and dolphins, as well as among dolphin families (Fleming et al., 1981; Miller et al., 2002; Meisner et al., 2005; Plön and Bernard, 2006; Miller et al., 2007; Neuenhagen et al., 2007; Li et al., 2009). Those variations are related to differences in phylogenetics and mating strategies, as reported in other mammals.

The Amazon river dolphin (*Inia geoffrensis*, CETACEA: INIIDAE) is a freshwater dolphin endemic to South America and widely distributed in the rivers of

Grant sponsor: Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM); Grant number: PAPAC 020/2013 (Process no. 062.00888/2014).

*Correspondence to: Rodrigo S. Amaral, Instituto Federal de Educação, Ciência e Tecnologia do Amazonas (IFAM)/Campus Manaus Zona Leste (CMZL), Av. Cosme Ferreira 8045, Gilberto Mestrinho, Manaus 69086-475, Amazonas, Brazil. Tel: +55 (92) 3643-3183; Fax: +55 (92) 3643-3184. E-mail: rodrigo.amaral@ifam.edu.br

Received 9 August 2016; Revised 8 October 2016; Accepted 31 October 2016.

DOI 10.1002/ar.23585

Published online 3 March 2017 in Wiley Online Library (wileyonlinelibrary.com).

the Amazon and Orinoco basins (Best and da Silva, 1993). Although some aspects of Amazon river dolphin biology are well known, there is a lack of even basic information about reproductive anatomy of this species. Therefore, the aim of this study was to describe the morphological characteristics of the Amazon river dolphin spermatozoa.

MATERIALS AND METHODS

Semen samples were obtained opportunistically from voluntary ejaculation of seven adult *I. geoffrensis* (total body length: range 212–237 cm and body mass: range 122–173 kg) during a capture–recapture campaign of a research program in the Mamirauá Sustainable Development Reserve, Brazilian Amazon. All animal handling procedures were conducted under Brazilian Government permission (SISBIO 50544-2) and approved by the Animal Care and Use Committee (Protocol 034/2015). Samples were fixed in 10% formalin solution. A sample from one animal was also fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for transmission electron microscopy.

TABLE 1. Morphometric parameters of *Inia geoffrensis* spermatozoa (N = 700)

Parameter	Mean ± SD	Range
Head length (μm)	5.57 ± 0.12	4.75–6.37
Head width (μm)	2.23 ± 0.11	1.85–2.93
Head thickness (μm)	1.78 ± 0.21	1.39–2.26
Midpiece length (MP) (μm)	3.30 ± 0.23	2.67–4.20
Midpiece width (μm)	1.24 ± 0.13	0.73–1.75
Flagellum length (F) (μm)	52.93 ± 5.37	42.45–61.73
Tail length (MP+F) (μm)	56.53 ± 5.03	45.96–65.28
Total length (μm)	62.32 ± 5.61	51.34–71.32

A drop of each sample fixed in formalin was placed between slide and coverslip, then analyzed and photographed with a phase-contrast microscope (Zeiss, Oberkochen, Germany). Measurements of head length, width, and thickness, midpiece length and width, flagellum length, tail length, and total length were taken from 100 spermatozoa of each animal using the software Image Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD). A drop of fixed sample from two animals was spread on a coverslip, air-dried, and immediately dehydrated in a graded ethanol and critical point dried. The spermatozoa were coated with gold and observed using a scanning electron microscope (LEO 435vp, Zeiss).

The sample for transmission electron microscopy analysis was rinsed with a sodium cacodylate buffer (0.1 M), processed using a transmission electron microscopy standard protocol and embedded in Spurr epoxy resin. Ultrathin sections were cut, contrasted with uranyl acetate

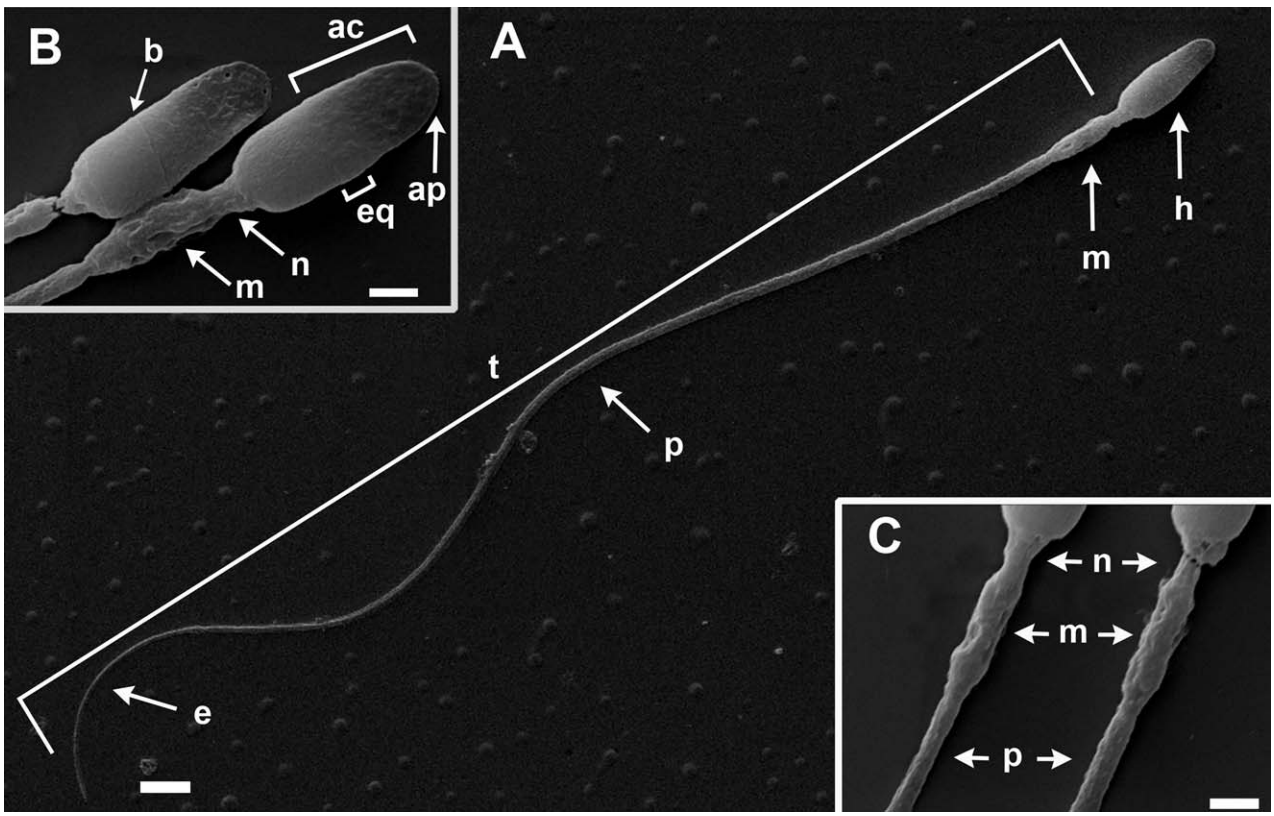

Fig. 1. Scanning electron micrographs of *I. geoffrensis* spermatozoa. Total view of the spermatozoon (**A**) and spermatozoa's head (**B**) and midpiece (**C**) details. h, head; m, midpiece; t, tail; p, principal piece; e, endpiece; ac, acrosomal region; ap, apical ridge; eq, equatorial segment; n, neck; b, boundary. Bar: 2 μm (A); 1 μm (B, C).

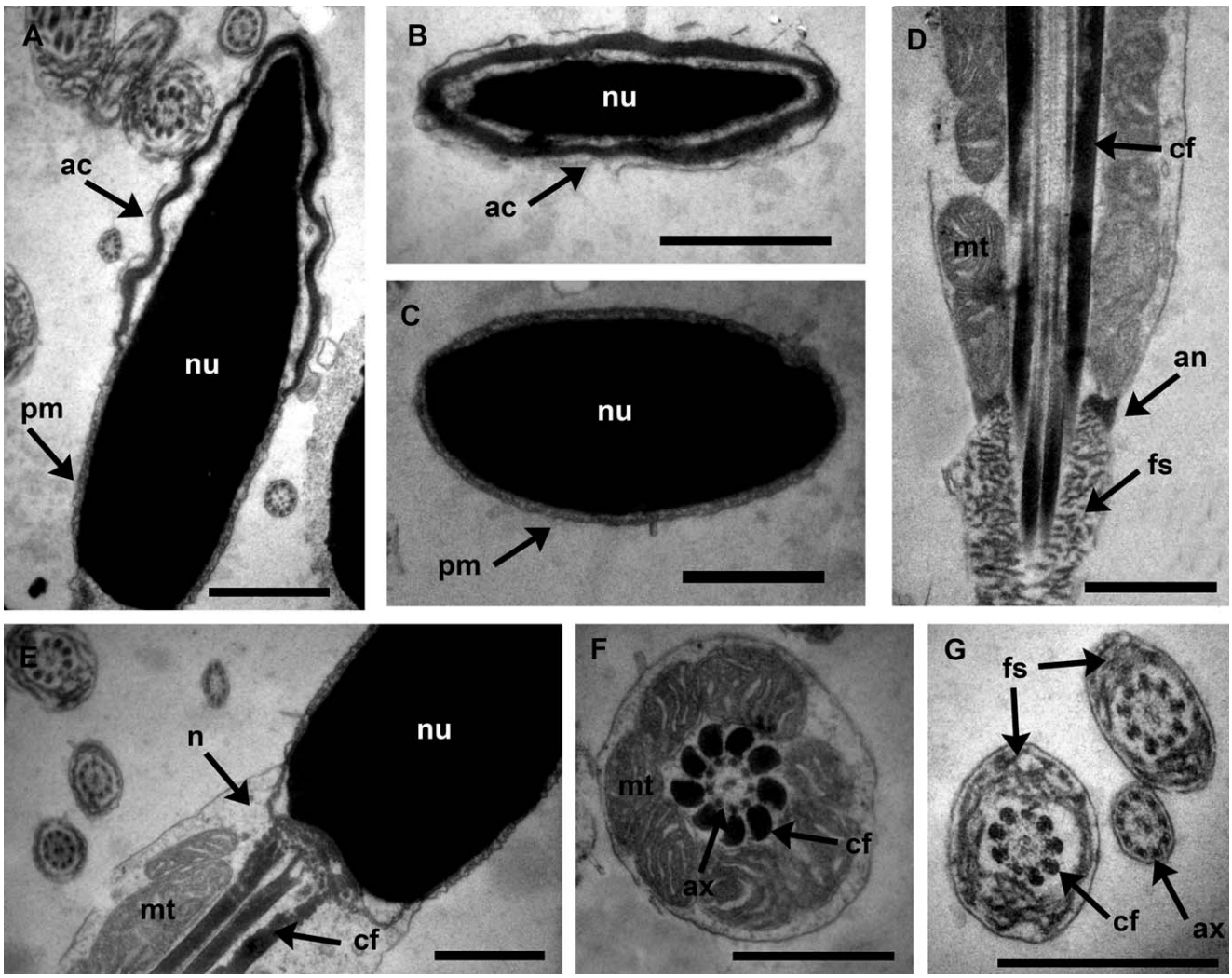

Fig. 2. Transmission electron micrographs of *I. geoffrensis* spermatozoa. Longitudinal sections of the head (**A**, **E**) and tail (**D**), and transversal sections of the upper portion (**B**) and basal portion (**C**) of the head, the midpiece (**F**), and different portions of the principal piece (**G**). ac, acrosome; nu, nucleus; pm, plasmatic membrane; mt, mitochondria; cf, coarse fibers; an, annulus; fs, fibrous sheath; ax, axoneme; n, neck. Bar: 1 μm.

and lead citrate, then observed and photographed using a transmission electron microscope (EM 109, Zeiss).

RESULTS

No statistical differences were observed in the morphometric parameters among animals (ANOVA: $P > 0.05$), so the samples were pooled. The mean values for the dimensions of the combined spermatozoa sample are shown in Table 1.

The spermatozoa showed an elongated ellipsoid shaped head, in which the anterior region is thinner than the posterior region, a well distinguishable midpiece (thicker part of the tail), and a long tail (Fig. 1A).

The acrosomal region was thin and flat, covering the anterior three-fifths of the head. This region showed a distinct apical ridge present along the anterior side (Fig. 1B). The equatorial segment was a narrow band located at the equator of the head, and a boundary was observed between the acrosomal and postacrosomal regions (a line that divides the anterior and posterior regions of the sperm head) (Fig. 1B). The postacrosomal region was thick with a smooth surface.

Ultrastructurally, the nucleus was composed of a compact mass of electron dense chromatin (Fig. 2A–C,E), following the format of the spermatozoa head, with the upper part thinner than the base (Fig. 2A–C), and a concave recess for tail insertion (Fig. 2E). The acrosome and the boundary were easily discernible (Fig. 2A,B).

The tail was attached to the head by a discernible neck (Fig. 1B,C), and it was characterized ultrastructurally as a region without mitochondria, in which coarse fibers were present binding the head to the tail (Fig. 2E). Axoneme fibers, organized as nine pairs of fibers surrounding a central pair (Fig. 2F,G), and coarse fibers, characterized as nine electron dense structures around

the axoneme (Fig. 2D–F,G), were present along the tail. The midpiece showed a cylindrical shape with indentations on the surface (Fig. 1B,C), with mitochondria surrounding the tail fibers in a random pattern (Fig. 2D–F). In longitudinal sections, four to five mitochondria were observed on either side of the tail fiber bundle. In transverse sections, four to six mitochondria were observed.

The flagellum showed a long principal piece and was characterized by the presence of a fibrous sheath around the tail fibers (Fig. 2D,G). The transition between the midpiece and principal piece was marked by the presence of the Jensen's ring (annulus) (Fig. 2D). The size of the coarse fibers varied along the midpiece, and the coarse fibers and the fibrous sheath became thinner along the flagellum (Fig. 2F,G). The end piece represented one-fifth of the flagellum length (Fig. 1A).

DISCUSSION

The general morphology of *I. geoffrensis* spermatozoa is consistent with the description by Fawcett (1975) for mammalian spermatozoa, although some morphological and morphometric differences were found between the spermatozoa of this species and other cetaceans.

The postacrosomal region of *I. geoffrensis* spermatozoa has a smooth surface like that of all cetacean sperm previously examined, with the exception of the Delphinidae family (Fleming et al., 1981; Mogoe et al., 1998; Kita et al., 2001; Miller et al., 2002; Meisner et al., 2005; Plön and Bernard, 2006; Miller et al., 2007; Neuenhagen et al., 2007; Li et al., 2009). The spermatozoa from all delphinids already examined, excluding killer whale (*Orcinus orca*), show several parallel longitudinal ridges in the postacrosomal region. The real function of these ridges remains unclear, but probably they are important for the fertilization process.

Usually, the mitochondria of the midpiece in mammalian sperm are elongated and arranged in an helical pattern around the tail fibers (Fawcett, 1975). Among cetaceans already evaluated, however, this helical pattern was only reported in the bowhead whale (*Balaena mysticetus*) (Miller et al., 2007). For other cetacean species, the mitochondria are apparently spherical, showing a random or in layered pattern (Fleming et al., 1981; Miller et al., 2002; Meisner et al., 2005; Plön and Bernard, 2006; Miller et al., 2007; Neuenhagen et al., 2007; Li et al., 2009). The midpiece of *I. geoffrensis* showed a random pattern of mitochondria with morphology similar to other cetaceans. This lack of helical pattern in most cetaceans is also related with the small size of the midpiece observed in those species. This morphological pattern looks to be a characteristic of odontocetes among cetaceans; however, an ultrastructural evaluation of the spermatozoa midpiece of other mysticeti species is necessary to confirm this hypothesis.

It is widely known that mitochondria provide energy for the spermatozoa (Fawcett, 1975); however, the explanation for the difference in midpiece shape and size, and mitochondrial arrangement between most cetaceans and other mammals is unknown. As suggested by Miller et al (2007), future studies regarding energy requirements of the spermatozoa could elucidate those differences.

Fleming et al (1981) also report the observation of two types of mitochondria in the midpiece of bottlenose dolphin (*Tursiops truncatus*) spermatozoa. This finding was not observed in *I. geoffrensis* spermatozoa, and there are no reports for other cetaceans. Miller et al (2007) suggest that mitochondria variation in *T. truncatus* could be related with freeze damage by cryopreservation.

The total length observed for *I. geoffrensis* spermatozoa (mean 62.32 μm) was within the range already reported for other cetaceans. However, sperm total length shows a wide variation among cetacean species, where sperm whale (*Physeter catodon*), bowhead whale, and dwarf sperm whale (*Kogia simas*) have the shortest spermatozoa (<50 μm total length) and killer whale, Risso's dolphin (*Grampus griseus*), Harbor porpoise (*Phocoena phocoena*), and short-finned pilot whale (*Globicephala macrorhynchus*) have the longest (>73 μm total length) (Ballowitz, 1907; Kita et al., 2001; Plön and Bernard, 2006; Miller et al., 2007).

The relationships between morphometric parameters or morphological characteristics of spermatozoa and body mass or mating systems in mammals have been the subject of speculation and remain controversial. Although Cummins and Woodall (1985) considered that there is a negative correlation between total length of sperm and body mass across mammals, Miller et al. (2007) did not find a significant correlation between body mass and sperm length after evaluation of 21 cetacean species.

Humphries et al. (2008) and Tourmente et al. (2011) investigated the possible link between sperm tail:head length ratio and sperm competition across mammalian species. Additionally, Anderson et al. (2005) suggested a link between midpiece volume and sperm competition. These authors indicated the influence of these morphological parameters on sperm swimming speed and, consequently, its role in sperm competition. However, none of these authors used data from cetaceans in their analyses. Due to the peculiar characteristic of cetacean sperm midpiece shape, these relationships may therefore not hold in cetaceans. Knowledge of sperm morphology in a larger number of cetacean species will be necessary before any link between morphology and sperm competition can be properly investigated.

This is the first description of morphological, morphometric, and ultrastructural aspects of *I. geoffrensis* spermatozoa. The Amazon river dolphin spermatozoa show particularities in the morphological characteristics, as in other cetaceans, when compared with other mammals. Further studies of sperm morphology in a wider spectrum of cetacean families than currently available could help to better understand the reproductive biology of these animals and the intergeneric and intrageneric relationships among them, as well as, among other mammals.

ACKNOWLEDGMENTS

This study was part of Projeto Boto, a research project set up under a cooperative agreement between the National Institute of Amazonian Research—INPA/MCTI and the Mamirauá Sustainable Development Institute—IDSM-OS/MCTI. The authors thank the Laboratório Temático de Microscopia Óptica e Eletrônica—LMTOE/INPA staff for their assistance in the sample analysis and Petrobras Socioambiental (Projeto Mamíferos Aquáticos da Amazônia: Conservação e Pesquisa) and Associação Amigos do Peixe-boi (AMPA) for their financial support. This scientific article was developed with Amazonas government support through the Fundação de

Amparo à Pesquisa do Estado do Amazonas (PAPAC 020/2013—Process no. 062.00888/2014).

LITERATURE CITED

Anderson MJ, Nyholt J, Dixton AF. 2005. Sperm competition and the evolution of sperm midpiece volume in mammals. J Zool 267:135–142.

Ballowitz E. 1907. Zur Kenntnis der Spermien der Cetaceen. Arch für Mikroskop Anat 70:227–237.

Best RC, da Silva VMF. 1993. Inia geoffrensis. Mamm Sp 426:1–8.

Cummins JM, Woodall PF. 1985. On mammalian sperm dimensions. J Reprod Fertil 75:153–175.

Fawcett DW. 1975. The mammalian spermatozoon. Dev Biol 44: 394–436.

Fleming AD, Yanagimachi R, Yanagimachi H. 1981. Spermatozoa of the Atlantic bottlenosed dolphin, *Tursiops truncatus*. J Reprod Fertil 63:509–514.

Humphries S, Evans JP, Simmons LW. 2008. Sperm competition: linking form to function. BMC Evol Biol 8:1–11.

Kita S, Yoshioka M, Kashiwagi M, Ogawa S, Tobayama T. 2001. Comparative external morphology of cetacean spermatozoa. Fish Sci 67:482–492.

Li HY, Zhang XF, Wang D, Chen DQ. 2009. Ultrastructure of the spermatozoa of the Yangtze finless porpoise (*Neophocaena phocaenoides asiaeorientalis*). Anat Histol Embryol 38:300–304.

Meisner AD, Klaus AV, O'Leary MA. 2005. Sperm head morphology in 36 species of artiodactylans, perissodactylans, and cetaceans (Mammalia). J Morphol 263:179–202.

Miller DL, Styer EL, Decker SJ, Robeck T. 2002. Ultrastructure of the spermatozoa from three odontocetes: a killer whale (*Orcinus orca*), a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and a beluga (*Delphinapterus leucas*). Anat Histol Embryol 31: 158–168.

Miller DL, Styer EL, Kita S, Menchaca M. 2007. The mature cetacean spermatozoon. In: Miller DL, editor. Reproductive biology and phylogeny of Cetacea: whales, dolphins and porpoises, 1st ed. Enfield: Science Publishers. p 245–280.

Mogoe T, Fukui Y, Ishikawa H, Ohsumi S. 1998. Morphological observations of frozen-thawed spermatozoa of Southern minke whales (*Balaenoptera acutorostrata*). J Reprod Dev 44: 95–100.

Neuenhagen C, García Hartmann M, Greven H. 2007. Histology and morphometrics of testes of the white-sided dolphin (*Lagenorhynchus acutus*) in bycatch samples from the northeastern Atlantic. Mamm Biol 72:283–298.

Plön S, Bernard RTF. 2006. A review of spermatozoan morphology in Cetacea with new data for the genus *Kogia*. J Zool 269:466–473.

Tourmente M, Gomendio M, Roldan ERS. 2011. Sperm competition and the evolution of sperm design in mammals. BMC Evol Biol 11:1–10.