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Comparative sperm ultrastructure of two tegu lizards (genus *Salvator*) and its relation to sperm competition

Running Head: Sperm ultrastructure in *Salvator* lizards

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Abstract

The knowledge of sperm ultrastructure of Squamata provides informative traits for phylogenetic analyses. Furthermore, several sperm ultrastructure traits are important for sperm motility and

longevity. Here, we provided a detailed ultrastructural description of the spermatozoa of two closely related teiid lizards, *Salvator rufescens* and *S. merianae*. We carried out an interspecific comparison of sperm ultrastructure traits and discussed their possible relation to sperm competition risk. Sperm ultrastructure of the two species shared patterns with other lizards previously described. The lack of interspecific differences in most sperm subcellular components, suggested that sperm ultrastructure traits are conserved within the genus *Salvator* and thus pointed out an important phylogenetic influence. However, we detected interspecific differences in the number of mitochondria and dense bodies sets, suggesting differences in the amount of energy available for sperm motility and longevity. Further comparative studies are urgently needed to understand the sperm metabolic pathways in neotropical lizards in general.

Keywords: Lizards, mitochondria, sexual selection, spermatozoa, ultrastructure

1. Introduction

Sperm ultrastructure studies provide information about subcellular mechanisms that may influence the reproductive success, supplying useful traits to elucidate phylogenetic relationships among species (Texeira et al., 1999; 2002; Giuliano et al., 2002; Tavares-Bastos et al., 2002; Tourmente et al., 2006; Colli et al., 2007; Tourmente et al., 2008). Furthermore, several sperm ultrastructure traits are important for sperm motility and longevity (Eddy et al., 2003; Turner et al., 2006). The outer dense fibers (ODFs) that surround the axoneme, in particular numbers 3 and 8, are enlarged in Squamata spermatozoa, providing flagellum rigidity during sperm movement (Gastman et al., 1993; Turner et al., 2006). Moreover, the number of mitochondria in the midpiece have a key role to provide energy for sperm survival and movement (Tavares-Bastos et al., 2002; Turner et al., 2006; Tourmente et al., 2009). Finally, the fibrous sheath (FS) is involved in sperm movement, influencing flagellum flexibility and the flagellar beat pattern (Fawcett, 1975; Lindemann et al., 1992; Eddy et al., 2003). In addition, it is considered an important source of energy for the spermatozoa, because it has enzymes involved in glycolysis, allowing the production

of ATP throughout the flagellum length (Narisawa et al., 2002; Miki et al., 2004; Turner et al., 2006). Tourmente et al., (2009) documented that species with higher levels of sperm competition, where selection on sperm performance may be particularly intense, the area of ODFs and FS are larger, suggesting an improvement in sperm quality in snakes.

Salvator merianae and *S. rufescens* (Daudin, 1802) (formerly *Tupinambis merianae* and *T. rufescens*) are excellent model systems to study the sperm ultrastructure in the context of sperm competition risk. They are closely related species (Cabaña et al., 2014), share many bioecological traits (Cardozo et al., 2012) and have a partially overlapping distribution (Cardozo et al., 2012; Lanfri et al., 2013), where reciprocal hybridization between these species and introgression by backcrossing occurs (Cabaña et al., 2014). In *Salvator* lizards, females can copulate with different males, even on the same day (Lopes and Abe, 1999). Moreover, follicular development is completed about 20 days after mating, suggesting sperm retention in female genital ducts (Garcia Valdez et al., 2011). Therefore, there is a high opportunity for sperm competition to occur in these species. Indeed, Blengini et al., (2014) confirmed that *S. rufescens* is exposed to higher levels of sperm competition risk than *S. merianae* and evidenced a large among- and within- male variation in sperm morphometric and dynamic traits, suggesting that males vary in sperm competitive ability. Variation in the secondary sexual character, the relative testis mass and the length of sperm component was observed between allopatry and sympatry in each species, suggesting differences in the investment of reproductive traits (Naretto et al., 2016). Furthermore, a negative relationship between secondary sexual character with sperm principal piece length and seasonal flexibility of male reproductive strategies were observed in *S. rufescens* (Blengini et al., 2016). Then, we expect to find variation in sperm ultrastructure traits, being S. *rufescens* the species that could present greater number of mitochondria and larger area of outer dense fibers and fibrous sheath than S. *merianae*. Even though, the sperm ultrastructure of *S. merianae* has been previously described (Tavares-Bastos et al., 2002), there is evidence that sperm traits could vary among populations in different geographical areas (Lüpold et al., 2011). Then, the aim of our study is to provide a detailed ultrastructural description of the spermatozoa of *S. rufescens and S. merianae* to compare sperm ultrastructure traits between these species.

2. Materials and Methods

2.1 Study species

Salvator merianae and *S. rufescens* are similar in body size and live in the southernmost area of genus distribution in South America (Lanfri et al., 2013). Both species are seasonal breeders, that reproduce from October to December (Fitzgerald et al., 1993; Naretto et al., 2014). They are included in the Appendix II of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES 2008). We are authorized to perform animal capture for scientific purposes by the government environmental agencies. Specimens were killed for the legal skin trade, in accordance with AVMA Guidelines on Euthanasia (AVMA 2007). Sampling was conducted at two sites of allopatry for each species (*S. merianae*: 31°28´W, 63°38´S to 31°45´W, 63°15´S; *S. rufescens*: 29°30´W, 64°15´S to 29°57´W, 63°55´S).

2.2 Sperm sampling procedure

Spermatozoa were obtained from the terminal portion of the epididymis (Depeiges and Dacheux, 1985). All the samples obtained were collected in a 1.5 mL plastic tube containing approximately 90 μL of phosphate buffered saline (PBS).

2.3 Spermatozoa ultrastructure

Sperm ultrastructure data was obtained from 3 males of each species of *Salvator*. Semen samples were washed twice with PBS and centrifuged for 7 min at 700G. The supernatant was eliminated and the resulting pellet was fixed for 3 h at room temperature in a 2% glutaraldehyde and 4% formaldehyde solution. Subsequently, the samples were postfixed in 1% osmium tetroxide in cacodylate buffer during 2 h at room temperature. The samples were washed with distilled water and dehydrated in a series of ascending acetone concentrations (50, 70, 90 and 100%), then transferred to a solution of 50% acetone and 50% Araldite epoxy resin followed by two final steps in 100% resin. The samples were then embedded in Araldite epoxy resin at 60°C for 24–72 h. Ultrathin sections (approximately 60 nm) were mounted on 250 mesh nickel grids, and stained with a saturated solution of uranyl in ethanol, and in lead citrate. Finally, the samples were observed

under a Zeiss LEO 906E transmission electron microscope. Ultrastructural morphometry was performed in the micrographs at different magnifications. The area (μm2) of the fibrous sheath and the outer dense fibers in position 3 and 8 was measured using Image J version 1.48g (NIH, USA) in one spermatozoa per male. The area of the fibrous sheath was quantified making a measure of the area of two concentric circles, then the subtraction of the external circle less internal circle was made. Differences between species in the area of fibrous sheath and outer dense fibers were determined by non- parametric Kuskal-Wallis test. These statistical tests were conducted using InfoStat software (version 2012; Universidad de Cordoba, Argentina).

3. Results

Since there were no differences between species in the most subcellular components analyzed, a single schematic drawing including the major findings in both species was provided (Fig. 1).

Acrosome complex. In both species, the acrosome complex, which was located in the anterior most region of the head, was comprised of an external and elongate acrosome vesicle, an internal cap, the subacrosomal cone, and the perforatorium (Fig. 2A, B, H-J). In cross section, it was depressed and increasingly circular toward the base (Fig. 2C-G, K-O). The acrosome vesicle was divided into: a narrow cortex and a wide medulla at its anterior portion. The cortex consisted of two layers with different electron densities: one external showing a thin electron-dense layer that surrounded an electron-lucent layer with a tubular organization (Fig. 2C, D, K, L). The medulla appeared as an electron-dense structure, filling the interior of the acrosomal vesicle (Fig. 2C, D, K, L). The perforatorium was a slender rod with a pointed tip, which extended anteriorly from the subacrosomal cone into the acrosomal vesicle (Fig. 2B, H). At the perforatorium posterior end, the base plate was cylindrical in both species (Fig. 2B, H). The subacrosomal cone extended posteriorly to the acrosome and had a paracrystalline structure (Fig. 2B, I, J). Both species had a well-developed epinuclear electron-lucent zone from the anterior portion of the nuclear rostrum to the apex of the subacrosomal cone, being larger in *S. rufescens* (1,1 μm) than in *S. merianae* (0,121

μm) (Fig. 2J, I). The basal portion of the subacrosomal cone covered the tapered rostrum of the nucleus, ending at the level of the nuclear shoulders (Fig. 2B, J).

Nucleus. The nucleus was a cylindrical, slightly curved and highly electron-dense structure, consisting of homogenous condensed chromatin (Fig. 2B, I, J). In transverse section, the nucleus was circular (Fig. 2G, O). The anterior pole of the nucleus formed a tapered rostrum that entered the subacrosomal cone (Fig. 2B, I, J). The basal end of the rostrum was marked by slight and rounded nuclear shoulders (Fig. 2B, I). The basal pole of the nucleus held a semicircular depression, the nuclear fossa, which was associated with the centriolar apparatus (Fig. 3B, 4B,C).

Neck region. This region formed the junction between the midpiece and the head of the sperm. In both species, it contained the proximal and distal centrioles, the first ring of mitochondria and dense bodies. The proximal centriole was being partially surrounded by the pericentriolar material that extends posteriorly between the two centrioles (Fig. 3B, C; 4C). There were no electrondense structures within any of the proximal centriole (Fig. 3C, 4C). The distal centriole formed the basal body of the axoneme and consisted of nine triplets of microtubules, nine outer fibers that partially cover the triplets, and two central singlets of the axoneme, occupying a small segment of the midpiece without projecting into the fibrous sheath (Fig. 3A, 4A).

Midpiece. This part of the flagellum consisted of mitochondria and the axoneme surrounded by a fibrous sheath and rings of dense bodies (Fig. 3A, 4A). The axoneme was characteristically arranged in a 9 + 2 pattern, with each doublet was close to an outer dense fiber (Fig. 3E, 4E). The fibers at doublets 3 and 8 were enlarged, detached from their corresponding doublet (Fig. 3E, 4E). In both species, mitochondria were columnar in longitudinal section (Fig. 3A, B, 4A, B), while in cross section, they appeared as irregular structures with linear cristae (Fig. 3E, 4E). The mitochondria were separated by rings of dense bodies. Dense bodies were well developed and appear as solid structures (Fig. 3A, 4A). There were differences in the number of mitochondria and set of dense bodies between species; *S. merianae* presented eight six sets of mitochondria around the axoneme (Fig. 3A) and *S. rufescens* had seven sets (Fig. 4A). However, we found no differences in the area of fibrous sheath (Kruskal-Wallis $H_{1,4}$=0.43; P= 0.70) and the area of outer dense fibers

in positions 3 and 8 (Kruskal-Wallis $H_{1,4}$=0.05; P= 0.99) between species. Posteriorly, the midpiece terminated at the annulus, marking the beginning of the principal piece (Fig. 3A, 4A).

Principal Piece. The principal piece started behind the midpiece. It consisted of the axoneme, surrounded by the fibrous sheath and the plasma membrane. The axoneme had no outer dense fibers (Fig. 3F, 4F).

4. Discussion

The sperm ultrastructure of two tegu species of *Salvator* was similar to other lizard species described (Texeira et al., 1999; 2002; Ferreria and Dorder, 2003; Colli et al., 2007), and to other species of the genus *Salvator* (Tavares-Bastos et al., 2002). Spermatozoa of *S. merianae* and *S. rufescens* showed ultrastructural traits characteristic of Squamata (Oliver et al., 1996), as a single perforatorium, the subacrosomal cone well developed, epinuclear lucent zone, fibrous sheath surrounding the axoneme at the midpiece, and enlarged outer dense fibers 3 and 8. Moreover, like other lizards, they had dense bodies well developed and placed at regular intervals between the mitochondria along the midpiece (Teixeira et al., 1999; Giugliano et al., 2002; Tavares-Bastos et al., 2002; Teixeira et al., 2002; Ferreira and Dolder, 2003; Vieira et al., 2004; Colli et al., 2007), which have been proposed as mitochondrial derivatives (Oliver et al., 1996).

Postcopulatory sexual selection drives the evolution of the sperm morphology (Gomendio and Roldan, 1991; 2008; Fitzpatrick et al., 2009; Lüpold et al., 2009; Tourmente et al., 2009; 2011) and sperm velocity (Kleven et al., 2009; Lüpold et al., 2009; Tourmente et al., 2011). Hence, sperm competition could be considered as a pressure of sexual selection over sperm ultrastructure to maximize male fertilization success (Snook, 2005; Pizzari and Parker, 2009; Tourmente et al., 2009). Blengini et al., (2014), reported that these species show differences in sperm component length and sperm velocity. Therefore, we expected to find differences in sperm ultrastructure traits as well. However, we found no differences in the most subcellular components analyzed. These results suggest that sperm ultrastructure traits are phylogenetically conserved within *Salvator*. Moreover, the similarities in sperm ultrastructure traits suggest that post-copulatory isolation mechanisms between these lizards are apparently relaxed, and could not prevent the hybridization between them when they are in sympatry. A previous study of two closed related species of snakes

that differ in the length of the sperm acrosome, sperm head and sperm midpiece, found no differences in sperm ultrastructure traits (Tourmente et al., 2008). Conversely, in a comparative study of five snake species of that differ considerably in relative testis mass and sperm length, significant differences in fibrous sheath area and outer dense fibers were found (Tourmente et al., 2009). We found interspecific differences in the number of mitochondria and dense bodies sets in *Salvator*. Our results are in agreement with the range of variation from six to eight set of mitochondria reported by Tavares-Bastos et al., (2002) for other species of *Salvator*.

Although mitochondria in the midpiece have traditionally been regarded as the main site of energy production in sperm, the glycolytic activity of the fibrous sheath has recently been highlighted as an additional energetic source for sperm motility (Ruiz-Pesini et al., 2007; Storey, 2008; Paoli et al., 2011; Tourmente et al., 2015). *Salvator rufescens* presented fewer mitochondria in the midpiece but higher sperm velocity than *S. merianae* (Blengini et al., 2014). Moreover, Blengini et al., (2014) reported a negative relationship between sperm velocity and midpiece length for both these *Salvator* species. Considering these results, *Salvator* sperm may have an additional source of energy besides mitochondria. In addition to the number of mitochondria, changes in mitochondria function can affect the amount of energy available for the sperm (Ruiz-Pesini et al., 1998; Anderson et al., 2007; Amaral et al., 2013). Then, the sperm midpiece would contribute with an increase in the energetic reserves for the sperm because it contains mitochondria that are essential for energy metabolism (Ruiz- Pesini et al. 2007) and the fibrous sheath where the anaerobic glycolysis occurs, a complementary metabolic pathway to produce sperm energy, allowing energy transmission along the principal piece (Ruiz-Pesini et al. 2007; Tourmente et al. 2015). Further studies are needed to understand the sperm energetic metabolism in these taxa.
This study provides evidence of similarities and differences in sperm ultrastructure traits in two closely related species of lizards, given the basis for future research to understand how subcellular traits may influence the reproductive success.

5. *Acknowledgments*

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Figure captions

Fig.1 Schematic representation of the spermatozoon of *Salvator merianae* and *S. rufescens* in longitudinal section with its corresponding transverse sections. The scale of some sections and structures has been changed for clarity.

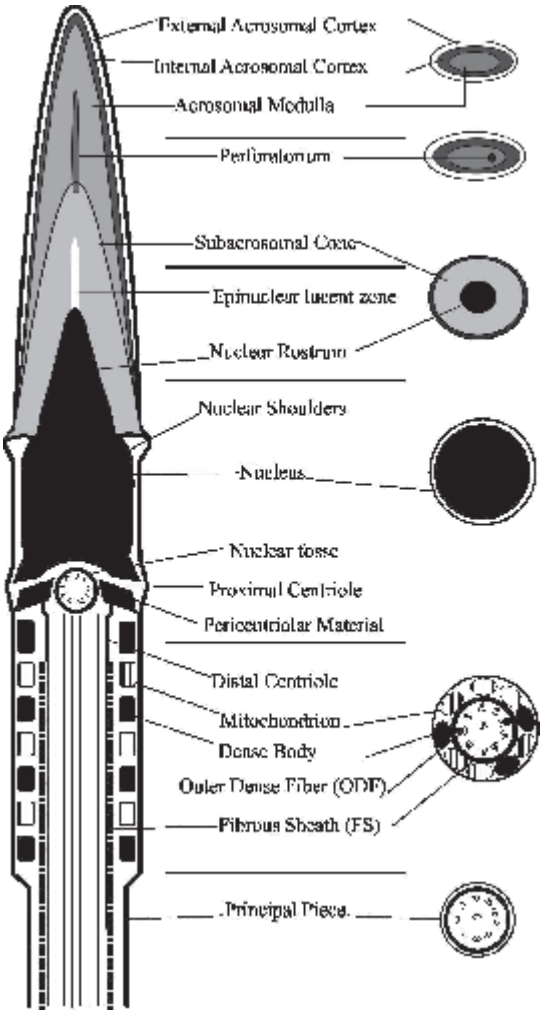

Fig. 2 Transmission electron micrographs of the head (acrosome complex and nucleus) of spermatozoa of *Salvator merianae* (A-G) *and S. rufescens* (H-O). A, B, H-J. Longitudinal section of the acrosomal complex presenting the acrosome and the subacrosomal cone. White arrows mark the nuclear shoulders and black arrows indicate perforatorium base. C-G, K-O. Transversal section of the nucleus. C-F, K-N Transversal section of the acrosomal complex. Abbreviations: a: acrosome; sac: subacrosomal cone; co1: external acrosomal cortex; co2: internal acrosomal cortex; m: medulla; n: nucleus; p: perforatorium; nr: nuclear rostrum; elz: epinuclear lucent zone.

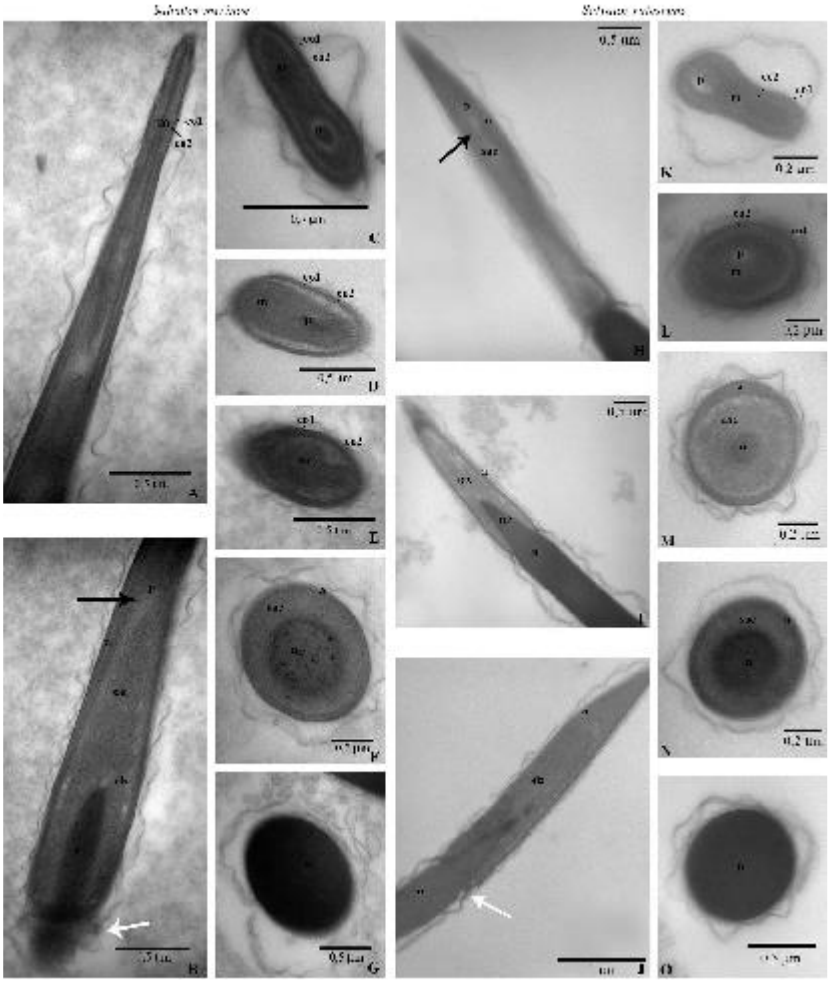

Fig. 3 Transmission electron micrographs of midpiece and principal piece of spermatozoa of *Salvator merianae.* A. Longitudinal section of midpiece. B, C. Longitudinal section or neck region, detailing centriolar apparatus. D. Transversal section of neck region showing distal centriole (black arrow indicates ODF). E. Transversal section of the midpiece (White arrow indicates ODF in position number 3). F. Transversal section of principal piece. ax: axonema; db: dense bodies; distC: distal centriole; pC: proximal centriole; nf: nuclear fose; ODF: peripheral fibers; m: mitochondria; pcm: pericentriolar material; n: nucleus; FS: fibrous sheath.

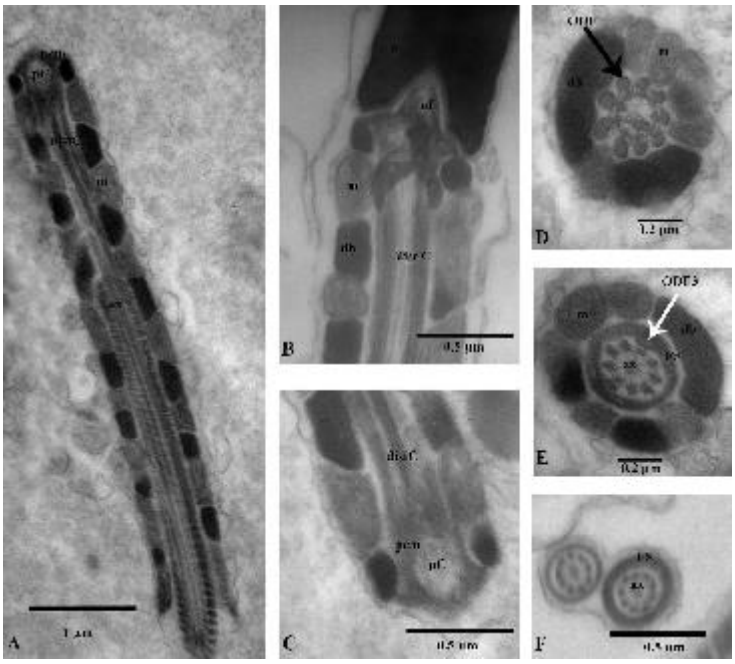

Fig. 4 Transmission electron micrographs of midpiece and principal piece of Spermatozoa of *Salvator rufescens.* A. Longitudinal section of midpiece. B, C. Longitudinal section or neck region, detailing centriolar apparatus. D. Transversal section of neck region. E. Transversal section of the midpiece (white arrow indicates ODF in position number 3). F. Transversal section of principal piece. ax: axonema; db: dense bodies; distC: distal centriole; pC: proximal centriole; nf: nuclear fose; ODF: peripheral fibers; m: mitochondria; pcm: pericentriolar material; n: nucleus; FS: fibrous sheath.