

## SHORT COMMUNICATION

### Morphology of the male reproductive tract of the harvestman *Mischonyx cuspidatus* (Roewer, 1913) (Gonyleptidae: Opiliones: Chelicerata)

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**Abstract.** Arachnida evolved different reproductive strategies in the terrestrial habitats. Knowledge of the morphology of reproductive systems varies depending on the group, and for Opiliones only a few studies exist addressing this topic. Here, we describe the morphology of the male reproductive tract and the spermatozoon of the harvestman *Mischonyx cuspidatus* (Roewer, 1913). In this species, males have a single testis, a pair of deferent ducts, a seminal vesicle, a propelling organ and a penis. The lumen of the folded seminal vesicle and testis follicles are filled with spermatozoa, suggesting a storage of sperm related to a possible reproductive strategy involving multiple matings. The spermatozoa are aflagellate and ca. 6.5 µm in length. This study sheds light on the knowledge of the harvestman's reproductive biology and life strategy, which can be used in future studies involving Opiliones behavior and systematics.

**Keywords:** Arachnida, aflagellate spermatozoa, Laniatores, reproduction.

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The Opiliones, harvestmen, is a diverse group of arachnids and characterized by the fusion of the prosoma and the opisthosoma, presence of a penis and a pair of defensive glands in the anterior-lateral region (Pinto-da-Rocha et al. 2007). Opiliones are divided into five suborders: Tetrophthalmi (fossil), Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores, the latter one being the most diverse with 35 families and 4183 species (Kury 2013, 2018; Kury et al. 2019).

The male reproductive system and spermatozoa have been described for representatives of all arachnid groups, including Scorpiones (e.g., Michalik & Mercati 2010), Acari (e.g., Alberti & Klompen 2002; Florek & Witalinks 2010), Araneae (e.g., Alberti 2000; Lipke et al. 2014; Lipke & Michalik 2015; Michalik & Ramirez 2014), and Solifugae (e.g., Alberti & Peretti 2002). The morphology of the penis of harvestmen is an important source of characters for taxonomic studies since this has interspecific morphological variations (Martens 1976, 1986, 1988; Pinto-da-Rocha et al. 2007; Macías-Ordóñez et al. 2010; Bennett & Townsend 2013; Machado et al. 2015; Pérez-González & Werneck 2018; Proud & Townsend 2019). However, in Opiliones, the internal morphology of the reproductive tract and spermatozoa was only described for few representatives of Cyphophthalmi (Janczyk 1956; Juberthie et al. 1976; Juberthie & Manier 1978; Gutjahr et al. 2006), Eupnoi (Reger 1969; Tripepi 1983), Dyspnoi (Juberthie & Manier 1976, 1977a,b) and Laniatores (Juberthie & Manier 1977c; Jones & Cokendolpher 1985; Moya et al. 2007).

In order to contribute to our knowledge of the male reproductive system of harvestmen, we investigated a representative, *Mischonyx cuspidatus* (Roewer, 1913), of the diverse family Gonyleptidae, which consists of ca. 300 genera with a wide distribution in the Brazilian Atlantic rainforest.

#### METHODS

**Animals.**—Twenty adult males of *Mischonyx cuspidatus* (Fig. 1A) were collected directly under rocks and logs in a fragment of Mata Atlântica, at Parque Municipal Juarez Frotté, Nova Friburgo (22°16'

S 42°31' W), Rio de Janeiro, Brazil. Voucher specimens were deposited at the National Museum of the Federal University of Rio de Janeiro (MNRJ-19771).

**Anatomy and histology of the reproductive system.**—The reproductive systems were dissected in 0.1 M NaCl and transferred to 2.5% glutaraldehyde in sodium cacodylate buffer, 0.1 M pH 7.2 for 2 hours at room temperature. Subsequently, they were washed in the buffer, dehydrated in a graded ethanol series (50, 70, 90 and 95%) and embedded in histoResin (Leica, Germany). Semi-thin sections (2 µm) were obtained in a rotating microtome (Leica, Germany) with a glass knife, stained with 1% toluidine blue in borate sodium buffer or hematoxylin and eosin and analyzed under a light microscope.

For anatomical analysis, shortly after fixation, some reproductive systems were photographed using an Olympus BX-60 microscope (Japan) and then drawn with Indian ink and graphite.

**Spermatozoa measurements.**—After dissection, the seminal vesicle was ruptured with the aid of forceps and the sperm spread on a glass slide with Bouin's fixative for 20 min. After washing, the samples were photographed under a BX-60, Olympus light microscope (Japan). Some samples were stained for 15 minutes with 0.2 µg/mL of 4,6-diamino-2-phenyl-indole (DAPI) in 0.1M sodium phosphate buffer and examined with an epifluorescence microscope BX-60, Olympus (Japan), equipped with a BP360-370nm excitation filter. All measurements were performed using the Image Pro-Plus software (Media Cybernetics, USA).

#### RESULTS

The male reproductive tract of *Mischonyx cuspidatus* (Fig. 1A) consists of a single long U-shaped testis connected to two deferent ducts ending in a large folded seminal vesicle (Fig. 1B). Following the seminal vesicle, there is a propulsive organ and an eversible penis (Figs. 1B–D).

The spermatozoa of *M. cuspidatus* were dispersed in the lumen of the testis and the seminal vesicle (Fig. 2A) and showed no apparent mobility. These aflagellate cells measured 6.5 µm in length and 1.7 µm

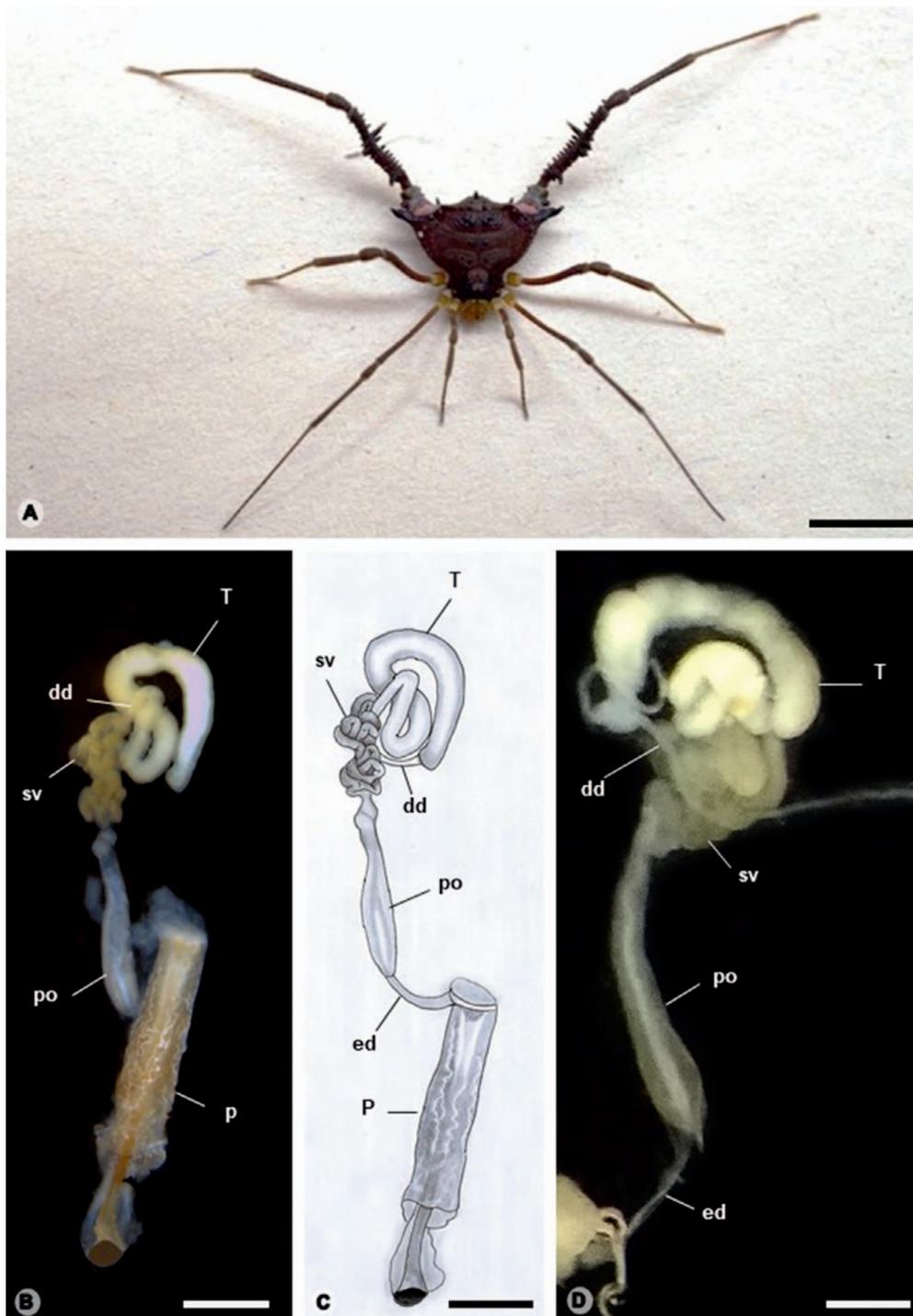


Figure 1.—*Mischonyx cuspidatus*. A. Habitus of the adult male (dorsal). B–D. Images (B and D) and schematic drawing of the anatomy of the reproductive system (C), showing the testis (T), deferent ducts (dd), seminal vesicle (sv), propulsive organ (po), ejaculatory duct (ed) and penis (p). Bars: A = 1.5 cm; B and C = 600  $\mu$ m and D = 400  $\mu$ m.

in width. The nucleus with tapered tips occupied almost the entire spermatozoa volume (Fig. 2B).

The single U-shaped testis follicle was connected to the deferent ducts, one at each end (Figs. 1B–D, 2C). The testis follicular epithelium was cubical with spherical nuclei in the median position of the cells (Figs. 2C, 3A). The epithelium of the deferent duct has flattened nuclei and a thick muscular layer (Fig. 3B). The deferent

ducts end in a dilated and folded region filled with sperm forming a single seminal vesicle (Figs. 2C, 3B,C). The epithelium of the seminal vesicle was formed by a single-layered squamous epithelium with flattened nuclei (Figs. 3B,C).

The propulsive organ was long and covered with several layers of muscles (Fig. 4A,B). An internal duct ran through the entire length of the propulsive organ (Figs. 1B,C, 4A,B) and in its terminal region was

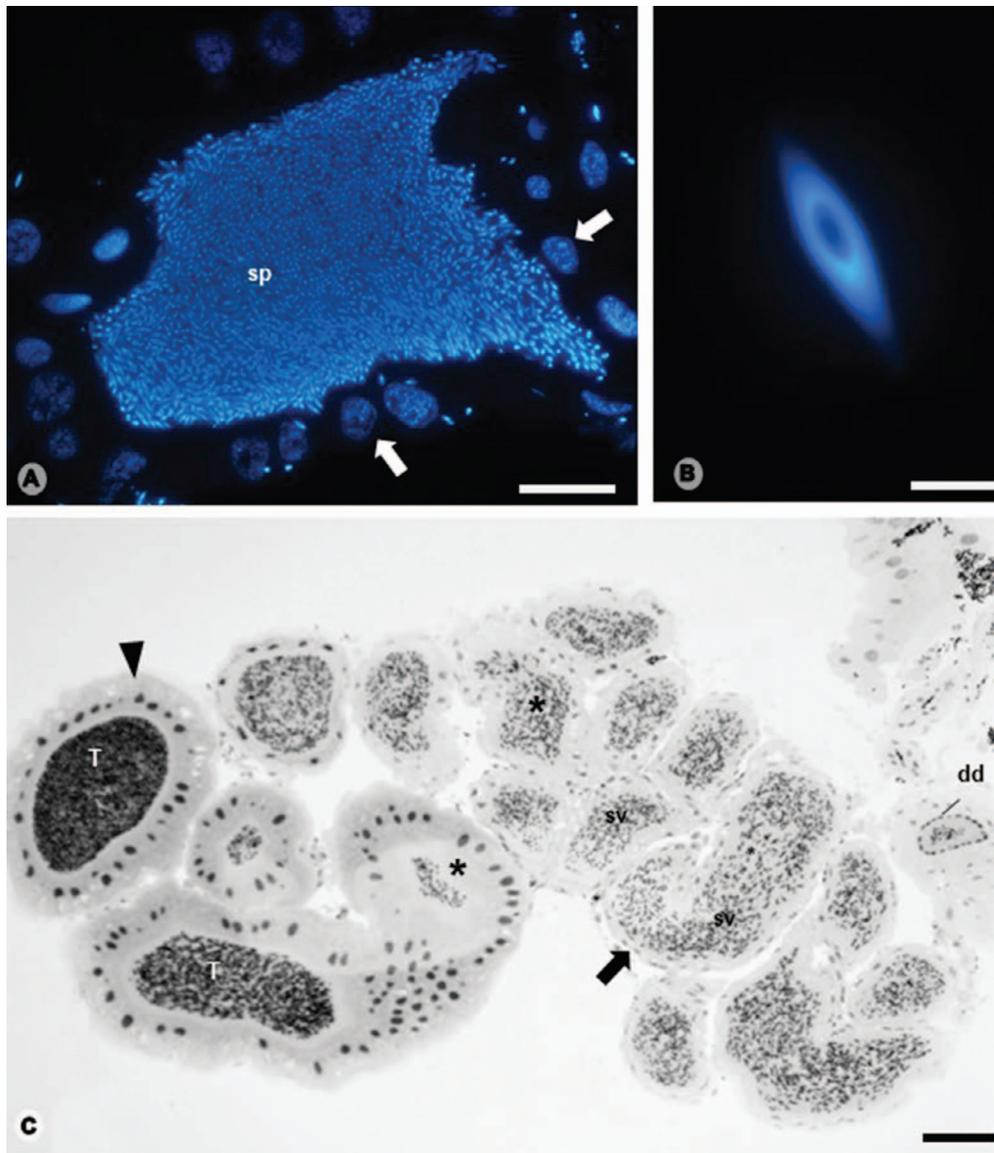


Figure 2.—Morphology of the reproductive tract and spermatozoa of *Mischnonyx cuspidatus*. **A–B.** Cross-section of the testis (**A**) and sperm (**B**) stained with DAPI, showing the lumen filled with spermatozoa (sp) and the spherical nuclei of the follicular epithelium (arrows). **C.** Cross-section showing testis (T), seminal vesicle (sv), deferent duct (dd) with sperm (asterisk) and the arrow points to the nucleus of the epithelium of the seminal vesicle. Bars: A = 30  $\mu\text{m}$ ; B = 2  $\mu\text{m}$  and C = 100  $\mu\text{m}$ .

connected to the ejaculatory duct (Fig. 4C). The ejaculatory duct had a thick muscular layer with a cuticle lining the lumen (Figs. 4D–F). The propulsive organ was connected to the eversible penis by the ejaculatory duct (Fig. 1B,C).

#### DISCUSSION

The morphological characters derived from the male reproductive tract and spermatozoa of arachnids have shown interspecific variations but some features are conserved at the family or subfamily level (e.g., Jamieson et al. 1999; Alberti & Peretti 2002; Alberti 2005; Pérez-González et al. 2016). The morphology of the harvestmen male reproductive tract has been described for the four extant suborders (Janczyk 1956; Juberthie 1965; Silhavy 1966; Suzuki 1966; Reger 1969; Juberthie et al. 1976; Juberthie & Manier 1976, 1977a,b,c, 1978; Martens 1976; Tripepi 1983; Jones & Cokendolpher 1985; Cokendol-

pher & Jones 1991; Gutjahr et al. 2006; Moya et al. 2007) and generated some characters with some inconsistent terminology. In Gonyleptidae, the reproductive tract of *Vonones sayi* (Simon, 1879) was described considering the *vasa deferentia* as *vasa efferentia* and the seminal vesicle divided into two regions, *vas deferens* and seminal reservoir (Cokendolpher & Jones 1991). In this study, we show that *M. cuspidatus* had the ground plan for harvestmen with a single testis, two deferent ducts, a single unpaired seminal vesicle, a propulsive organ, and an eversible penis.

The long and enlarged testis follicle and the folded seminal vesicle, with the lumen filled with spermatozoa, suggests that *M. cuspidatus* store sperm cells and, possibly, its reproductive strategy may involve multiple mating such as found in other Gonyleptidae (Birkhead & Moeller 1998).

The aflagellate spermatozoa measuring 6.5  $\mu\text{m}$  in *M. cuspidatus* are similar to that found for other Laniatores (Juberthie & Manier 1977c;

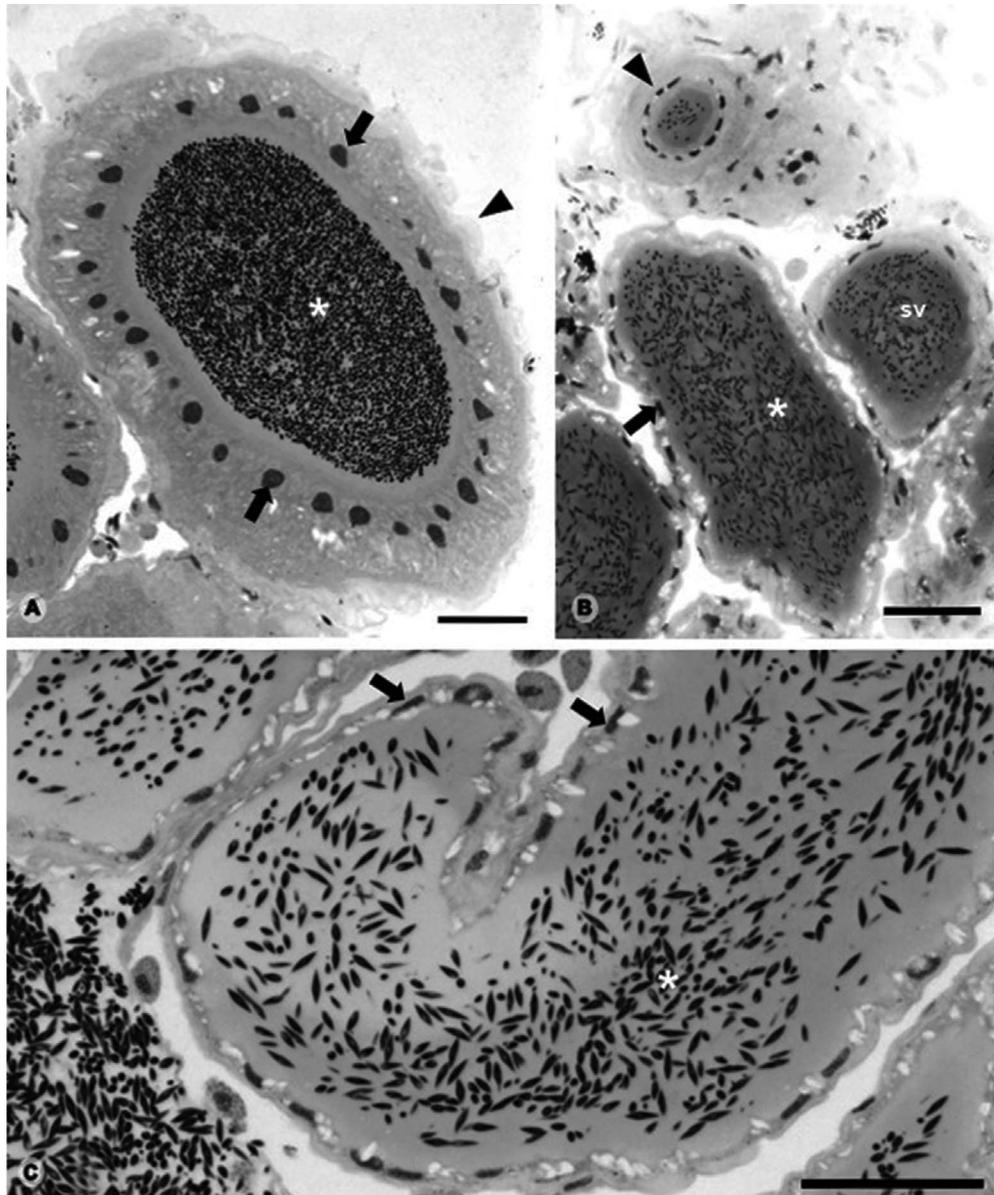


Figure 3.—Histology of the reproductive tract of *Mischnonyx cuspidatus*. A. Transverse section of the testis showing sperm in the lumen (asterisk), nuclei of the epithelium (arrows) and muscle layer (arrowhead). B-C. Seminal vesicle cross-section (sv) showing a flattened nucleus of the epithelium (arrows), and sperm (asterisks). In (B) detail for the deferent duct with flattened nuclei and a thick muscular layer (arrowhead). Bars: A-C = 50  $\mu$ m.

Jones & Cokendolpher 1985; Morrow 2004; Moya et al. 2007). Aflagellate spermatozoa seem to have evolved independently in at least 36 animal groups, including many orders of Arthropoda (Morrow 2004). For Opiliones, only Cyphophthalmi representatives keep sperm with flagella during spermatogenesis, but they are already immobile (Alberti 1995, 2000, 2005; Alberti et al. 2009), whereas all other species investigated, to date, have aflagellate spermatozoa (Moya et al. 2007). Spermatozoa with oval shapes and without flagella are also found in Acari, Palpigradi, and Solifugae (Alberti 2000).

A strong selective pressure led to the evolution of complex mechanisms, such as sperm competition, in arthropods (Birkhead & Moeller 1998). In Opiliones, this selective force may be responsible for the gradual loss of the flagellum (Morrow 2004). Whereas groups more closely related to the ancestor, such as Cyphophthalmi, retained

an immobile axoneme in the flagellum (Alberti 1995), the more derived taxa (i.e., Eupnoi, Dyspnoi, and Laniatores) retract the flagellum during spermiogenesis (Jones & Cokendolpher 1985; Alberti 1995, 2000; Moya et al. 2007). Some hypotheses try to explain the loss of the flagellum in Opiliones spermatozoa: (i) basal groups (i.e., similar to the ancestor) transfer sperm through spermatophores, which are stored closely to the ovipositor of the females without need for spermatozoa motility (Juberthie & Manier 1978); (ii) the spermathecae (seminal receptacles) of females are small compared with those from Scorpiones (Sissom 1990) and Araneae (Foelix 1996), resulting in a selective pressure for the spermatozoa with small size to be stored in large quantities in the female reproductive tract (Moya et al. 2007); (iii) the spermathecae are filled with aflagellate spermatozoa to avoid additional mating (Swallow & Wilkinson 2002); and (iv) extracellular projections in the spermatozoa of Laniatores may play a

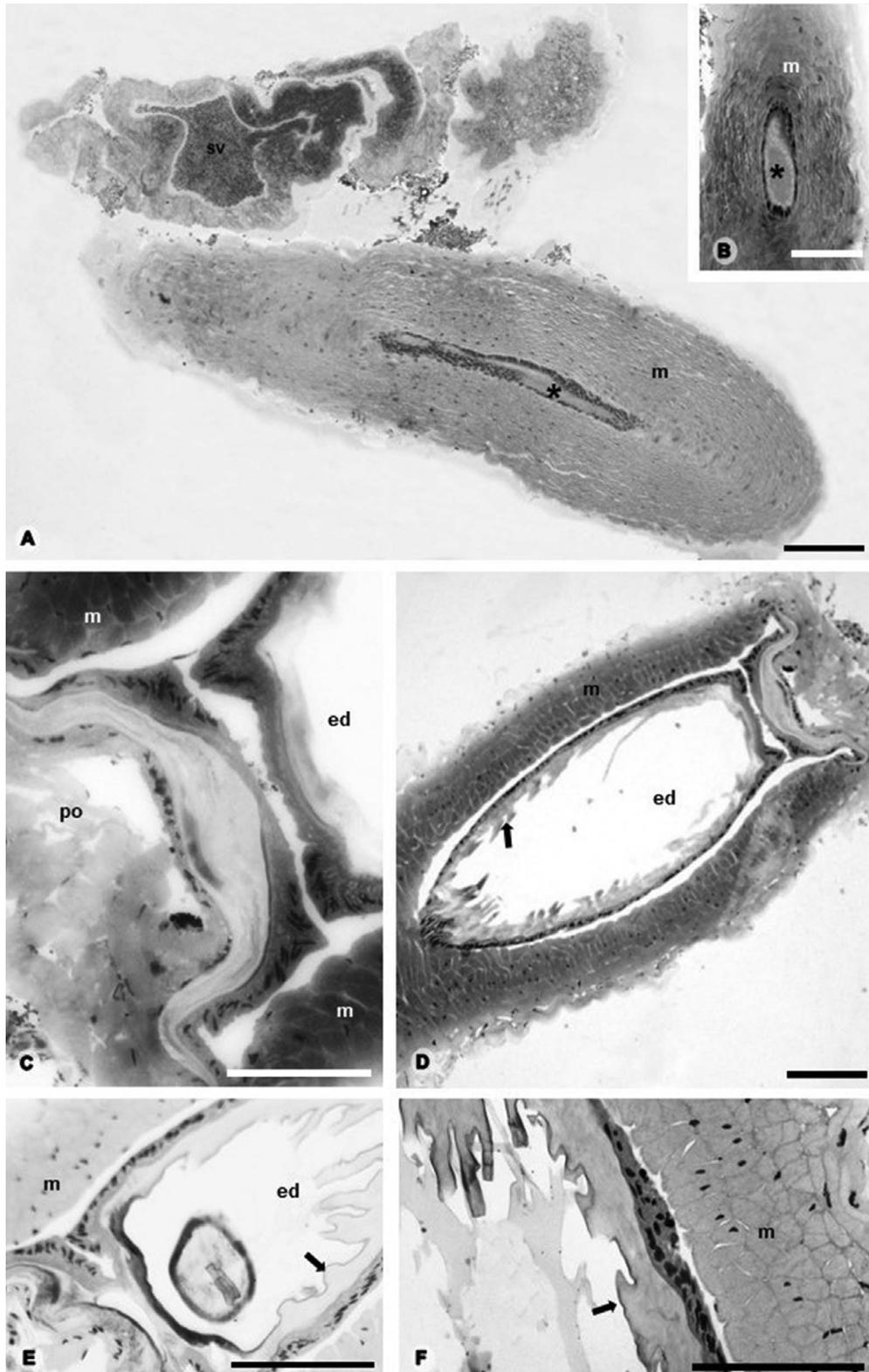


Figure 4.—Histology of the reproductive tract of *Mischonyx cuspidatus*. A, B. Cross section of the seminal vesicle (A) and propulsive organ (A–B) showing lumen of the duct with spermatozoa (asterisks) and thick muscular layer (m). C. Transition region from the propulsive organ (po) to the ejaculatory duct (ed). D–F. Longitudinal sections of the ejaculatory duct region (ed) showing the cuticle (arrows) and the muscular layer (m). Bars: A, C–F = 100  $\mu$ m and B = 60  $\mu$ m.

role in the transport of these cells along the ducts in the male reproductive tract and their attachment in the spermathecae (Jones & Cokendolpher 1985; Moya et al. 2007).

The presence of the propulsive organ anterior to the penis in *M. cuspidatus* is a common feature in Eupnoi, Dyspnoi, and Laniatores, whereas Cyphophthalmi have a small and non-intromittent organ called a spermatopositor (Macías-Ordóñez et al. 2010; Garwood et al. 2014; Burns & Shultz 2015). The eversion and inversion of the penis probably occur due to the action of extrinsic and hydraulic muscles (Shultz 2000; Shultz & Pinto-da-Rocha 2007). The propulsive organ close to the basal region of the penis in Laniatores may be responsible for the strength of the muscular contraction, which releases the sperm through the ejaculatory duct (Martens 1976; Shultz & Pinto-da-Rocha 2007). The structures associated with the penis in Laniatores have morphological differences being an important source of characters for taxonomy and systematics (Macías-Ordóñez et al. 2010; Pérez-González & Werneck 2018).

This study describes the structure of the male reproductive tract and spermatozoa of Neotropical harvestman, *M. cuspidatus*. Data on the reproductive biology of Opiliones are important because the structural characters may be useful for understanding the evolution of reproductive system in the group.

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