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Original article

Morphology and late embryonic development of the male reproductive tract of an Andean onychophoran (Peripatidae) population

Morfología y desarrollo embrionario tardío del sistema reproductor de los machos de una población de onicóforos andinos (Peripatidae)

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Abstract

We examined the male reproductive tract development in a population of onychophorans from the Colombian Cordillera Oriental, describing the macroscopic and histological features during late embryonic development in neonates and in males of different body sizes collected in the field. We described the general anatomy of the reproductive system and found it similar to that of other species from the Peripatidae family. During intra-oviductal development, the reproductive system reached full morphological differentiation at the embryonic stage VII, located in the most distal portion of the mother's ascending oviduct. The embryos occupying the anterior region of the maternal descending oviduct possessed partially pigmented bodies and exhibited active spermiogenesis, although they did not yet produce spermatozoa. The advanced pigmented embryos, located more distally in the maternal descending oviduct, produced spermatozoa and were, therefore, classified as mature individuals, though not reproductively active, as they lacked spermatophores in the lumen of the deferent duct. Neonates and males of all sizes were potentially reproductively active, evidenced by continuous sperm production and the formation of spermatophores ready for transfer to the female gonopore.

Key words: Deferent ducts; seminal vesicle; spermatogenesis; sexual maturity; testes; velvet worm.

Resumen

Examinamos el desarrollo del tracto reproductivo masculino de una población de onicóforos de la cordillera Oriental colombiana para describir las características macroscópicas e histológicas durante el desarrollo embrionario tardío en neonatos y en machos de diferentes tamaños corporales recolectados en campo. Describimos la anatomía general del sistema reproductivo y encontramos que es similar a la conocida en otras especies de la familia. Durante el desarrollo intraoviductal se alcanzó la diferenciación morfológica completa del sistema reproductivo en los embriones machos del estado embrionario VII, ubicados en la porción más distal del oviducto ascendente de la madre. Los embriones alojados en la región anterior del oviducto descendente materno exhibían cuerpos parcialmente pigmentados y espermiogénesis activa, aunque sin producción de espermatozoides aún. Los embriones pigmentados avanzados, localizados más distalmente en el oviducto descendente materno, producían espermatozoides y por ello se clasificaron como individuos maduros, aunque no reproductivamente activos, ya que carecían de espermatóforos en la luz del conducto deferente. Los recién nacidos y machos de todos los tamaños eran reproductivamente activos en potencia, lo que se evidenciaba por la producción continua de espermatozoides y la formación de espermatóforos listos para su transferencia al gonoporo de la hembra.

Palabras clave: conductos deferentes; vesícula seminal; espermatogénesis; madurez sexual; testículos; gusano de terciopelo.

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Introduction

Onychophorans (velvet worms) are terrestrial invertebrates characterized by nocturnal predatory behavior and a strict dependence on humid microhabitats. They are commonly found beneath decaying logs, rocks, and leaf litter (**Manton**, 1946; **Barclay *et al.***, 2000). The phylum Onychophora currently comprises 237 described species, including five fossil taxa of uncertain affinity to extant lineages, distributed across two extant families: Peripatidae (92 species) and Peripatopsidae (140 species) (**de Sena Oliveira**, 2023). The family Peripatidae shows a circumtropical distribution in eastern Africa, Central America, northern South America, and Southeast Asia. Peripatopsidae occur mainly in temperate regions of southern Australia, New Zealand, Chile, and South Africa (**Bouvier**, 1904; **Evans**, 1901; **Monge-Nájera**, 1995; **Reid**, 1996; **de Sena Oliveira *et al.***, 2012; **de Sena Oliveira *et al.,*** 2023).

Although both families exhibit a generally conserved morphology, onychophorans display considerable diversity in mating systems and reproductive modes (**Marotta & Ruhberg**, 2004). Sexes are separate, and reproduction is typically amphimictic, except for *Epiperipatus imthurni* (Peripatidae), in which parthenogenesis has been reported (**Read**, 1988). Sex ratios are often skewed, with females outnumbering males, and sexual size dimorphism is common, as males are smaller than females (**Leishman & Eldredge**, 1990; **Hebert *et al.***, 1991; **Sunnucks *et al.***, 2000). Additional sex-specific differences have been documented in growth patterns, mortality, and the timing of sexual maturity within Peripatopsidae (**Sunnucks *et al.***, 2000) and Peripatidae (**Lavallard & Campiglia**, 1975). For example, in males of *Peripatus acacioi* (Peripatidae), sperm production begins immediately after birth, whereas females require a longer period to attain sexual maturity, typically during their second or third year (**Lavallard & Campiglia**, 1975). Females exhibit a broad range of reproductive and embryonic nutritional modes (**Mayer *et al.***, 2015). Parity modes and embryonic nutritional modes are distinct reproductive traits. Because each parity mode can be associated with different patterns of embryonic nutrition, **Mayer *et al.*** (2015) combined both classifications to provide a comprehensive overview of all reproductive strategies known in Onychophora, including: oviparity + lecithotrophy, ovoviviparity + lecithotrophy, viviparity + lecithotrophy, viviparity + matrotrophy, viviparity + placentotrophy, and combined lecithotrophic/matrotrophic viviparity. Placentotrophic viviparity occurs in Neotropical Peripatidae.

Studies on male onychophorans include analyses of sperm ultrastructure and spermatogenesis, as well as phylogenetic inferences based on sperm characters within Peripatopsidae (**Baccetti *et al.***, 1976; **Baccetti & Dallai**, 1977; **Camatini *et al.***, 1979a; **Storch & Ruhberg**, 1983), and investigations of the ultrastructure of the reproductive tract and the formation of spermatozoa and spermatophores in species of Peripatidae (*Peripatus sedgwicki*, *Epiperipatus biolleyi*) (**Storch & Ruhberg**, 1990; **Storch *et al.***, 1995). **Gaffron** (1885 a,b) provided an early description of the anatomy and histology of the male reproductive apparatus in *Peripatus* (Peripatidae).

In both families, the male reproductive tract consists of a pair of testes, seminal vesicles with their efferent ducts, and an unpaired vas deferens (deferent duct) and ejaculatory duct (**Kemp**, 1914; **Storch & Ruhberg**, 1977; **Storch & Ruhberg**, 1990; **Storch *et al.***, 1995). Spermatophores may occur in the distal region of the reproductive tract, within the vas deferens, and are released through the ejaculatory duct, which opens at the genital opening or gonopore.

Embryonic development of the reproductive system in Neotropical Peripatidae has been described for *Epiperipatus edwardsii* (**von Kennel**, 1885), *Macroperipatus torquatus* (**von Kennel**, 1885), *Eoperipatus weldoni* (**Evans**, 1901), and *Epiperipatus trinidadensis* (**Anderson & Manton**, 1972). These studies indicate that the earliest stages of gonadogenesis occur during the gastrula phase, when primordial germ cells (PGCs) initiate proliferation and migration. At more advanced developmental stages, the formation of the posterior-most pair of somites begins. In this region, PGCs lie in close association with the

endoderm and mesoderm before becoming incorporated into the coelomic cavities (**von Kennel**, 1888; **Evans**, 1901; **Anderson**, 1973). Within these cavities, PGCs attach to the splanchnic walls of the dorsolateral compartment of each somite. As the dorsolateral walls fuse with the pericardial floor, the PGCs become enclosed within a vesicle situated beneath the pericardial floor (**von Kennel**, 1885; **Anderson**, 1973). The paired vesicles containing PGCs subsequently fuse to form two longitudinal, dorsolateral tubes. Once PGCs enter the coelomic lumina of these tubes, they resume proliferation as oogonia or spermatogonia (**von Kennel**, 1885; **Anderson**, 1973). The posterior ends of the developing gonads then join the paired genital ducts (gonoducts), which extend within the pre-anal segments and later differentiate into the deferent ducts in males or the oviducts in females. The terminal somite pair of the anal region forms male-specific accessory structures —the anal glands— which are absent in females (**von Kennel**, 1885; **Anderson**, 1973).

Although early or near-prenatal onset of male sexual maturity has been noted in a few species, developmental studies on the male reproductive system remain remarkably scarce, and no detailed analyses exist for Andean Peripatidae, creating significant gaps in our understanding of how and when males become reproductively competent. This lack of comparative data limits our ability to evaluate how reproductive modes, ecological context, and phylogenetic history influence the onset of gametogenesis and the differentiation of male reproductive structures. Studying these processes in an Andean Peripatidae species offers a valuable opportunity to clarify the developmental mechanisms underlying male sexual maturity, address long-standing research gaps, and refine our understanding of reproductive diversity within Onychophora.

Methodology

Species taxonomic identity

Today, we lack a comprehensive taxonomic revision of Andean Onychophora. This population was previously identified as *Macroperipatus geayi*; however, *M. geayi* was originally described from a single specimen (**Bouvier**, 1905), without the explicit designation of a holotype, resulting in uncertainty regarding its taxonomic identity. Furthermore, the species name has been subject to orthographic inconsistencies in the literature (**Jerez-Jaimes & Bernal-Pérez**, 2009; **de Sena de Oliveira *et al.***, 2012), and *Macroperipatus geayi* is currently regarded as a *nomen dubium* (**de Sena de Oliveira *et al.***, 2023), precluding an unequivocal taxonomic assignment. More recently, studies have investigated key reproductive traits associated with its placentotrophic viviparity, referring to it as *Undescribed Species 1* (**Treffkorn *et al.***, 2019). Available morphological and genetic evidence suggests that the Andean population analyzed in this study may correspond to an undescribed Peripatid genus and species (**Hernández-Lagos**, 2018). Because the taxonomic identity of this population is unresolved and no formal generic assignment exists, we refer to the specimens exclusively by their collection locality. No additional onychophoran species have been recorded at this site, reducing the likelihood of species overlap within the sampled material.

Specimen collection and tissue processing

Specimens were manually collected from leaf litter and decaying logs in the municipality of Los Santos, on the western slopes of the Colombian Andes Cordillera Oriental (Santander Department, Colombia; 07°00’52.86’’ N, 72°53’48.05’’ W; 2300 m a.s.l.). A broad range of body sizes was obtained for both females and males. Live specimens were transported to the laboratory and maintained in a terrarium placed inside a wine cooler at 16°C, following the husbandry conditions described by **Hernández-Lagos** (2014). Individuals were euthanized by immersion in a lethal 2% v/v lidocaine solution. Body length and width were measured using a digital caliper (0.02 mm precision), and sex was determined under a stereomicroscope following the criteria of **Sunnucks *et al.*** (2000) and **Hernández-Lagos** (2014).

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As in other Peripatidae, this species exhibits placentotrophic viviparity and superfetation, enabling the study of embryogenesis without the need for large sample sizes. In the largest gravid females, two distinct regions of the oviduct are evident. The proximal portion of the ascending oviduct contains the earliest developmental stages: morulae, gastrulae, and chambered embryos in segmentation stages that remain attached to the oviductal wall via a stalk. In contrast, the distal chambers, primarily within the descending oviduct, contain more advanced embryos that lie free within the lumen (**Figure S1A, B,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). From these females, we obtained embryonic series as well as neonates fixed immediately after parturition. Additionally, males representing a wide range of body sizes collected in the field were included in the study. All specimens were fixed in 10% v/v buffered formalin and subsequently transferred to 70% v/v ethanol for long-term storage.

Macroscopic analysis of the male reproductive tract

Embryos were examined macroscopically without dissection due to their small size. Neonates and field-collected individuals spanning the observed body size range (14–50 mm in length) were dissected ventrally, from the genital opening to the mouth, using microsurgical scissors. Observations and photographic documentation of the reproductive organs were conducted under a stereomicroscope (Nikon® SMZ-1000) to characterize the reproductive system across developmental stages. The reproductive tracts were removed from the hemocoelic cavity and placed in Petri dishes for detailed examination, description, and imaging of their component structures. Morphological descriptions were compared with previously published accounts for both onychophoran families (**Brockmann *et al.***, 1999; **Mayer & Tait**, 2009; **Storch & Ruhberg**, 1990; **Storch *et al.***, 1995). Selected photographs were used to prepare schematic illustrations of the female and male reproductive tracts following fixation. Images were captured using a Nikon® stereomicroscope equipped with a Canon EOS Rebel XS® digital camera.

Histological analysis

Once the reproductive tracts of females and males were removed, each tract was subdivided according to its constituent organs. In gravid females, early embryonic chambers were dissected together with the corresponding portion of the oviduct in which they were enclosed, whereas advanced, free embryos were extracted and fixed independently of the oviduct. Tissue samples and whole-body preparations were dehydrated through a graded ethanol series, cleared in xylene, and embedded in Paraplast (McCormick®). Transverse sections (5–7 μm thick) were obtained and stained with hematoxylin–eosin (H–E) for general morphological assessment. For histochemical characterization of glycoproteins, we used periodic acid–Schiff (PAS), Alcian blue (AB) at pH 2.5, and Masson's trichrome stain (MT), following **Luna** (1968). We examined the prepared slides using a Nikon® H-550S microscope to document the structure of gonads and associated ducts and to determine their reproductive condition. Adult males were considered reproductively active when spermatozoa were present within the seminal vesicle and efferent duct, and spermatophore formation was evident along the deferent duct.

The anatomical and histological terminology applied in the description of embryonic chambers and oviductal structures follows the conventions established for other Neotropical Peripatidae, including *Epiperipatus acacioi* (**Campiglia & Walker**, 1995; **Walker & Campiglia**, 1998), *E. biolleyi* (**Brockmann *et al.***, 1999), *E. edwardsii* (**von Kennel**, 1885), *E. imthurni* (**Sclater**, 1887), *E. trinidadensis* (**Anderson & Manton**, 1972), *Macroperipatus torquatus* (**von Kennel**, 1885; **Anderson & Manton**, 1972), and *Plicatoperipatus jamaicensis* (**Huebner & Lococo**, 1994). For the male reproductive system, comparative terminology was drawn from descriptions representing both extant families, including *Peripatus sedgwicki* (**Storch & Ruhberg**, 1990), *Epiperipatus biolleyi* (**Storch *et al.***, 1995), and *Opisthopatus cinctipes* (**Storch & Ruhberg**, 1977).

Results

Five gravid females were dissected (**Figure S1A,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425), which ranged from 32 to 70 mm in length. From these females, embryos occupying the embryonic chambers of the ascending oviduct during early development (stages I–VI according to **Walker & Tait**, 2004) showed no visible evidence of gonadal differentiation. Embryos with evident gonads were only observed from stage VII onward. Seven embryos at advanced developmental stages (stage VII, non-pigmented embryos, and pigmented embryos) were studied. As usual, at stage VII of development (≈5 mm body length), organogenesis is advanced, the papillae on the body surface are fully developed, and the claw morphology of each lobopod is evident (**Figure S2A,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425); likewise, the genital opening is located ventrally between the penultimate pair of legs. At this stage of development, the embryos occupy the most distal portion of the ascending oviduct, and the body may appear bent, partially occupying the terminal portion of the ascending oviduct and the anterior portion of the descending oviduct. Here, the embryonic stages we analyzed corresponded to this final stage of the ascending oviduct, as well as to the most advanced embryos (partially, ≈10 mm of body length, or fully pigmented, ≈20 mm of body length) occupying the descending oviduct (**Figure S1A, B,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425).

We analyzed eight neonates and 20 field-collected males. Pigmented embryos (near-birth male embryos) measured 16–22 mm in body length, whereas male neonates measured 22–26 mm, and field-collected males ranged from 14 to 50 mm. Consequently, there was substantial size overlap among advanced embryos, neonates, and the series of field-collected males. The number of oncopod pairs ranged from 26 to 34 among the individuals analyzed.

Macroscopic description of the adult male reproductive tract

Males possess a reproductive tract consisting of a pair of testes and a pair of seminal vesicles with their associated efferent ducts, whereas the deferent duct and the ejaculatory duct are unpaired (**Figure 1A-C**) (**Figure S1C,** https://raccefyn.co/index.php/raccefyn/

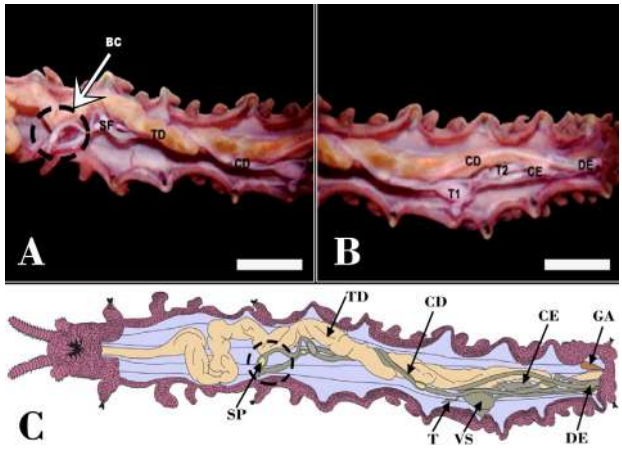

Figure 1. General morphology of the adult male reproductive tract. **A)** Anterior body region in the body cavity (BC); after longitudinal dissection, a loop (dotted line) of the vas deferens is observed, where it changes course toward the posterior region of the body. **B)** Posterior region of the body showing the paired structures (testes and efferent ducts), the vas deferens, and the ejaculatory duct. **C)** Diagram of the hemocoelic cavity of a male of the species showing the relative position of the digestive and reproductive tracts and their respective organs. The anal glands in the gonopore region are paired and parallel to the ejaculatory duct. CE: efferent duct; CD: vas deferens; DE: ejaculatory duct; GA: anal glands; SP: spermatophore; T: testis; TD: digestive tract; T1: testis one; T2: testis 2; VS: seminal vesicle

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article/view/4091/5425). Each testis is an elongate tubular structure, in some cases exhibiting slight folding (**Figure 2A**), which connects to the seminal vesicle through a narrow duct, and both organs are situated in the right posterolateral region of the alimentary canal within the hemocoelic cavity (**Figures 1C; 2B**). The seminal vesicle is oval and of greater diameter than the testis (**Figures 1C; 2B**). Posteriorly, each seminal vesicle joins its corresponding efferent duct (**Figures 1C; 2B**).

The efferent duct arises from the posterior region of the seminal vesicle and extends anteriorly, becoming progressively narrower along its course (**Figure 1C**) (**Figure S1C,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). The efferent ducts are convoluted and tortuous, and their distal portions fuse at the level of the seventh pair of oncopods to form the deferent duct (**Figure 2B, C**). The deferent duct is remarkably long, appearing to approximate the total body length of the individual (**Figures 1A-C, 2D**), and is divided into an ascending portion that runs anteriorly and a more distal descending portion that extends posteriorly to connect with the ejaculatory duct and gonopore (**Figure S1C,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). In the anterior region of the ascending portion, the duct forms a loop that redirects its course posteriorly (descending portion), and it is within this region that spermatophores become visible (**Figure 1A, C;**

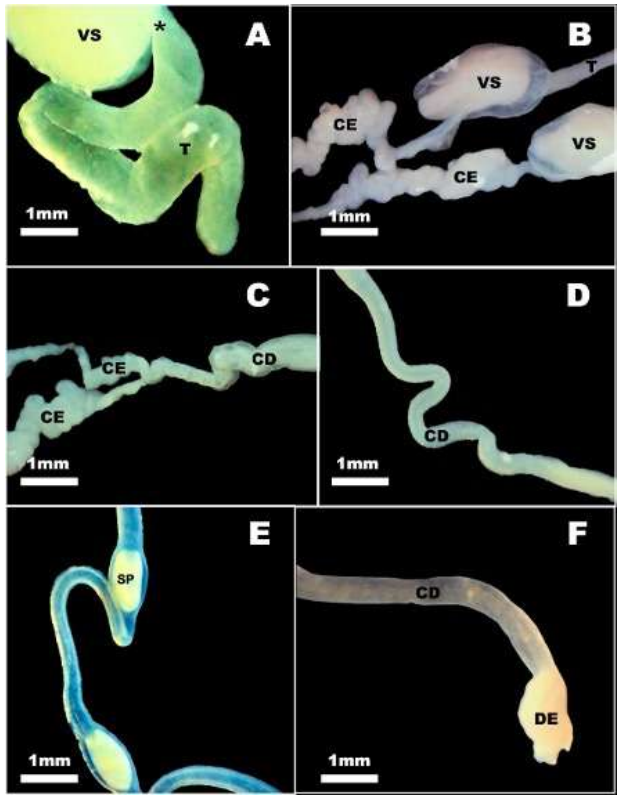

Figure 2. Stereoscopic photographs of the different organs comprising the reproductive tract of a reproductive male. **A)** Dorsal view of the testis, which is tubular and folded, and connected to the seminal vesicle through a narrow duct (asterisk). **B)** Lateral view of the seminal vesicle, with a larger diameter than the other organs of the tract. **C)** Lateral view of the efferent duct, which has a convoluted and tortuous shape. Toward the posterior region, both ducts fuse (asterisk) to give rise to the vas deferens. **D)** Dorsal view of the vas deferens, showing folds and a larger diameter compared to the efferent duct. **E)** Dorsal view of the descending portion of the vas deferens, showing two spermatophores (asterisks). **F)** Lateral view of the ejaculatory duct, which ends in a bulb-like structure toward its posterior-most region. T: testis; VS: seminal vesicle; CE: efferent duct; CD: vas deferens; SP: spermatophore; DE: ejaculatory duct

2E). Spermatophores occur within the lumen of the deferent duct in the anterior and middle regions of the descending portion; they are elliptical structures measuring approximately 1.1 mm in length (**Figure 2E**). Along the deferent duct, two to three spermatophores were observed in the 28 field-collected and in newborn individuals, indicating that neonates already possessed spermatophores within their deferent ducts.

The ejaculatory duct constitutes the most distal portion of the reproductive tract and opens externally at the genital opening, which is positioned at the twenty-eighth oncopod pair (**Figures 1C, 2F**). A pair of accessory glands is situated laterally to the gonopore; these glands are slender, tubular, and brownish in coloration, and they lie freely within the body cavity between the gonopore and the twenty-eighth oncopod pair (**Figure 1C**) (**Figure S1C,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). The spatial relationship of the reproductive tract and the alimentary canal varies across developmental stages, being ventral in advanced embryos and neonates and shifting to a lateral position in adults (**Figure 1A-C**) (**Figure S2B,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425).

Microscopic description

Testis. The testis exhibited a similar organization in embryos VII (**Figure S2B-D, Figure S3A;** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425), partially pigmented and pigmented embryos, neonates (**Figure S4A-B,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425), and field-collected individuals. They are surrounded by a thin layer of connective tissue, beneath which numerous germinal epithelial cells are present, consisting of spermatogonia and spermatocytes (**Figure 3**). Spermatogonia are polygonal cells with large, rounded nuclei containing conspicuous chromatin granules. Primary spermatocytes are large, rounded cells with large, irregular nuclei, and their chromatin is observed either in interphase or forming mitotic figures. Although both cell types appear intermingled throughout the tissue, primary spermatocytes are more abundant toward the periphery of the organ (**Figure 3A, B, D**). Both cell types were found in the testes of all studied individuals (**Table 1**).

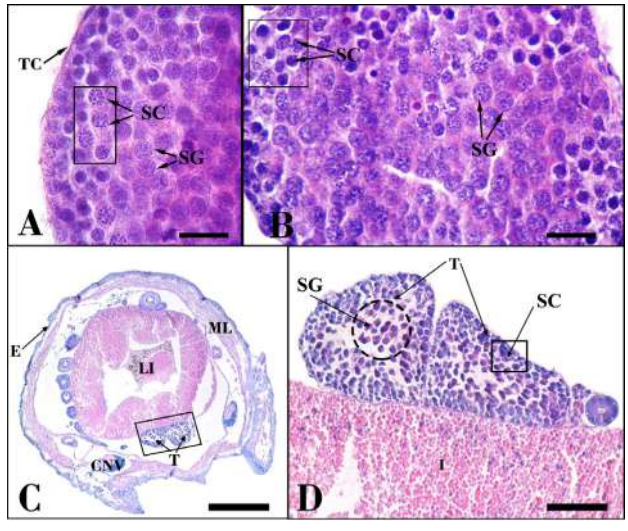

Figure 3. Testis histology**. A)** Cross-section of the testis of an adult male. **B)** Spermatogonia are dispersed in the center of the testis, while primary spermatocytes are in the peripheral region of the organ. **C)** Transverse section of the body of a pigmented male embryo. The testes (T) are located ventrally relative to the alimentary canal. **D)** Spermatogonia are observed in the central region of the testis and spermatocytes in the periphery. SG: spermatogonia; SC: spermatocytes; TC: connective tissue; E: ectoderm; CNV: ventral nerve cord; LI: intestinal lumen; ML: longitudinal musculature; T: testis. Scale bars: **A** = 50; **B** = 40 µm; **C** = 140 µm; **D** = 100 µm

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Table 1. Comparative progression of germline cells within the male reproductive tract across developmental stages

Germline cell type and reproductive organ	Stage VII embryo	Partially pigmented embryo	Fully pigmented embryo	Neonate	Adult
Testis (spermatogonia and spermatocytes)	Present	Present	Present	Present	Present
Seminal vesicle	Spermatogonia and spermatocytes	Spermatogonia, spermatocytes, and spermatids	All spermatogenic cell types	All spermatogenic cell types	All spermatogenic cell types
Efferent ducts with sperm in the lumen	Absent	Absent	Present	Present	Present
Spermatophores in the deferent duct	Absent	Absent	Absent	Present	Present

Seminal vesicle. The seminal vesicle is larger in diameter than the testis and is lined by a simple cuboidal luminal epithelium with acidophilic cytoplasm. Beneath the epithelium lies a thin layer of connective tissue containing collagen fibers, and externally, a delicate layer comprising scattered smooth muscle fibers (**Figure 4A;** in adults, and in pigmented embryos **Figure 5**). Within the lumen of the seminal vesicle in neonates (**Figure S4D,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425) and field-collected males (**Figure 4**) (**Figures S4C, D**; https://raccefyn.co/index.php/raccefyn/article/view/4091/5425), all germ-line cell types were present, including spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa (**Figure 4B–D**). These cells occurred dispersed and loosely arranged in the lumen, accompanied by an eosinophilic secretion. The seminal vesicle of the individuals examined contained the full spectrum of spermatogenesis and spermiogenesis, with spermatids at multiple maturation stages readily observed (**Figure 4D**). In the posterior region of the seminal vesicle, maturing spermatids and spermatozoa were more abundant. Spermatozoa were filiform and embedded in an eosinophilic secretion (**Figure 4C**). Therefore, spermatogonial mitosis appears to occur in the testis, whereas spermatogenesis and spermiogenesis take place within the seminal vesicle.

Advanced embryos at stage VII already exhibited evident testes and seminal vesicles (**Figure S2B-G,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). The testes were surrounded by a thin layer of connective tissue. Toward the innermost portion of this tissue, a germinal epithelium layer was observed, composed of spermatogonia and primary spermatocytes (**Figure S2C-D,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). Spermatogonia are polygonal in shape, with circular nuclei in which some chromatin granules are visible. Primary spermatocytes are large, rounded cells with irregular nuclei; the chromatin may be in interphase or forming mitotic figures. Within the testis, these two cell types were observed randomly distributed, with primary spermatocytes being more abundant than spermatogonia. The seminal vesicles are wider than the testes and are lined by a luminal epithelium composed of cuboidal cells (**Figure S2E-F,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). At this developmental stage, males lack spermatids and spermatozoa (**Table 1**); however, the presence of primary spermatocytes was high, and it was also observed in the testes.

In the advanced embryos of the descending oviduct, the seminal vesicle was morphologically similar to that of neonates and all field-collected males (**Figures 5B, C**). However, we observed variations in the cellular composition of the germinal epithelium in the more advanced embryonic stage, fully pigmented embryos (**Figure 5A**). In the lumen, the complete series of germ-line cells up to mature spermatozoa was present (**Figure 5D**), as

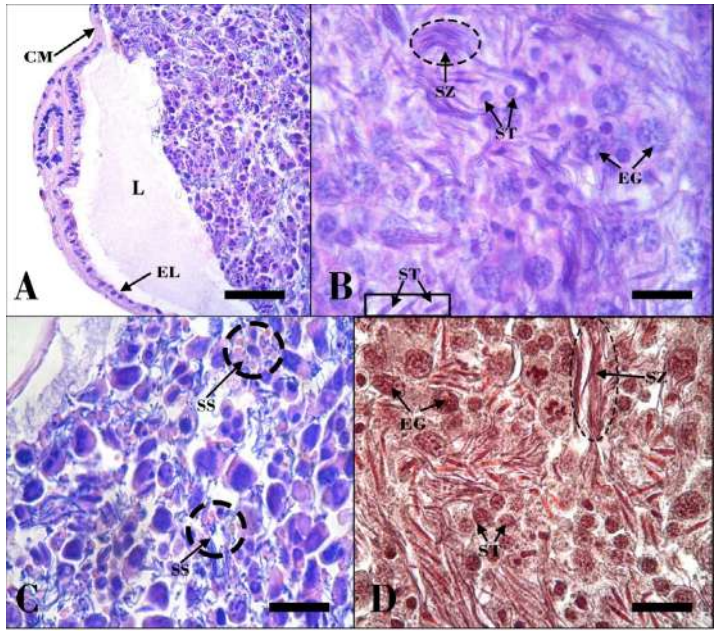

Figure 4. Seminal vesicle of an adult male. Transverse sections showing: **A)** The cuboidal luminal epithelium surrounded by a thin layer of connective tissue and muscle fibers. **B)** Spermatogenic epithelium in different stages of spermatogenesis and spermiogenesis. **C)** Toward the lumen of the seminal vesicle, secretions associated with germline cells are observed. **D)** Detailed view of germline cells in different stages, with a higher presence of spermatids and spermatozoa (trichrome staining). CM: muscle layer; L: lumen; EL: cuboidal epithelium; SG: spermatogonia; ST: spermatids; SZ: spermatozoa; SS: secretions. Scale bars: **A** = 50 µm; **B**, **C**, and **D** = 20 µm

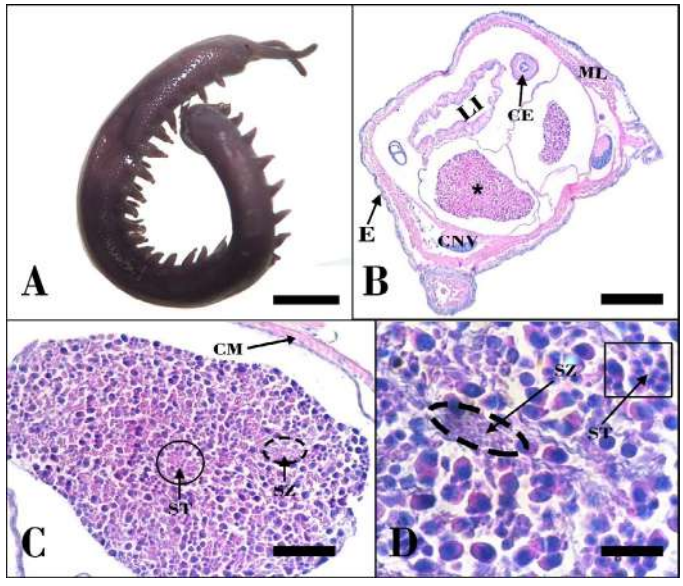

Figure 5. Transverse section of the body of **A)** a pigmented male embryo whose transverse section exhibits **B)** the large seminal vesicle (asterisk) in a ventral position relative to the alimentary canal (LI), and **C)** within its lumen, spermatogonia, spermatocytes, and early spermatids seen in detail. **D)** Toward the posterior region of the seminal vesicle, maturing spermatids and spermatozoa are observed. CE: ejaculatory duct; CM: muscle layer; CNV: ventral nerve cord; E: ectoderm; LI: intestinal lumen; ML: longitudinal musculature; ST: spermatids; SZ: spermatozoa. Scale bar: **A** = 1 mm; **B** = 140 µm; **C** = 40 µm; **D** = 20 µm

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also observed in neonates and adults. In slightly earlier embryos (only partially pigmented) (**Figure 6A**), gametes were absent; nevertheless, spermatogenesis and spermiogenesis were already underway (**Figure 6B, C**) (**Table 1**).

Accordingly, the earliest individual in which spermatozoa were detected in both the seminal vesicle and the efferent duct was an unborn, fully pigmented embryo measuring 19 mm in body length. This means that males are capable of sperm production while still within the maternal oviduct, and they are born already sexually mature.

Efferent duct. The efferent ducts of advanced embryos, neonates, and adults exhibit a simple cuboidal luminal epithelium with rounded basal nuclei and acidophilic cytoplasm. Beneath the epithelium lies a thin layer of connective tissue containing collagen fibers, which is externally surrounded by a delicate layer of smooth muscle fibers (**Figure 7A-D, F**). The lumen widens toward the posterior region. Within the lumen, abundant spermatozoa accompanied by eosinophilic secretion were observed in the anterior–middle portion of the efferent duct (**Figure S5A,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). In advanced embryos, the efferent duct showed a similar organization but with a narrower lumen (**Figure 7D**); fully pigmented embryos already contained spermatozoa (**Figure 7C,D**), whereas embryos at stage VII and partially pigmented embryos lacked luminal sperm (**Figure 7E-F**) (**Figure S3B,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425) (**Table 1**).

Deferent duct. Deferent ducts in neonates and field-collected males were bounded externally by four to five layers of striated muscle fibers, followed internally by a thin layer of loose connective tissue containing collagen fibers and a simple columnar luminal epithelium composed of tall cells with basal nuclei and eosinophilic cytoplasm. In the anterior portion of the duct, an intensely eosinophilic secretion was observed in the lumen (**Figure 8A, B**) (**Figures S4A, B; S5,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). In the mid-posterior portion of the duct, spermatophores were present within the lumen. The luminal epithelium appeared distended to accommodate the spermatophore, resulting in a noticeable reduction in epithelial height (**Figure 8C, D**). Spermatophores are large, oval structures consisting of a thin, acellular, eosinophilic wall and an internal matrix filled with spermatozoa embedded within a paler, vacuolated eosinophilic secretion (**Figure 8E**) (**Figure S4A, B,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425).

In contrast to neonates and field-collected males, the deferent ducts of advanced embryos were surrounded by two to three layers of smooth muscle and lined by a simple columnar epithelium with basophilic basal nuclei (**Figure S3C,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). As in the other stages, an eosinophilic secretion was present within the lumen of the anterior portion of the duct (**Figure 9A, B**); however, spermatophores were not observed in any embryonic specimen (**Table 1**).

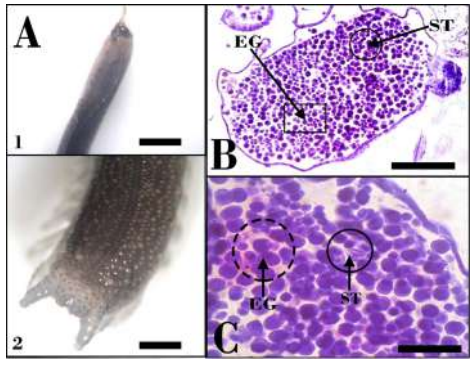

Figure 6. Partially pigmented embryo showing (**A**) the anterior region (above) and the posterior region (below); **B)** the transverse section of the seminal vesicle, where germline cells are observed, except for spermatozoa; **C)** the posterior region of the seminal vesicle showing maturing spermatids. EG: spermatogonia; ST: spermatids. Scale bar: **A** 1 = 1 mm, 2 = 1 mm; **B** = 30 µm; **C** = 10 µm

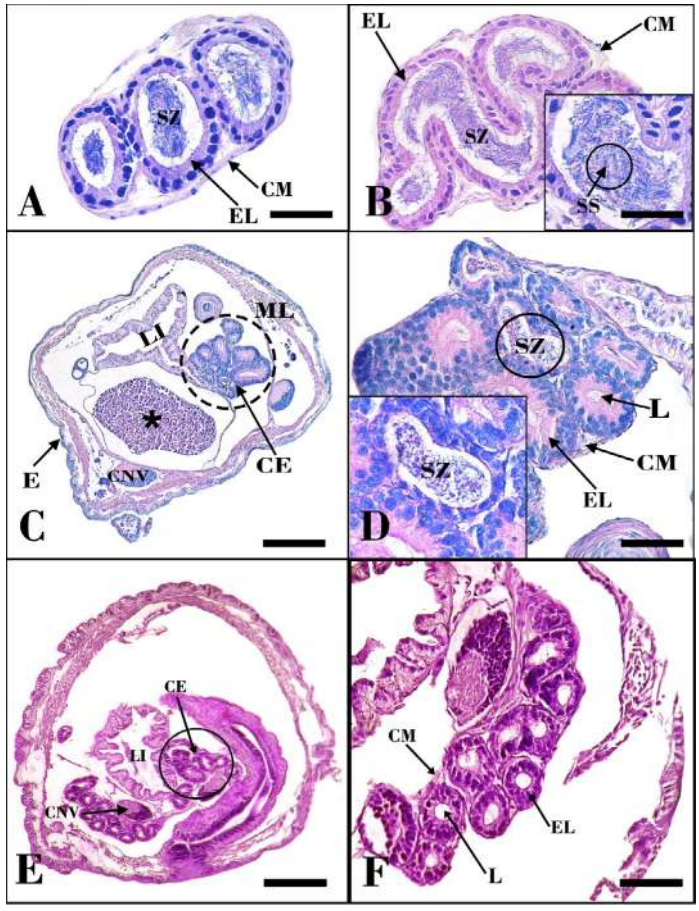

Figure 7. Transverse section of the efferent duct in an adult male. **A)** Cuboidal luminal epithelium surrounded by a muscle layer; spermatozoa are observed toward the lumen, accompanied by a pink secretion. **B)** In the posterior portion of the efferent duct, the lumen is distended and shows a higher concentration of spermatozoa together with an eosinophilic secretion. **C)** Transverse section of a pigmented fetus; the efferent ducts are located ventrally relative to the alimentary canal. **D)** The luminal epithelium is cuboidal, and spermatozoa are already observed toward the lumen (inset). **E)** Transverse section of partially pigmented embryos; the efferent ducts are located ventrally relative to the alimentary canal. **F)** Absence of spermatozoa in the luminal region. *: seminal vesicle; CE: efferent duct; CM: muscle layer; CNV: ventral nerve cord; E: epidermis; EL: luminal epithelium; L: lumen; LI: intestinal lumen; ML: longitudinal musculature; SZ: spermatozoa. Scale bar: **A** and **B** = 30 µm; **C** = 140 µm; **D** = 30 µm; **E** = 140 µm; **F** = 30 µm.

The deferent ducts of advanced embryos, neonates, and field-collected males were PAS-positive along the luminal border of the epithelium, indicating the presence of neutral carbohydrates (**Figure 10**). Alcian blue staining was positive in the spermatophore wall and the granular matrix surrounding the spermatozoa, which signals carboxylated mucopolysaccharides (**Figure 10A, B**).

Ejaculatory Duct. The ejaculatory duct consists of a simple cuboidal to columnar luminal epithelium, underlain by a thin layer of connective tissue containing collagen fibers, and surrounded by a very thick layer composed of several bands of striated muscle (**Figure 11A, B, E, F**) (**Figure S3D, S5C,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425). The lumen of this duct contains a strongly eosinophilic secretion that is weakly basophilic along the luminal epithelial margins and devoid of spermatozoa. In advanced embryos, the morphology is similar (**Figure 11C, D**) (**Figure S3D,** https://raccefyn.co/index.php/raccefyn/article/view/4091/5425.

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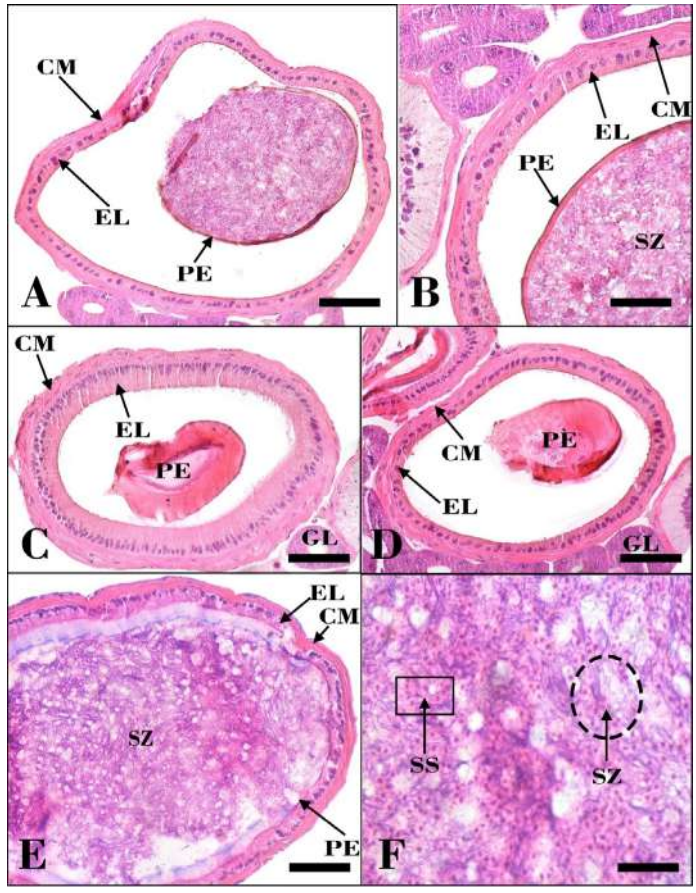

Figure 8. Transverse sections of the deferent duct in a newborn male. **A)** Presence of an eosinophilic secretion in the lumen; columnar epithelium surrounded by a thin layer of connective tissue and a muscle layer. **B)** The luminal epithelium is distended toward the posterior portion of the duct. Eosinophilic luminal secretion is seen. **C)** and **D)** A spermatophore sheath wall is observed within the lumen, with spermatozoa present inside it. **E)** The spermatophore completely occupies the luminal space. **F)** Interior of the spermatophore sheath showing a high concentration of spermatozoa accompanied by a vacuolar eosinophilic secretion. CM: muscle layer; EL: luminal epithelium; GL: anal glands; PE: spermatophore sheath wall; SZ: spermatozoa; SS: secretion accompanying spermatozoa. Scale bars: **A** = 50 µm, **B** = 40 µm; **C** and **D** = 30 µm; **E** = 40 µm; **F** = 20 µm

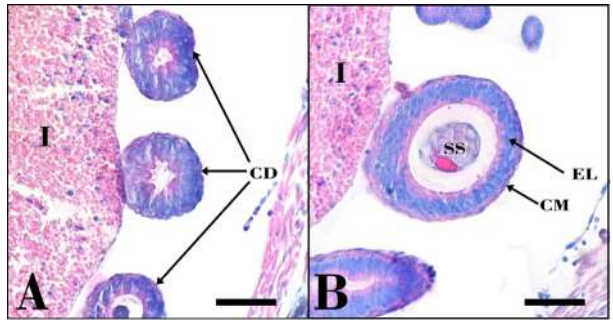

Figure 9. Transverse sections of the mid-body region of a pigmented male embryo. **A)** The deferent duct is located ventrally relative to the alimentary canal. **B)** Deferent duct showing luminal secretion in the mid-posterior region. CD: deferent duct; CM: muscle layer; EL: cuboidal epithelium; I: alimentary canal; SS: eosinophilic secretion. Scale bars: **A** = 40 and **B** = 30 µm

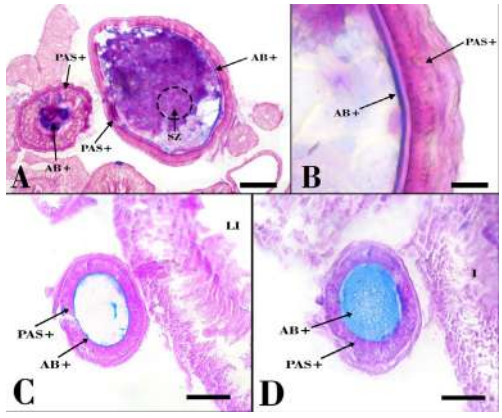

Figure 10. Deferent duct with spermatophore sheath in a reproductive male. Periodic acid–Schiff (PAS), Alcian Blue (AB) (see Methods). **A)** Deferent duct PAS-positive for the border of the luminal epithelium and for the secretion associated with spermatozoa. **B)** AB-positive reaction for the spermatophore sheath wall. **C)** and **D)** PAS- and AB-positive reactions in the anterior and posterior portions of the deferent duct for the spermatophore sheath in neonates. LI: intestinal lumen; I: intestine. Scale bars: **A** = 30, **B** = 20 µm; **C** and **D** = 30 µm

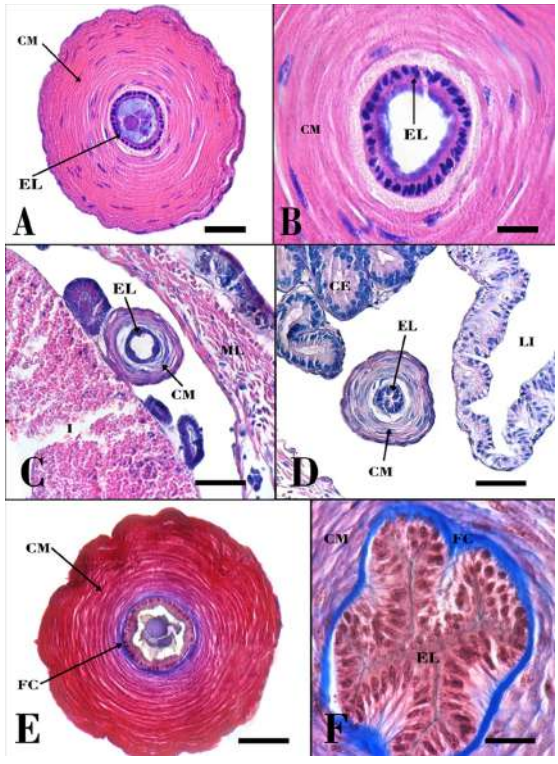

Figure 11. Transverse section of the ejaculatory duct in males. **A)** Luminal epithelium surrounded by connective tissue and followed by a thick layer of skeletal muscle in an adult male. **B)** Detail of the cuboidal to columnar luminal epithelium and a very dense layer of skeletal muscle. **C)** Transverse section showing the anterior portion of the ejaculatory duct in embryos; layers of skeletal muscle are observed. **D)** In the posterior portion, the characteristic cruciform shape of the genital opening is observed. Transverse section of the ejaculatory duct (trichromic staining: MT). **E)** Transverse section of the ejaculatory duct in an adult male with MT staining. The luminal epithelium is surrounded by connective tissue containing collagen fibers and a dense layer of skeletal muscle fibers. **F)** Luminal epithelium in the most distal portion of the duct, showing the typical cruciform shape of the gonopore opening. CM: muscle layer; EL: luminal epithelium; CE: efferent duct; FC: collagen fibers; LI: intestinal lumen. Scale bars: **A** = 40 µm and **B** = 10 µm; **C** and **D** = 30 µm; **E** = 40 µm; **F** = 10 µm

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Discussion

The male reproductive tract of the species under study exhibited an organization broadly consistent with that described for other Peripatidae, such as *Epiperipatus biolleyi* (**Storch *et al.***, 1995) and *Peripatus sedgwicki* (**Storch & Ruhberg**, 1990). As reported for these taxa, the testes, seminal vesicles, and efferent ducts showed positional variability within the hemocoelic cavity, with a tendency to be laterally displaced in larger males (**Campiglia & Walker**, 1995). This contrasts with Peripatopsidae, whose efferent duct is proportionally longer and functions as the primary site of spermatophore formation (**Pflugfelder**, 1968; **Storch *et al.***, 2000), whereas the deferent duct is comparatively short. In Peripatidae, mating involves alignment of the posterior oncopods and direct deposition of the spermatophore onto the female gonopore via the ejaculatory duct (**Lavallard & Campiglia**, 1975; **Walker & Campiglia**, 1998). Peripatopsidae, in contrast, display a wider diversity of insemination mechanisms, most of them intradermal and involving a copulatory organ or cephalic dorsal structures (**Tait & Norman**, 2001; **Curach & Sunnucks**, 1999).

Testicular histology corresponded closely to that reported for other Peripatidae and Peripatopsidae (**Ruhberg & Storch**, 1976). The testes represent the site of mitosis and early spermatogenesis, whereas completion of spermatogenesis, spermiogenesis, and spermiation occur in the seminal vesicle, as described for *Epiperipatus biolleyi* (**Storch *et al.***, 1995). While spatial dissociation of mitotic and meiotic phases (compartmentalization) occurs in some invertebrates, certain polychaetes, for example (**Daly**, 1974), with mitosis occurring in specialized zones (e.g., germinal sacs) and meiosis proceeding elsewhere (e.g., coelom), this is not a universal or recurring pattern across all invertebrate groups. Likewise, many mollusks exhibit distinct proliferative and meiotic territories within ramified gonads (**Li *et al.***, 2022). Insects maintain a mitotic germarial niche proximally and meiotic–spermiogenic regions distally along the testis tubule (**Koçakoğlu *et al.***, 2019; **Lago *et al.***, 2020). Together, these comparisons highlight the recurrent evolutionary solution of separating stem-cell proliferation from sperm maturation. Nevertheless, most of the invertebrates retain both processes (mitosis and meiosis) within the testis proper, and the seminal vesicle and the vas deferens are only involved in storage and transport of mature sperm (**Roosen-Runge**, 1977). In this sense, the seminal vesicle served as a storage or modification site in all surveyed groups, not as the primary location for spermatogenesis. Onychophorans are exceptional in having complete spermatogenesis in a different organ, the seminal vesicle.

While several studies describe embryonic development processes in Peripatidae and related Onychophora —particularly for body segments, nephridia, and uterine structures (**Walker**, 1995; **Walker & Campiglia**, 1988; **Mayer & Koch**, 2005; **Campiglia & Walker**, 1995; **Mayer *et al.***, 2015)— there remains a notable gap regarding the specific compartmentalization of reproductive organs such as the testes and seminal vesicles. Our study did not include earlier developmental stages that could clarify the development of both organs and how the compartmentalization is established for spermatogenesis. Further targeted research is therefore needed to address these aspects in Onychophora.

The efferent ducts exhibited a histological organization comparable to that of previously studied species (**Storch & Ruhberg**, 1976; **Storch *et al.***, 1995). Spermatozoa occurred freely within the lumen and did not form spermatophores at this site, differing from *Opisthopatus cinctipes*, whose sperm cells are embedded in a developing spermatophore (**Storch *et al.***, 2000). The eosinophilic and vacuolated secretions within the seminal vesicle lumen were characteristic of Onychophora (**Willey**, 1898; **Camatini *et al.***, 1979b) and originated from maturing spermatocytes (**Storch & Ruhberg**, 1977). These secretory droplets coalesced into masses that migrated toward the efferent and deferent ducts and contributed to spermatophore wall formation, as also described for *Paraperipatus* and *Austroperipatus* (**Tait & Briscoe**, 1990). In the anterior deferent duct, PAS and Alcian Blue positivity indicates mucopolysaccharides and acidic carbohydrates involved in spermatophore construction and possibly sperm nutrition, as suggested

by **Storch *et al.*** (1995) and **Sherbon & Walker** (2004). Notably, males of some *Paraperipatus* species lack these secretory zones and instead retain sperm freely within the tract (**Ruhberg**, 1985).

The spermatophores observed displayed an ellipsoidal morphology comparable to that of Neotropical Peripatidae (**Walker & Campiglia**, 1998; **Sherbon & Walker**, 2004) but differed from the forms described for Asian Peripatidae (*Typhloperipatus*, *Eoperipatus*) (**Kemp**, 1914) and the multilayered spherical spermatophores characteristic of several Peripatopsidae (**Pflugfelder**, 1968; **Storch & Ruhberg**, 1977). This diversity across Onychophora is tightly associated with variation in sperm-transfer modes (**Walker & Tait**, 2004; **Elliot *et al.***, 1993).

The ejaculatory duct consisted of a secretory epithelium surrounded by collagen fibers and a thick layer of striated muscle, a configuration consistent with Peripatidae that enables voluntary control during copulation (**Campiglia & Walker**, 1995; **Storch *et al.***, 1995). In Peripatopsidae, genera such as *Paraperipatus* and *Austroperipatus* rely on a penis-like structure to transfer sperm (**Ruhberg**, 1976; **Tait & Briscoe**, 1990).

Pigmented embryos contained spermatozoa within the seminal vesicles and efferent ducts, a pattern previously reported for *Plicatoperipatus jamaicensis* and *Oroperipatus eisenii* (**Havel *et al.***, 1989; **Rucker**, 1900). This has been interpreted as evidence for extremely early male sexual maturation, consistent with **Ghiselin**'s (1974) hypothesis of the "male-dispersion strategy", which favors early reproduction over somatic growth.

Across invertebrates, early male sexual maturation has evolved repeatedly, often as an adaptive response to life-history constraints or intense reproductive competition. For instance, in cephalopods such as *Loligo pealeii*, the small, sneaker males complete spermatogenesis and produce functional spermatophores earlier than the consort males (**Marian *et al.***, 2005). In insects, *Drosophila melanogaster* males finish spermatogenesis during late pupation and eclose with a full complement of mature sperm (**Seong *et al.***, 2023). These convergent patterns highlight the evolutionary significance of accelerated male reproductive development and help contextualize the unusually early maturity reported in Peripatidae. However, existing research on invertebrates indicates that final spermatogenesis —the production of mature, functional sperm— does not occur during larval or embryonic stages. Rather, the formation of mature sperm is consistently observed during pupal or adult development. The occurrence of final spermatogenesis during late embryogenesis in some onychophorans would therefore represent a unique condition among invertebrates.

All examined males, starting from the pigmented embryos, exhibited spermatogenesis and spermiogenesis in their seminal vesicles; from neonates onward, spermatozoa were found in the efferent ducts along with spermatophores in the deferent duct, indicating that once males initiate sperm production, it is likely continuous throughout their lifetime. Variation in male body size was evident, with embryos equaling or surpassing the length of newborn males. Previously, other authors have proposed that such variation correlates with maternal body size, as larger females tend to produce larger offspring (**Bouvier**, 1915; **Cuénot**, 1949). Maternal provisioning and developmental plasticity likely shape intrauterine growth and might modulate the timing of reproductive maturation.

Recent phylogenomic studies have improved the resolution of relationships within Onychophora (**Baker *et al.***, 2021) and expanded the comparative framework. A global checklist (**de Sena Oliveira *et al.***, 2023) and increased species discovery (**Trewick**, 2024) further highlight the importance of evaluating reproductive variation across taxa. The draft genome of *Epiperipatus broadwayi* (**Sato *et al.***, 2023) provides a new basis for exploring the molecular pathways, for example, underlying spermatophore formation and reproductive secretions —topics historically understudied in velvet worms (**Mayer**, 2007; **Monge-Nájera *et al.***, 1993).

Significant gaps remain in our understanding of male reproductive maturation and highly disbalanced sex ratios in Onychophora. Despite isolated reports of early or near-prenatal sexual maturity in a few species, there are no broad comparative studies

examining the morphology or histology of the male reproductive tract across embryonic, neonatal, and juvenile stages. Quantitative data on the prevalence of early spermatogenesis are lacking, and the relationship between reproductive mode —ranging from oviparity to various forms of viviparity— and the timing of male maturity has not been systematically evaluated. Furthermore, hypotheses linking early maturation to ecological pressures, sperm competition, or life-history strategies remain largely untested, underscoring the need for integrative, multi-species analyses.

Supplementary information

See the supplementary information in https://raccefyn.co/index.php/raccefyn/article/view/4091/5425

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Author contributions

This study partially contributed to FFG's undergraduate thesis. NHD and MPRP: conception, documentation, and study design; FFG: histological work, analysis, image preparation, and documentation; FFG and NHD: results review and analysis; FFG and NHD: drafting of the initial manuscript; NHD and MPRP: review and editing of the manuscript. MPRP: project resources and supervision. All authors reviewed and approved the final version of the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Artificial Intelligence Use declaration

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