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Research Article

Reproductive system of the insular yellow crab *Johngarthia lagostoma* (H. Milne Edwards, 1837) (Brachyura: Gecarcinidae): from production to the storage of seminal fluid

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Abstract

This study describes the reproductive systems of male and female *Johngarthia lagostoma*, a land crab endemic to South Atlantic oceanic islands, focusing on spermatozoa production and storage. Specimens from Trindade Island (Brazil) were analysed for anatomy, histology, and histochemistry. The male system includes a pair of tubular testes showing different stages of spermatogenesis and spermiogenesis, leading to mature spermatozoa. These move to the anterior vas deferens (AVD), which has proximal and distal portions, the latter containing coenospermic spermatophores surrounded by secretion type I, reactive to proteins and acidic and neutral polysaccharides. The median (MVD) and posterior (PVD) vas deferens produce type II (strongly protein-reactive) and type III (weakly protein-reactive) secretions. Accessory glands between the MVD and PVD produce the same secretions plus a secretion type IV, reactive to neutral polysaccharides. These mix with the spermatophores and other vas deferens secretions, increasing the PVD's secretion volume, crucial for the initial release of spermatophores into the seminal receptacle. The female reproductive system features voluminous seminal receptacles connected to the ovary, comprising mesodermal and ectodermal regions classified as ventral-type connection. Plugs and sperm packets are absent, with the seminal receptacles filled with free spermatozoa, suggesting dehiscence occurs shortly after sperm transfer. The influence of male and female secretions on this process is suggested, alongside their roles in sperm maintenance and fertilization facilitation.

Introduction

Reproduction in Brachyura is modulated by a series of factors, ranging from life history aspects and complex reproductive strategies to peculiar behaviours in courtship and mating. In this context, numerous morphological characteristics have evolved within the group, contributing to its reproductive success (see McLay and Becker, 2015). The morphology of the reproductive system of some Brachyura species has already been described in the literature, but the vast majority have focused on Heterotremata crabs, with few studies on Podotremata and Thoracotremata (see Antunes et al., 2016; Assugeni *et al.*, 2021; Becker *et al.*, 2011; Castilho *et al.*, 2008; Diesel 1989; Erkan *et al.*, 2009; Garcia-Bento *et al.*, 2019; Nascimento and Zara, 2013; Shinozaki-Mendes *et al.*, 2011; Simeó *et al.*, 2009; Souza *et al.*, 2013; Zara *et al.*, 2012).

The male reproductive system of brachyurans is bilateral, H-shaped, located in the cephalothorax, and consists of a pair of testes, vasa deferentia and ejaculatory ducts (Watanabe *et al.*, 2022). Histologically, the testes of brachyurans can be classified as lobular, which is the common pattern recorded for most crabs, although they can also be tubular, as described for some species of Grapsoidea MacLeay, 1838, Majoidea Samouelle, 1819 and Xanthoidea MacLeay, 1838 (Nagao and Munehara 2003; Simeó *et al.*, 2009). Spermatozoa produced in the testes, once mature, are transported to the vas deferens, which is typically divided into three regions: anterior (AVD), median (MVD), and posterior (PVD) (Krol *et al.*, 1992). In the AVD, seminal fluid is produced, which is related to the aggregation of spermatozoa, leading to the formation of coenospermic spermatophores (with several spermatozoa), which is the most common type observed in Eubrachyura, in contrast to cleistospermic spermatophores (with a single sperm), more common in freshwater crabs (Anilkumar *et al.*, 1999; Klaus and Brandis, 2011; Tiseo *et al.*, 2014). The other two regions (MVD and PVD) are responsible for the storage of spermatozoa, as well as the production of additional seminal fluid (Adiyodi and Anilkumar, 1988; Beninger *et al.*, 1988; Johnson 1980; McLay and Becker, 2015). Besides, in some species, these regions may present accessory glands, ceca, diverticula, or outpockets, which function to produce secretions for the formation of spermatic layers or packages (Majoidea), or sperm plugs (Portunoidea) (Diesel, 1989; Zara *et al.*, 2012, 2014; Antunes et al., 2018; Watanabe *et al.*, 2022).

Among female eubrachyuran crabs, the reproductive system may have an expansion of the oviduct, leading to an internal connection with the ovary, where sperm storage and fertilization occur, called the seminal receptacle (SR), with a dorsal mesodermal region and a ventral ectodermal region, continuous with the vagina and vulva, whose opening is mesial on the sixth thoracic segment (McLay and López-Greco, 2011). The connection of the oviduct with the SR can differ according to the eubrachyuran species (Diesel, 1991; McLay and López-Greco, 2011), ranging from a dorsal position (opposite to the insertion with the vagina) to a ventral position, juxtaposed to the vagina, still in the mesodermal region (Assugeni and Zara, 2022; McLay and Becker, 2015). This expansion and connection of the ovary with the oviduct do not occur in Podotremata crabs, where the sperm storage structure, the spermatheca, is exclusively ectodermal, surrounded by cuticle, and derived from the 7th and 8th thoracic segments, forming a suture line on the female thoracic sternum (Garcia-Bento *et al.*, 2019; Guinot *et al.*, 2013).

Female Eubrachyura store sperm until fertilization (McLay and Becker, 2015; McLay and López-Greco, 2011), with the maintenance cost supported by female secretions (see Adiyodi and Anilkumar, 1988; Beninger *et al.*, 1993; McLay and Becker, 2015). In contrast, male secretions can influence the process of sperm competition, possibly having a gel-like consistency (Antunes *et al.*, 2016; Diesel, 1991; Ryan, 1967) or hardening into a seminal plug (Assugeni *et al.*, 2021; Zara *et al.*, 2014, 2012). Gel-like secretions surround the spermatic package, acting to displace ejaculates from previous matings (Antunes *et al.*, 2016), while the seminal plug externally occludes the vulva opening in females, preventing other males from introducing their gonopods and transferring their genetic material (Hartnoll, 1969; Hines *et al.*, 2003; Zara *et al.*, 2014). Moreover, the combined secretions from males and females may have other functions, such as aiding in spermatophore dehiscence, protecting gametes (e.g., bacteriostatic properties), and supporting anaerobic sperm metabolism (Beninger *et al.*, 1993; Jeyalectumie and Subramoniam, 1991; Sant'Anna *et al.*, 2007).

In Gecarcinidae MacLeay, 1838, few studies describe aspects of the morphology or histochemistry of the reproductive system, particularly concerning *Cardisoma guanhumi* Latreille in Latreille, Le Peletier, Serville & Guérin, 1828 (see Shinozaki-Mendes *et al.*, 2011, 2012; Souza *et al.*, 2013). Nevertheless, the available studies are relatively superficial, especially concerning the male reproductive system, resulting in considerable morphological gaps that hinder meaningful comparisons with other species of the family. Additionally, within this family, different selective pressures act on continental species (e.g., genus *Cardisoma* Latreille, 1828) and insular species (e.g., genera *Gecarcoidea* H. Milne Edwards, 1837, *Gecarcinus* Leach, 1814, and *Johngarthia* Türkay, 1970), which have contrasting life histories (Guinot *et al.*, 2018; Marin and Tiunov, 2023; Pkl *et al.*, 2008). To date, no descriptions of the male and female reproductive systems of insular gecarcinids exist, except for the external morphology of the gonads and their use in studies of sexual maturity (Hartnoll *et al.*, 2017; João *et al.*, 2021). Among the insular species, the genus *Johngarthia* stands out, encompassing six endemic species of oceanic islands: *Johngarthia malpilensis* (Faxon, 1893), *Johngarthia planata* (Stimpson, 1860*), Johngarthia cocoensis* Perger, Vargas and Wall, 2011, and *Johngarthia oceanica* Perger, 2019 in the Eastern Pacific; *Johngarthia lagostoma* (H. Milne Edwards, 1837) in the Central-West Atlantic; and *Johngarthia weileiri* (Sendler, 1912) in the Eastern Atlantic (Perger *et al.*, 2011; Pkl *et al.*, 2008).

Johngarthia lagostoma (H.Milne Edwards, 1837), commonly known as the yellow crab, has its global distribution restricted to four small islands in the Atlantic Ocean: Rocas Atoll, Fernando de Noronha, Ascension, and Trindade. In Brazil, this species is categorized as 'Endangered' (EN) by the IUCN criteria due to its reduced range of occurrence, declining habitat quality, and the introduction of exotic species (Pinheiro *et al.*, 2016). The literature on *J. lagostoma* is still scarce, focusing on its reproductive dynamics (Hartnoll *et al.*, 2010; João *et al.*, 2022, 2021; Mosna et al., 2025), larval development (Colavite *et al.*, 2021; Lira *et al.*, 2021), population structure (Hartnoll *et al.*, 2009, 2006; João *et al.*, 2023a), and evolution (Hartnoll *et al.*, 2017; João *et al.*, 2023b). However, studies are still needed to describe the morphology of the male and female reproductive systems, facilitating a better understanding of its reproductive history.

This study describes the male and female reproductive systems of *J. lagostoma*, analysing their anatomy, histology, and histochemistry to elucidate the production and storage of seminal fluid and spermatozoa, aiming to understand the coevolution of reproductive systems in this important island crab. Studies on the reproduction of endangered species are crucial for developing conservation strategies (Pinheiro *et al.*, 2016). Therefore, the results of this study are key to advancing reproductive knowledge of *J. lagostoma*, which belongs to one of the most terrestrial brachyuran families on our planet (Marin and Tiunov, 2023).

Materials & methods

Specimens of *J. lagostoma* ($n = 10$) were manually collected during expeditions to Trindade Island (Brazil) from February to April 2019 and from April to June 2022. Five specimens of each sex were obtained, with carapace width (CW) sizes exceeding physiological maturity (CW ≥ 56 mm), as estimated by João *et al.*, (2022). In the laboratory, the animals were weighed (WE, total weight) using a precision balance (0.01 g) and measured with a digital caliper (0.01 mm). Subsequently, the specimens were anesthetized by cooling and then dissected. The reproductive systems of each sex were fixed in 4% paraformaldehyde solution in 0.2 M phosphate buffer in seawater. The samples were kept in this fixative until the end of the expedition and transported to the laboratory.

In the laboratory, the materials were subjected to three 30-minute washes in 0.2 M sodium phosphate buffer (pH 7.2) and then photographed under a stereomicroscope for anatomical descriptions. The samples were subsequently dehydrated in an ascending series of ethanol concentrations (70–95%) and embedded in Leica® glycol methacrylate historesin. Serial sections of 4–7 μm thickness were obtained using a rotary microtome.

For general histological description, the slides were stained with hematoxylin and eosin (Junqueira and Junqueira, 1983). For histochemistry, the slides were subjected to the following techniques: Xylidine Ponceau for proteins (Mello and Vidal, 1980); periodic acid-Schiff (PAS) for neutral polysaccharides; and Alcian Blue for acidic polysaccharides (Junqueira and Junqueira, 1983). To better visualize different stages of spermiogenesis, testicular slides were treated with the PAS technique combined with hematoxylin (Junqueira and Junqueira, 1983).

Results

Five males ranged in carapace width (CW) from 91.3 to 99.6 mm (mean ± SD: 95.0 ± 3.4 mm) and in body weight (WE) from 259.4 to 342.6 g (305.9 ± 33.5 g). Similarly, five females ranged in CW from 81.6 to 94.3 mm (90.5 ± 5.1 mm) and in WE from 235.6 to 259.4 g (246.7 ± 10.1 g).

Male reproductive system

Anatomy

The male reproductive system of *Johngarthia lagostoma* (Figure 1) exhibits bilateral symmetry with an 'H'shape, consisting of a pair of testes. These are located on the upper margin (right and left) of the cephalothorax, connected to the vasa deferentia, which extend longitudinally over the hepatopancreas, ending at the posterior region of the body. The testis is highly convoluted (Figure 1A and B), starting at the anterior periphery of the cephalothorax and traversing the central region of the cephalothoracic cavity, where this paired organ joins through a commissure located just below the intestine.

The vas deferens was divided into three distinct regions: anterior (AVD), median (MVD), and posterior (PVD) (Figure 1A). The AVD is a highly convoluted, slender tubular structure (Figure 1B). The MVD is long, slightly convoluted, with a smooth surface and a wider diameter compared to the AVD (Figure 1C). At the transition between the MVD and PVD, tubular accessory glands with multiple branches are observed (Figure 1D). The PVD appears as a single slender tube, slightly convoluted and smooth like the MVD, positioned just after the accessory glands (Figure 1E).

Histology and histochemistry

The testes of *J. lagostoma* are of the tubular type (Figure 2A), showing cells at different stages of spermatogenesis (Figure 2B–E) and spermiogenesis (Figure 2F–H). Generally, spermatogonia are located at the periphery of the seminiferous tubule (Figure 2B), forming the germinative zone, with cells displaying large basophilic nuclei. Primary and secondary spermatocytes are mainly observed in the maturation zone. Primary spermatocytes have large nuclei in different stages of meiotic prophase (Figure 2C–D), while secondary spermatocytes show small nuclei with homogeneous chromatin (Figure 2E). Spermiogenesis begins with early spermatids that display rounded basophilic nuclei and an acrosomal vesicle stained with PAS (Figure 2F). In the final stages, spermatids have more reduced crescent-shaped nuclei, while the acrosomal vesicle appears more heterogeneous, strongly stained with the PAS-H technique, and positioned opposite the nucleus (Figure 2G). Mature spermatozoa are located in the evacuation zone, displaying slender nuclei seemingly surrounded by the acrosomal vesicle with its central portion, the perforatorial chamber, showing weaker staining for neutral polysaccharides (Figure 2H).

Mature spermatozoa are transported to the vas deferens, where in the AVD, due to the presence or absence of the spermatophore wall compacting sperm masses, this region was divided into two parts – proximal (AVDp) and distal (AVDd). Both parts exhibit simple cuboidal epithelium (Figure 3A–B). In the lumen of the AVDp, a large mass of free spermatozoa immersed in basophilic secretion is observed (Figure 3B), slightly positive for proteins (Figure 3C) and for neutral and acidic polysaccharides (Figure 3D–E, respectively), termed secretion type I (SI). Additionally, at the periphery of the AVDp, another type of acidophilic secretion is noted, termed secretion type II (SII). SII is strongly reactive for proteins (Figure 3C), positive for neutral polysaccharides (Figure 3D), and negative for acidic polysaccharides (Figure 3E). In the AVDd, SII becomes thicker and starts enveloping the spermatozoa, forming the spermatophore wall (Figure 3F–G). The spermatophore wall is basophilic (Figure 3G–H), strongly proteinaceous (Figure 3I), and positive for neutral (Figure 3J) and acidic polysaccharides (Figure 3K). Inside the spermatophores, the spermatozoa are immersed in SI, which remains basophilic and slightly positive for proteins, neutral, and acidic polysaccharides. SII, which surrounds the spermatophores, retains the same chemical composition observed in the AVDp (Figure 3F–K).

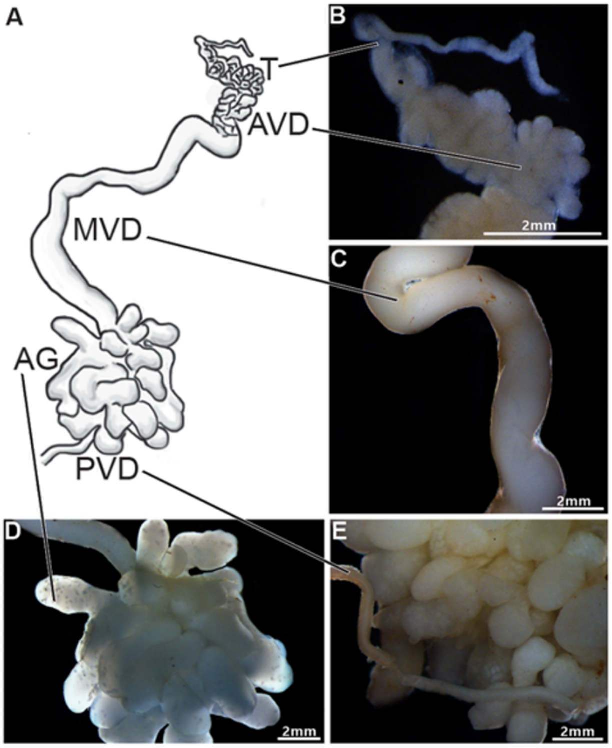

Figure 1. Anatomy of the male reproductive system of Johngarthia lagostoma. (A) Diagram of the male reproductive system (right side), showing the testis (T), different regions of the vas deferens (AVD, anterior; MVD, middle; and PVD, posterior), and the accessory glands (AG); (B) Detail of the testis and AVD, both convoluted regions; (C) Detail of the MVD, longer and less convoluted; (D) Detail of the accessory glands, highly branched tubular structures; (E) Detail of the PVD, which channels the various branches of the accessory glands into a single tubule.

In the MVD, the epithelium is simple squamous (Figure 4A). SII, located around the spermatophores, may serve as a structural matrix that supports the deposition of a new granular or globular secretion (SIII) (Figure 4B). SII remains acidophilic (Figure 4A–B), strongly proteinaceous (Figure 4C), weakly reactive for neutral polysaccharides (Figure 4D), and acidic polysaccharides (Figure 4E). SIII shows basophilic granules, weakly positive for proteins, neutral polysaccharides, and positive for acidic polysaccharides (Figure 4B–E). The chemical composition of the secretions from the wall and inside the spermatophores is identical to that of

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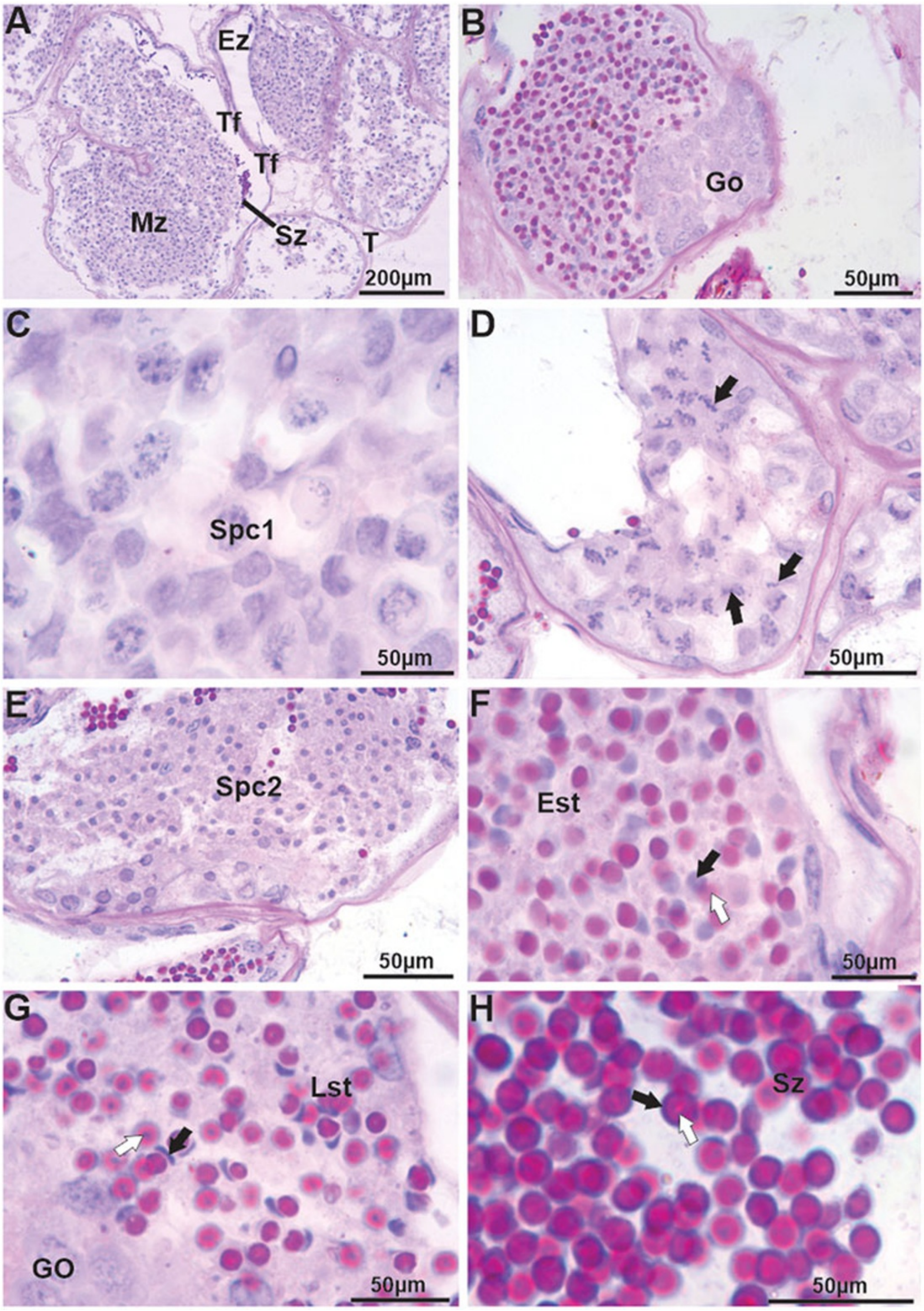

Figure 2. Histology of the testes, vas deferens, and germ cells (spermatogenesis and spermiogenesis) in *Johngarthia lagostoma*. (A) Tubular testes with visible spermatozoa in the seminiferous tubule; (B) Spermatogonia located at the periphery of the seminiferous tubule (germinal zone). These cells have large basophilic nuclei; (C) and (D) Detail of spermatocytes I with nuclei at different stages of meiotic prophase (black arrows); (E) Spermatocytes II consisting of small, homogeneous nuclei; (F) Beginning of spermiogenesis with early spermatids exhibiting basophilic, round nuclei (black arrow) and an acrosomal vesicle stained by PAS (white arrow); (G) Final stage of spermatid with the nucleus in a crescent shape (black arrow), with a more heterogeneous acrosomal vesicle positioned opposite the nucleus (white arrow); (H) Mature spermatozoa in the evacuation zone, with basophilic nuclei (black arrow) and a heterogeneous acrosomal vesicle stained by PAS (white arrow). Staining: PAS and hematoxylin. Est, early spermatids; Ez, evacuation zone; Go, spermatogonia; Lst, late spermatid; Mz, maturation zone; Spc1, spermatocytes I; Spc2, spermatocytes II; Sz, spermatozoa; Tf, seminiferous tubule.

the AVDd (Figure 4B–E). At the transition between the MVD and PVD, tubular accessory glands with branches open into the lumen. These glands produce a large amount of secretion, named secretion type IV (SIV), which is released through a merocrine mechanism, since there is no apical accumulation or epithelial dilation, nor goblet cell–like release or holocrine disintegration. SIV exhibits slightly basophilic spherical to elliptical granules (Figure 4F–G), weakly positive for proteins (Figure 4H), positive for neutral polysaccharides (Figure 4I), and negative for acidic polysaccharides (Figure 4J).

In the PVD, just after the glands and in the ejaculatory duct, the seminal fluid in the duct (spermatophores, SII, and

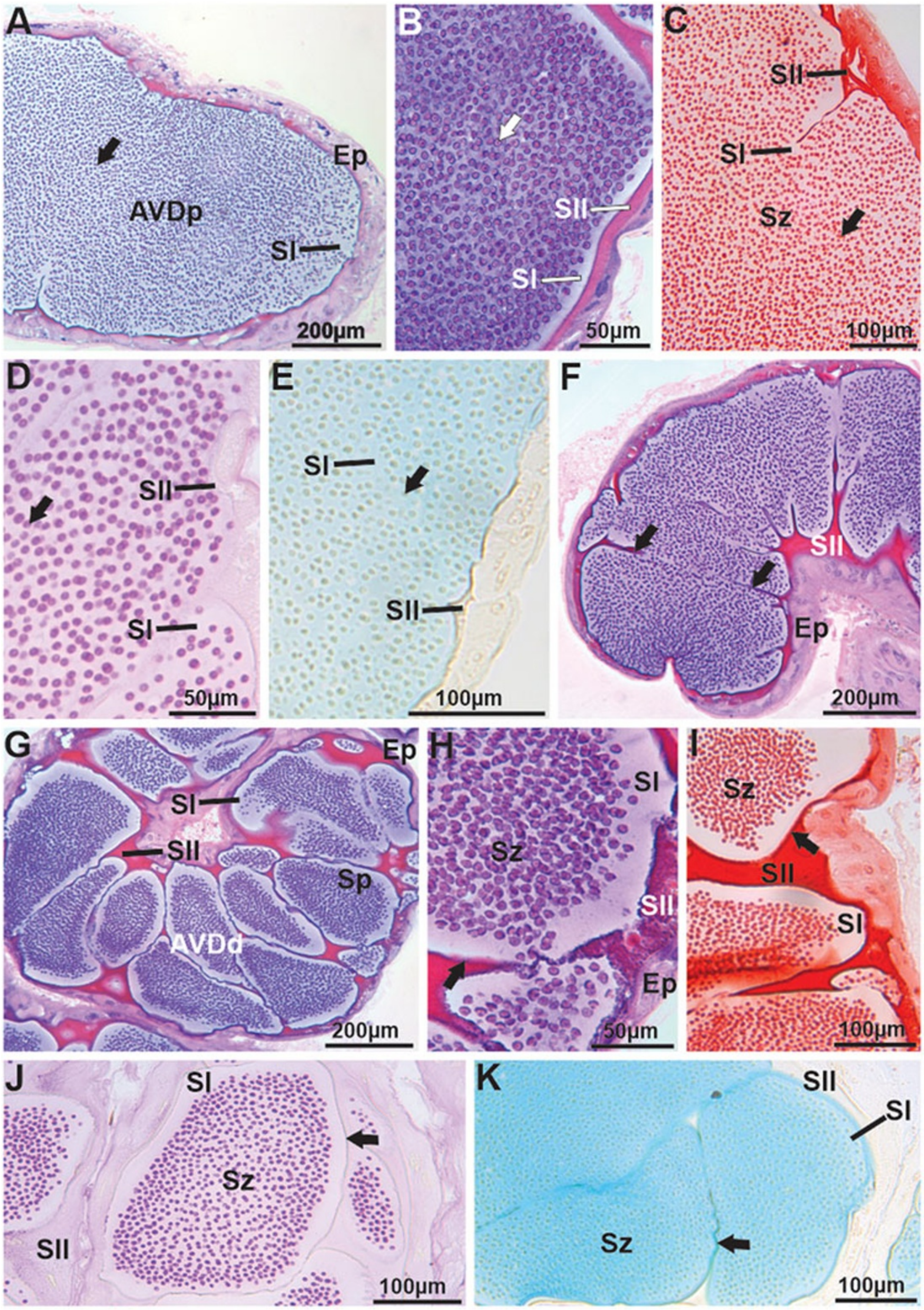

Figure 3. Histology and histochemistry of the anterior vas deferens (AVD) in *Johngarthia lagostoma*. (A) Proximal region of the anterior vas deferens (AVDp) in HE, with the lumen filled with free spermatozoa (arrow) immersed in type I secretions. (B) Detail of the AVDp in HE, showing free spermatozoa (arrow) and basophilic type I and acidophilic type II secretions. (C) Detail of free spermatozoa (arrow) in the AVDp, with secretions subjected to Xylidine ponceau (Secretion one: slightly positive; Secretion two: strongly positive for proteins). (D) and (E) AVDp subjected to PAS and Alcian blue (detection of neutral and acidic polysaccharides, respectively), presenting free spermatozoa (arrow). Note that secretion one is slightly positive for both stains, while secretion two is slightly positive for PAS and negative for Alcian blue. (F), (G), and (H) Distal region of the vas deferens (AVDd) in HE, showing the formation of spermatophores with spermatozoa. Secretion one remains basophilic, secretion two acidophilic, and the wall of the coenospermic spermatophores (arrow) is basophilic. (I) AVDd with the wall of the spermatophores (arrow) strongly proteic when subjected to Xylidine ponceau. (J) and (K) Spermatophores with walls (arrows) positive for neutral and acidic polysaccharides, stained with PAS and Alcian blue, respectively. Ep, simple cuboidal epithelium; Sp, spermatophores; Sz, spermatozoa; SI, secretion one; SII, secretion two.

SIII), along with the SIV from the glands, mix (Figure 5A–H). SIII and SIV are positive for neutral polysaccharides when stained with PAS (Figure 5E–F), while for acidic polysaccharides, SIV is slightly negative, and SIII is positive (Figure 5G–H). The SII, originating from the AVDp, remains non-reactive for acidic polysaccharides (Figure 5G–H). Additionally, the number of spermatophores in the PVD is clearly lower than in the MVD, and they are more dispersed among the secretions, with greater spacing between the spermatophores themselves.

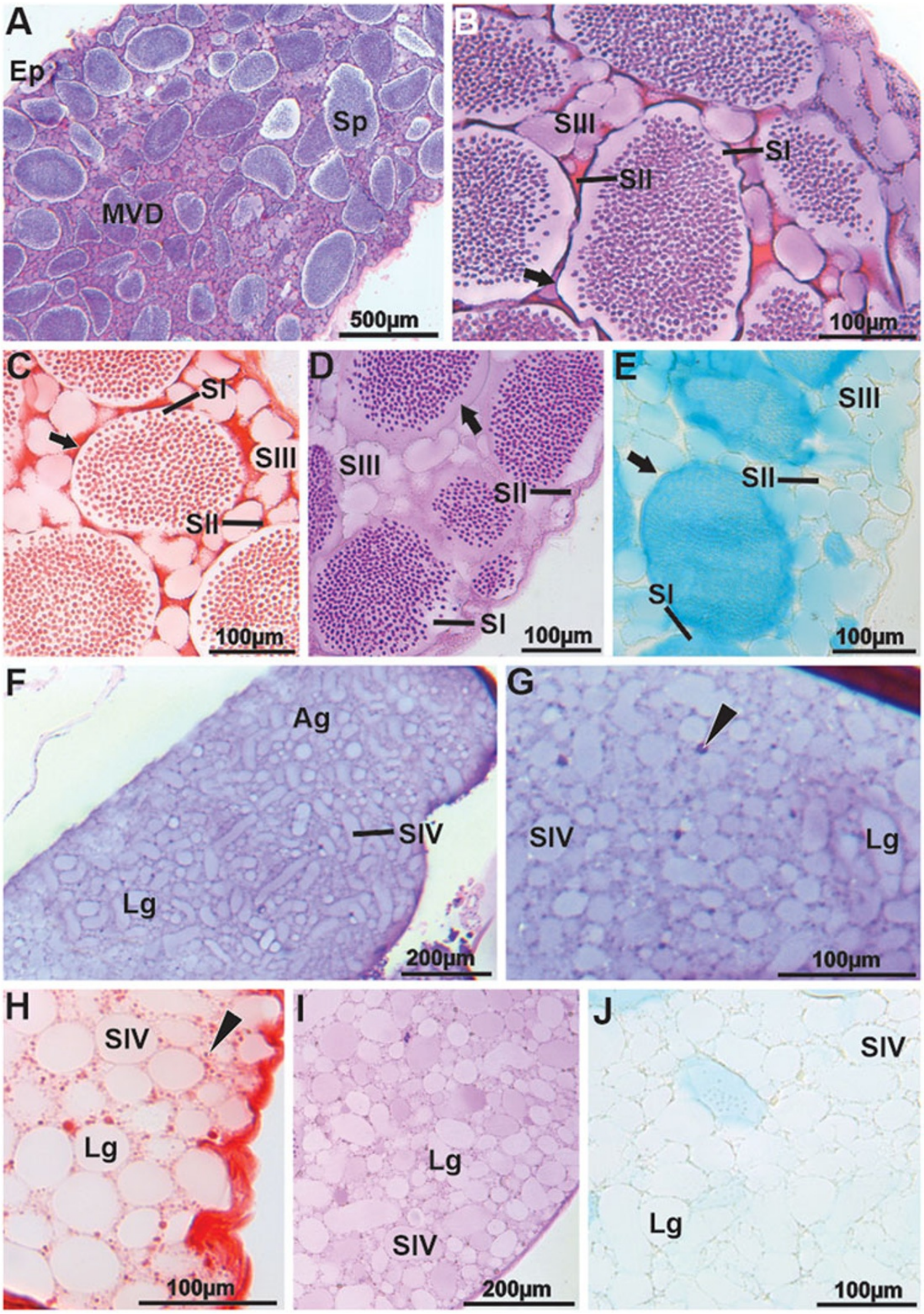

Figure 4. Histology and histochemistry of the middle vas deferens (MVD) and accessory glands (AG) in *Johngarthia lagostoma*. (A) General view of the MVD in HE, showing a large quantity of spermatophores immersed in secretion. (B) Detail of the MVD stained with HE. Note the spermatophores with basophilic walls (arrow), surrounded by acidophilic secretions one and two, which form the matrix for secretion three, containing basophilic granules. (C) In Xylidine ponceau, secretion two is strongly positive, and secretion three shows slight reactivity to the stain. The arrow points to the spermatophore wall. (D) and (E) MVD subjected to PAS and Alcian blue, respectively. The spermatophore wall (arrow) and secretions two and three are weakly reactive to the stains. (F) and (G) General view and detail of the accessory gland in HE, showing large basophilic granules that make up secretion four and small portions of secretion two (head of the arrow). (H) Granules of secretion four show weak positivity when subjected to Xylidine ponceau. Notice that secretion two is more strongly stained for proteins (head of the arrow). (I) and (J) Secretion four with granules weakly positive for neutral and acidic polysaccharides when stained with PAS and Alcian blue, respectively. Ag, accessory gland; Ep, simple squamous epithelium; Lg, basophilic granules; Sp, spermatophores; SI, secretion one; SII, secretion two; SIII, secretion three; SIV, secretion four.

Female reproductive system

Anatomy

In *J. lagostoma*, the female reproductive system consists of a concave-type vagina, a pair of transversely paired ovaries connected to a pair of SRs through an oviduct located adjacent to the vagina, which is considered a ventral-type receptacle (Figure 6A–C). The vagina forms a dilated structure, with one of its faces collapsed against the other and associated with longitudinal

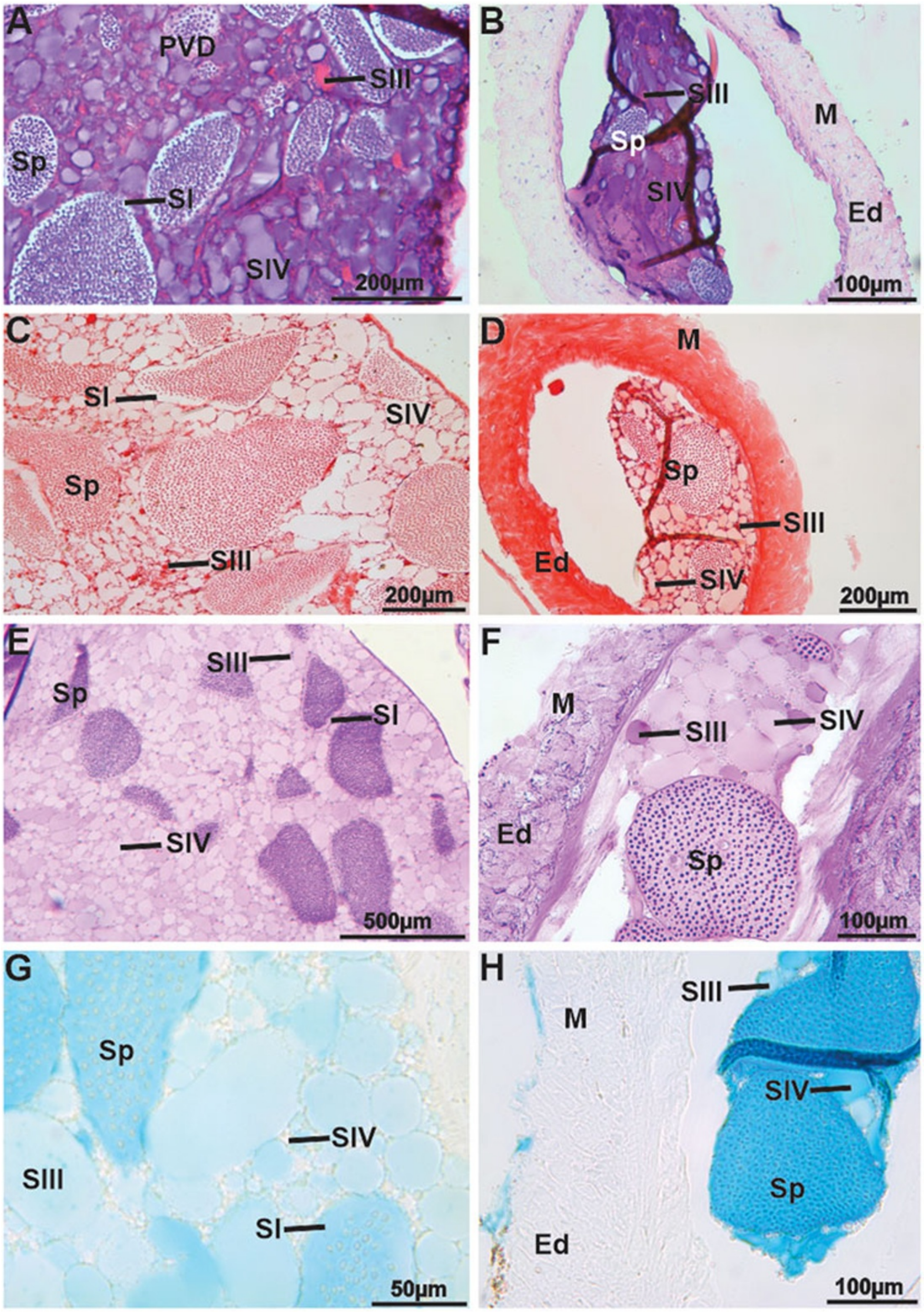

Figure 5. Histology and histochemistry of the posterior vas deferens (PVD) and ejaculatory duct (ED) in Johngarthia lagostoma. (A) and (B) PVD and ejaculatory duct in HE, showing a mixture of secretions from the vas deferens and accessory glands. Note the more spaced spermatophores, secretion types of SI and SIV (basophilic), and type SIII (acidophilic). (C) and (D) PVD and ejaculatory duct, respectively. In Xylidine ponceau, all secretions are weakly positive for proteins. (E) and (F) PVD and ejaculatory duct, showing that SIV becomes more positive with PAS. (G) and (H) PVD and ejaculatory duct, exhibiting SIV less positive for acidic polysaccharides when subjected to Alcian blue. Ed, ejaculatory duct; M, muscle bundles; Sp, spermatophores; SI, secretion one; SII, secretion two; SIII, secretion three; SIV, secretion four.

musculature attached to the opposite face of the oviduct opening, both in the ventral region and along one of the vagina's margins (Figure 6A–B). The vagina opens at the vulva (gonopores), which exhibit one of their faces protruding from the external surface (Figure 6A and 6D–E). In all females studied, the interior of the seminal receptacles was filled with a large amount of secretion; however, the SR did not have a rigid consistency (Figure 6E).

Histology and histochemistry

Histological sections confirmed the classification of the seminal receptacle as ventral-type, with the oviduct opening in the dorsal mesodermal region, near the transition area with the ventral ectodermal region (Figure 7A–C).

The dorsal region of the seminal receptacle is composed of a simple columnar or prismatic epithelium, seated on connective tissue forming the collagenous layer, containing many fibroblasts

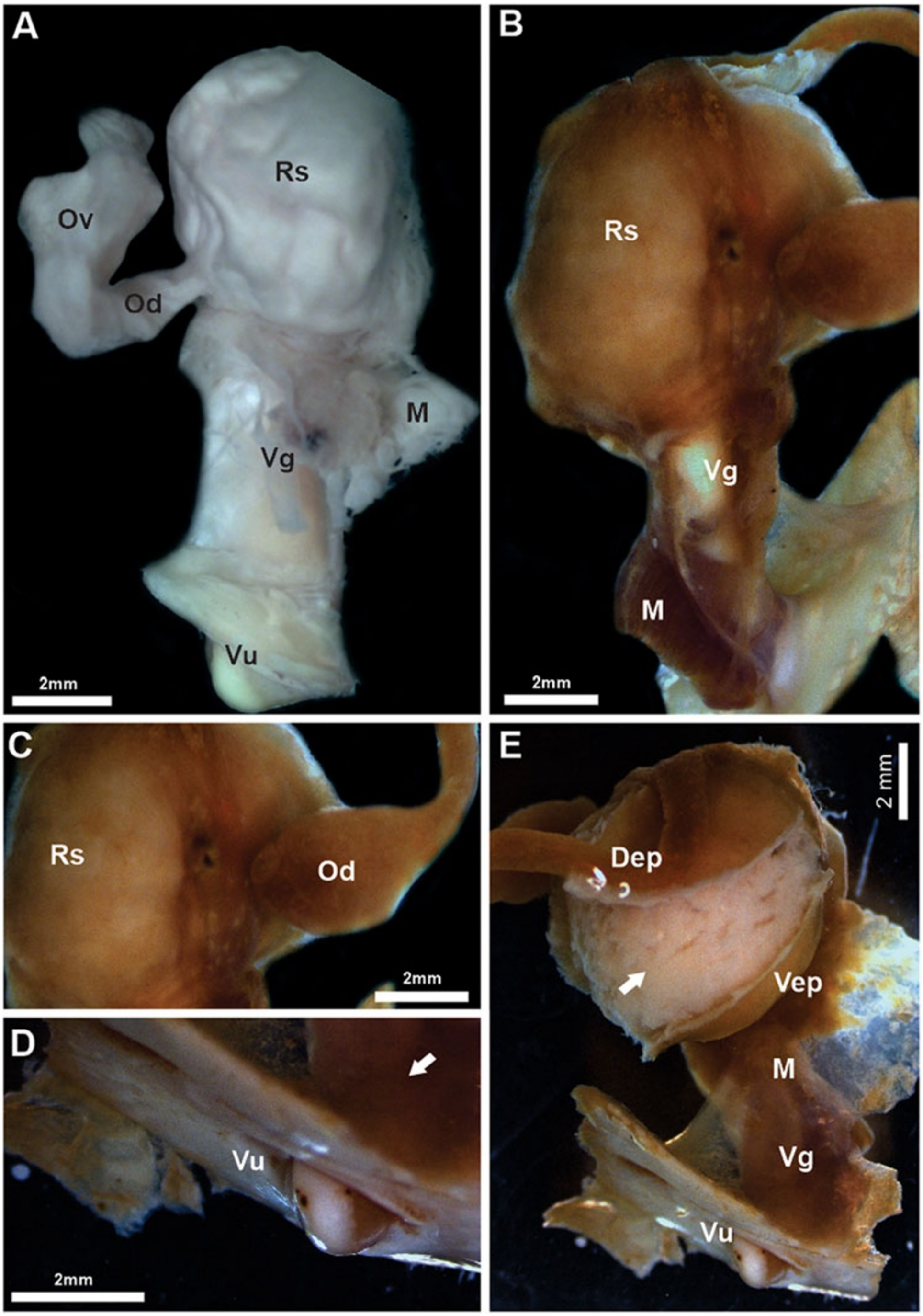

Figure 6. Anatomy of the female reproductive system (seminal receptacle) in *Johngarthia lagostoma*. (A) General view of the ventral-type seminal receptacle, with connection to the ovary through the oviduct. (B) Vagina with one face collapsed onto the other, showing longitudinal muscle attached to the oviduct opening. (C) Connection of the seminal receptacle to the oviduct. (D) Vagina opening into the vulva (arrow), near the gonopores. (E) Interior of the receptacles after rupture of the dorsal and ventral epithelium, showing a large amount of secretion, but with no rigid consistency (arrow). Dep, dorsal epithelium; G, gonopore; M, longitudinal musculature; Rs, seminal receptacle; Od, oviduct; Ov, ovary; Vep, ventral epithelium; Vg, vagina; Vu, vulva.

as well as collagen fibres (Figure 7D–F). Additionally, the epithelial cells of the dorsal region have cytoplasmic secretory material at the apical pole, producing an appearance of different heights in the cells. The secretory vesicles have acidophilic areas with a basophilic central core, possibly released by a merocrine mechanism (Figure 7D–E).

The dorsal region of the seminal receptacle ends abruptly at the epithelium of the ventral region, which is covered by a thin cuticular layer at this point (Figure 7F).

The ventral region of the seminal receptacle, particularly opposite the oviduct opening, is characterized by the presence of numerous voluminous folds with cells formed by simple epithelium resting on the collagenous layer, which in some areas is anchored to muscle fibres (Figure 7F–I). The ventral region is continuous with the vagina, which is obliterated, with one face meeting the opposite margin of the vagina, producing a C-shaped appearance, classified as concave-type. Thus, the vaginal lumen is significantly reduced (Figure 7A and I). The vaginal epithelium has the same

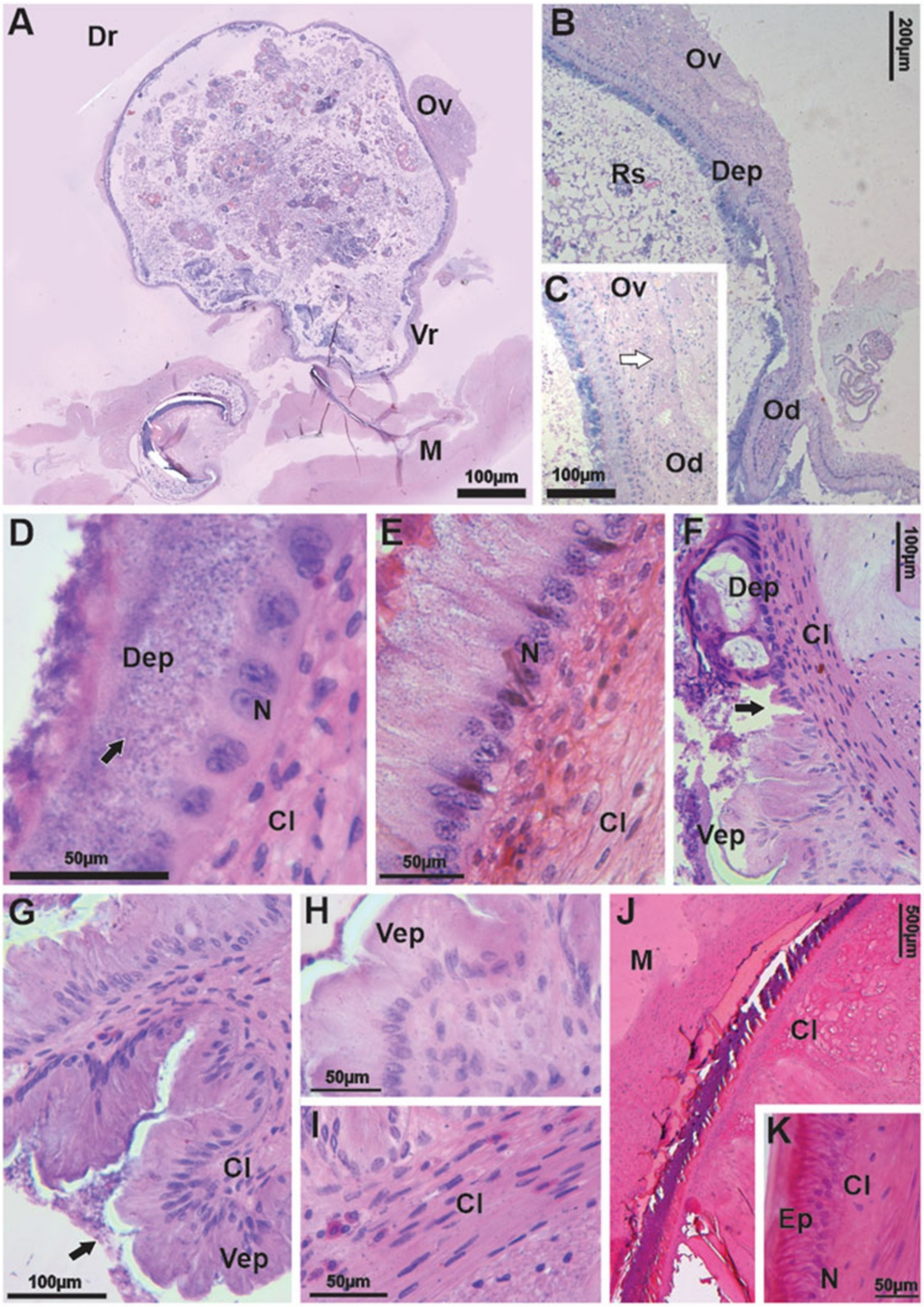

Figure 7. Histology of the seminal receptacle in female *Johngarthia lagostoma*. (A) General view of the seminal receptacle from the dorsal to the ventral region, associated with longitudinal musculature. (B) Detail of the ventral connection of the seminal receptacle with the oviduct, opening into the dorsal mesodermal region, near the area of transition to the ventral ectodermal region. (C) Nearly transverse section of the oviduct in the dorsal region of the ovary, highlighting the lining epithelium, as well as the presence of amorphous material filling the oviduct (arrow). (D) Detail of the dorsal region, composed of simple columnar or prismatic epithelium with elliptical nuclei. Note the presence of many fibroblasts (arrow) on the collagenous layer, stained with HE, along with a layer of connective tissue. (E) Detail of the dorsal epithelium showing varying heights due to the accumulation of secretory material. (F) Abrupt termination of the dorsal region (arrow). (G) Ventral region with numerous folds (arrow). (H) Detail of the folds in the ventral region, with cells formed by simple epithelium. (I) Detail of the epithelium in the ventral region with the connective tissue layer. (J) Epithelium of the vagina, with longitudinal muscle fibres present at the opening of the oviduct, associated with a collagenous layer. (K) Detail of the vaginal epithelium. Cl, connective tissue layer; Dep, dorsal epithelium; Dr, dorsal region; Ep, epithelium; M, longitudinal musculature; N, elliptical nuclei; Od, oviduct; Ov, ovary; Vr, ventral region.

characteristics as the region opposite the oviduct opening; however, associated with the collagenous layer, many oblique muscle fibres are observed, present only on one of the faces (Figure 7J–K). Longitudinal muscle fibres are also observed along the vagina, positioned on the face coinciding, with the oviduct opening (Figure 7J).

The lumen of the seminal receptacle is filled with three types of secretions, in which sperm masses (wall-less spermatophores)

Table 1. Histochemistry of the secretions of the vas deferens (males) and seminal receptacles (females) in Johngarthia lagostoma. Where: AVDp, anterior proximal vas deferens; MVD, median vas deferens; PVD, posterior vas deferens; SP, spermatophores; SI to SIV, secretion types I to IV; +++, strongly positive; ++, positive; +, weakly positive; and –, negative

		Coloration (Target)		
		Proteins	Neutral Polysaccharides	Acid Polysaccharides
Males	SI (lúmen of AVDp)	+	+	+
	SI (interior of the SP)	+	+	+
	SII (AVDp)	+++	+	–
	SII (wall of SP)	+++	+	+
	SIII (MVD)	+	+	+
	SIV (transition of MVD and PVD)	+	+++	++
Females	SI	++	+++	++
	SII	+++	+++	+

or free spermatozoa are immersed. The secretion produced by the dorsal columnar epithelium is basophilic (SI), occurring in association with an acidophilic secretion (SII) (Figure 8A–C). Through histochemical analysis, it was found that the dorsal epithelium, SI, free spermatozoa, and sperm masses are reactive to proteins, whereas SII is strongly positive (Figure 8D–F). When subjected to PAS, SI and SII were strongly positive for neutral polysaccharides (Figure 8G–I). Regarding the presence of acidic polysaccharides, only SI (produced by the dorsal epithelium) was reactive, whereas SII was weakly positive and SIII was negative for this compound (Figure 8J–L; Table 1). Furthermore, areas without reactivity were detected in the lumen of the seminal receptacle for any histological techniques used (Figure 8C, F, I e K).

Discussion

The present study provides the first anatomical, histological, and histochemical description of the male and female reproductive systems of the insular crab *Johngarthia lagostoma*. The male reproductive system of *J. lagostoma* is divided into a pair of testes, with their vasa deferentia and accessory glands arranged in an 'H' shape, similar to what has been described for the gecarcinid *C. guanhumi* (Shinozaki-Mendes *et al.*, 2011) and other brachyuran species (Castilho *et al.*, 2008; Krol *et al.*, 1992; Shinozaki-Mendes and Lessa, 2018; Wang *et al.*, 2015; Zara *et al.*, 2012). In turn, females exhibit paired gonads connected transversely, with a concave-type vagina (sensu Hartnoll, 1968) and a pair of seminal receptacles connected ventrally to the oviduct (sensu Diesel, 1991), with no presence of sperm packets or the formation of sperm plugs within. This morphological model is similar to the one described for *C. guanhumi* by Souza *et al.* (2013), and is widely common among crabs of the Thoracotremata Guinot, 1977 (for review, see McLay and López-Greco, 2011).

Male reproductive system

The testes of *J. lagostoma* was histologically classified as tubular, similar to the pattern described for other species of Thoracotremata, such as those of the genus *Pachygrapsus* Randall, 1840 (Chiba and Honma, 1972; Tiseo *et al.*, 2014). However, it differs from the lobular type reported for *C. guanhumi* by Shinozaki-Mendes *et al.* (2011), which is the testicular type typically observed in most crabs (Nascimento and Zara, 2013; Simeó *et al.*, 2009; Zara *et al.*, 2012). In *J. lagostoma*, cells at different stages of spermatogenesis and spermiogenesis are present within the same testicular tubule, suggesting that cellular development does not occur synchronously. Despite this, synchronization in the development of these cells has been previously reported by Johnson (1980), and it was also verified for *C. guanhumi*, where the germ cells are at the same stage in each tubule in the maturation zone (Shinozaki-Mendes *et al.*, 2011). At the beginning of spermatogenesis in *J. lagostoma*, spermatogonia are located at the periphery of the seminiferous tubule, forming the germinal zone, following the germinal centre pattern observed for portunids (Johnson, 1980; Nascimento and Zara, 2013; Ryan, 1967; Zara *et al.*, 2012). In *C. guanhumi*, spermatogonia were observed on opposite sides, not close to the evacuation zone, which in this case only contains spermatozoa (Shinozaki-Mendes *et al.*, 2011). The same pattern was found by Simeó *et al.* (2009) for *Maja brachydactyla* Balss, 1922. It is possible to analyse, both during spermatogenesis and spermiogenesis, a reduction in the cell nucleus until the formation of the mature spermatozoon, which is present in the evacuation zone. This same pattern has already been observed for other brachyurans, such as *Ucides cordatus* (Linnaeus, 1763), according to Castilho *et al.* (2008); *Callinectes sapidus* Rathbun, 1896, by Johnson (1980); *Portunus pelagicus* (Linnaeus, 1758), studied by Ravi *et al.* (2014); *Callinectes danae* (Smith, 1869), as per Zara *et al.* (2012); and *Callinectes ornatus* Ordway, 1863, according to Nascimento and Zara (2013). However, such nuclear reduction was not observed during the spermiogenesis of *M. brachydactyla* (Simeó *et al.*, 2009).

The division of the vas deferens into three regions (AVD, MVD, and PVD) for *J. lagostoma* is commonly proposed for other Brachyura species (Krol *et al.*, 1992). Additionally, based on histological and histochemical differences in the AVD, it was possible to subdivide it into proximal and distal regions (AVDp and AVDd, respectively), due to the storage of spermatozoa from the testes and the production of seminal fluid. This fluid, produced in AVDd, plays an important role in the formation of the wall of the coenospermic spermatophores, which house a large quantity of spermatozoa immersed in the extracellular matrix, being the predominant packing pattern in Thoracotremata. Several families within Brachyura exhibit coenospermic spermatophores, including: Grapsidae MacLeay, 1838 (Garcia and Silva, 2006Nicolau *et al.*, 2012; Tiseo *et al.*, 2014); Ocypodidae Rafinesque, 1815 (Castilho *et al.*, 2008); Pinnotheridae De Haan, 1833 (Becker *et al.*, 2013);

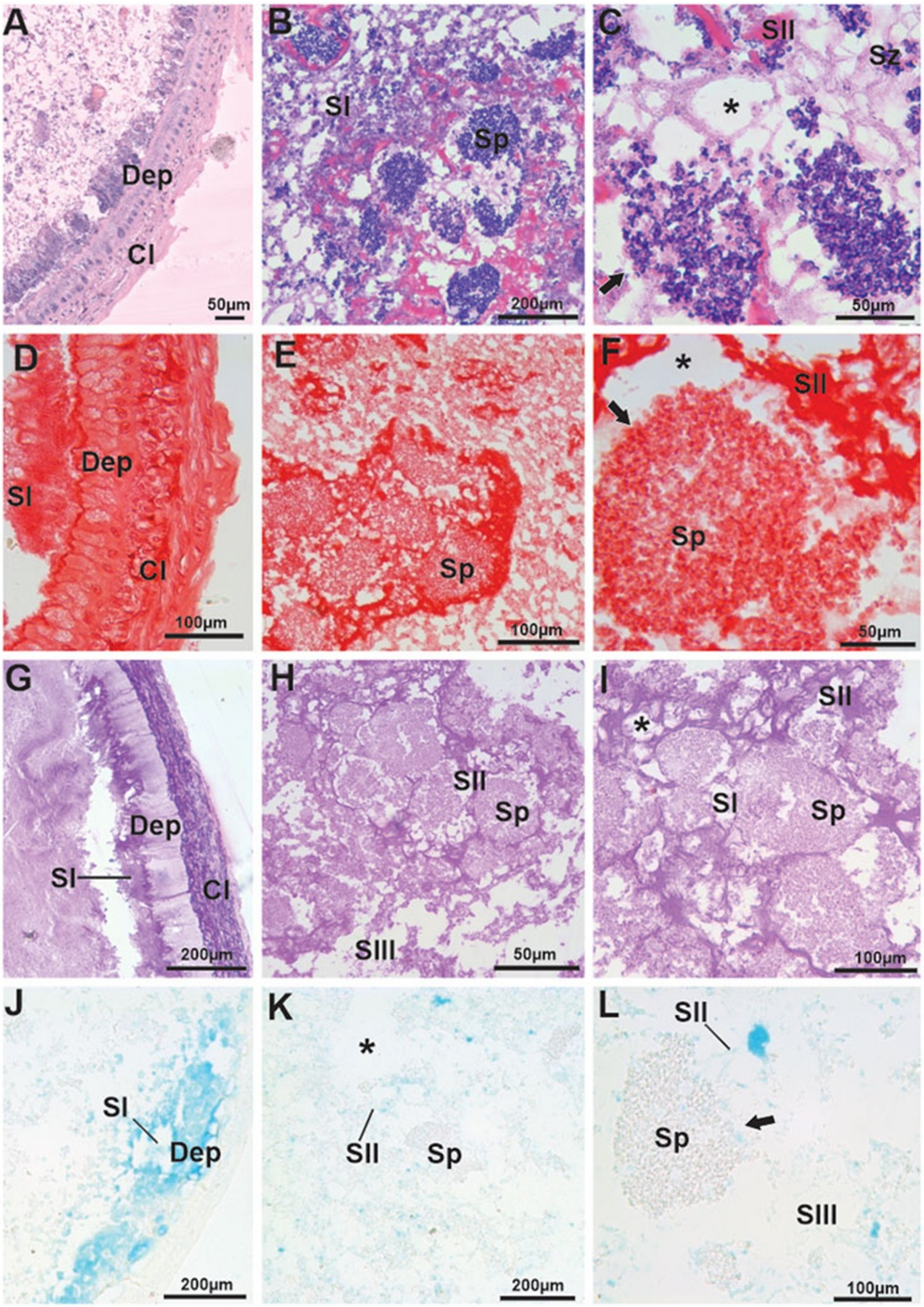

Figure 8. Histology and histochemistry of the secretions in the seminal receptacle of female *Johngarthia lagostoma*. (A) General view of the seminal receptacle, subjected to HE staining technique. (B) Detail of the seminal receptacle, with wall-less spermatophores immersed in secretion type one, produced by the dorsal columnar epithelium. (C) Seminal receptacle with spermatozoa and the additional presence of secretion one (acidophilic), showing reactivity to HE. (D) Seminal receptacle subjected to Xylidine Ponceau, with the dorsal epithelium and secretion one reacting for proteins. (E) Wall-less spermatophores reacting for proteins. (F) Secretion two strongly positive for proteins, surrounding the wall-less spermatophores. (G) Detail of the seminal receptacle subjected to PAS, with the dorsal epithelium and secretion one being strongly positive for neutral polysaccharides. (H) Secretion two surrounding the sperm masses, being strongly positive to the dye, while secretion three is negative. (I) Seminal receptacle showing the two secretions present and the sperm masses. (J) Seminal receptacle subjected to Alcian Blue, with secretion one produced by the dorsal epithelium reacting for acidic polysaccharides. (K) Spermatophores weakly positive, with secretion two weakly positive. (L) General view of the seminal receptacle, showing the two types of secretions and the sperm masses. Arrows indicate the loss of spermatophore walls. * indicate regions that do not show reactivity for any histological techniques used. Cl, connective tissue layer; Dep, dorsal epithelium; Sp, spermatophores; Sz, spermatozoa; SI, secretion one; SII, secretion two; SIII, secretion three.

and Sesarmidae Dana, 1851(Santos *et al.*, 2009). The differential dehiscence rate varies between types of spermatophores, being higher in cleistospermic ones, which contain only one germ cell per spermatophore, and are related to species-specific reproductive strategies (Guinot *et al.*, 1997; Klaus and Brandis 2011; Klaus *et al.*, 2009; Tiseo *et al.*, 2014). For *J. lagostoma*, analyses of SR contents revealed only the presence of free spermatozoa or masses of spermatozoa without the spermatophore walls, as also demonstrated in other *Stenorhynchus seticornis* (Herbst, 1788) (Antunes *et al.*, 2016). However, males showed both coenospermic spermatophores and seminal secretions (both internal and external to the spermatophores) in the distal regions of the vas deferens, which demonstrated reactivity to acidic polysaccharides, indicating a possible relationship with spermatophore dehiscence.

In the MVD and PVD of *J. lagostoma*, spermatophores are immersed in a large amount of seminal fluid, consisting of a homogeneous matrix, in which secretion granules are embedded. Additionally, the accessory glands, recorded in the MVD–PVD transition region, contribute to the production of a larger amount of granular material, which is released into the PVD after the glands open. A similarity can be observed between the secretions produced by the vas deferens and the accessory glands, suggesting that the process is related to the increased production and final volume of seminal fluid, which may serve distinct functions, such as assisting in the conduction of spermatophores and spermatozoa during sperm transfer (Benhalima and Moriyasu, 2000; Johnson 1980; Sainte-Marie and Sainte-Marie 1998; Zara *et al.*, 2012); enabling the formation of sperm packets (Antunes *et al.*, 2016; Garcia-Bento *et al.*, 2019; Watanabe *et al.*, 2022); and nourishing the spermatozoa in the seminal receptacles (Diesel 1989; Sant'Anna *et al.*, 2007; Zara *et al.*, 2014). In this regard, in the seminal receptacles of *J. lagostoma*, as in most Thoracotremata, a mixture of free spermatozoa in secretion occurs, indicating that the seminal fluid from the vas deferens is helping to increase the flow, optimizing the transfer of sperm to the female's seminal receptacle (Antunes *et al.*, 2016; Assugeni and Zara, 2022; Diesel, 1989; Sal Moyano *et al.*, 2010; Spalding, 1942).

Female reproductive system

Johngarthia lagostoma presents a concave type of vagina (*sensu* Hartnoll, 1968), following the characteristic pattern for Thoracotremata (Becker *et al.*, 2011; Hartnoll, 1968; Lautenschlager *et al.*, 2010; McLay and Becker 2015; Souza *et al.*, 2017; Vehof *et al.*, 2015). This type of vagina is also observed in some Heterotremata Guinot, 1977, including the majid crabs *Hyas araneus* (Linnaeus, 1758), *Hyas coarctatus* Leach, 1815 (Hartnoll, 1968), and *Inachus phalangium* (Fabricius, 1775) (Diesel, 1989). In the concave type of vagina, the muscles are predominantly attached internally to the flexible side of the vagina, forming a crescent-shaped configuration. This structure allows the female to exert control over the movement of the vaginal lumen during copulation and fertilization, assisting in the loss of fluids, an important function for crabs inhabiting terrestrial environments. This regulation also plays a key role in controlling the penetration of gonopods during sperm transfer and the transport of sperm deposited in the vagina towards the seminal receptacles (Becker *et al.*, 2011; Diesel, 1989; Hartnoll, 1968; Lautenschlager *et al.*, 2010; Souza *et al.*, 2017). Hartnoll (1968) described another type of vagina, referred to as simple, characterized by a stiffer, rounded lumen in cross-section, with this pattern occurring in other families within the Heterotremata subsection. The distribution of different types of vagina among brachyurans suggests that the simple form is more likely to be considered primitive, while the concave form would be a secondary derivation (Hartnoll, 1968; Vehof *et al.*, 2017).

Regarding the seminal receptacles (SR), a ventral connection with the oviduct was observed, aligning with the pattern seen in other Thoracotremata (Becker *et al.*, 2011; Lautenschlager *et al.*, 2010; López-Greco *et al.*, 2009, 1999; McLay and López-Greco, 2011; Souza *et al.*, 2017, 2013), particularly in more derived species within the Grapsoidea-Ocypodoidea clade (McLay and López-Greco, 2011). According to Brink and McLay (2009), the position of the connection to the receptacle influences the fertilization process of species. In those where this connection is ventral, the sperm from the last male to copulate with the female is preferred for fertilizing the oocytes, while sperm from earlier copulations are pushed further away and are more distant from the ventral region, where the oocytes are released into the seminal receptacle (McLay and Becker, 2015; McLay and López-Greco, 2011).

Some species employ different strategies to ensure paternity, such as the display of pre- and post-copulatory behaviors (e.g., guarding embraces) (for review, see Guinot *et al.*, 2013; McLay and Becker, 2015). Hartnoll *et al.* (2006) highlighted the presence of pre-copulatory guarding behaviour in *J. lagostoma*, although João *et al.* (2021) did not record any form of guarding by females before or after copulation. According to the latter authors, the differing findings can be explained by the species' behavioural plasticity on each island, which would be understandable given that on Trindade Island, *J. lagostoma* occupies a top predator position in the terrestrial environment, unlike on Ascension Island. However, guarding behaviors are not common in crabs with a higher degree of terrestriality (McLay and Becker, 2015), being confirmed in Thoracotremata almost exclusively for some varunid species, such as *Hemigrapsus sexdentatus* (H. Milne Edwards, 1837), where males embrace females until they extrude their eggs (Brockerhoff and McLay, 2005).

In Eubrachyura, the seminal secretion transferred during copulation can form sperm packages or spermatophores inside the seminal receptacles (Antunes *et al.*, 2016; Assugeni *et al.*, 2021; Beninger *et al.*, 1988; Diesel, 1989; Guinot *et al.*, 2013; Hartnoll, 1969; Zara *et al.*, 2014, 2012). These sperm packages contribute to sperm competition, depending on the positioning of the oviduct (dorsal, ventral, or intermediate), the fertilization of the oocytes may favour the gametes of a specific male (Antunes *et al.*, 2016; Beninger *et al.*, 1988; Diesel, 1989). The spermatophore plug, prevents the loss of sperm after copulation and also prevents subsequent copulations by temporarily occluding the seminal receptacles, vagina, and gonopores (Assugeni *et al.*, 2021; Guinot *et al.*, 2013; Hartnoll, 1969; Hines *et al.*, 2003; McLay and Becker, 2015; Zara *et al.*, 2014, 2012). Sperm packages and spermatophore plugs were not observed in *J. lagostoma*, but histological analysis revealed that the seminal receptacles were filled with free sperm immersed in secretions. Therefore, in *J. lagostoma*, there appears to be a simple transfer from a single male, or if the female accepts more than one male, with successive copulations – as theorized by João *et al.* (2021) – the male genetic material is mixed within the seminal receptacle. This is consistent with the absence of guarding behaviours, sperm package formation, and spermatophore plugs in the seminal receptacles. This knowledge gap highlights the need for further studies on this subject, directed at a possible selection process at the cellular level in this species, as well as exploring other strategies, such as the differential morphology of the gonopods and their use during copulation.

The dorsal region of the seminal receptacle in *J. lagostoma* terminates abruptly, without a transitional zone between the dorsal and ventral epithelia, such as the presence of a "velum" seen in a few majid crabs (Diesel, 1989; González-Pisani *et al.*, 2012) or modified dorsal epithelium, as recorded in portunid crabs (see Assugeni *et al.*, 2021; Zara *et al.*, 2014). However, *J. lagostoma* exhibits prominent folds in the ventral region of the seminal receptacle, particularly opposite the oviduct opening. These cuticular folds were also observed in other Thoracotremata crabs, such as *Pinnotheres pisum* (Linnaeus, 1767), *Pinnotheres pectunculi* Hesse, 1872, *Nepinnotheres pinnotheres* (Linnaeus, 1758), *Cyrtograpsus angulatus* Dana, 1851, *Neohelice granulata* (Dana, 1851), and *O. quadrata* (Fabricius, 1787) (see Becker *et al.,* 2011; López-Greco *et al.,* 2009). According to these studies, the folds in the cuticular region may aid in the mixing of sperm and oocytes, as well as allowing the expansion of the seminal receptacle, maximizing its sperm storage capacity and fertilization potential.

In the lumen of the seminal receptacle of *J. lagostoma*, two secretions (SI and SII) were observed, along with free sperm or small sperm clusters. SI appears to originate from the dorsal columnar epithelium of the seminal receptacle, associated with the production of polysaccharides while the SII seem to come from the males eyaculate, considering the histochemical similarity to those recorded in the vas deferens. Polysaccharides secretions may help maintain sperm during sperm transfer and storage in the seminal receptacles (Anilkumar *et al.,* 1999; Diesel, 1989). According to Anilkumar *et al.* (1999), a temporal analysis of the seminal receptacle of newly copulated females of the grapsoid *Metopograpsus messor* (Forskål, 1775) showed that 72 hours after mating, the spermatophore wall dissolved, leaving only free sperm, suggesting that this process is triggered by female fluids. On the other hand, for the varunid *Eriocheir sinensis* H. Milne Edwards, 1853, it is described that the dissolution of the spermatophore wall was more efficient when the seminal fluids produced by the male accessory glands were mixed with the fluids from the females (Hou *et al.,* 2010). For *J. lagostoma*, the presence of male and female secretions in the lumen of the seminal receptacle was verified, as well as the absence of spermatophores with walls, similar to what was observed for *E. sinensis*. However, the authors provided empirical evidence supporting the efficiency of spermatophore dissolution for the species, and further studies similar to those for *J. lagostoma* are needed to verify whether the secretions found in the lumen reflect this process.

In addition to the secretions found in the seminal receptacles (SR), colourless areas with no chemical reactivity to the histological techniques used were also detected. It is supposed that these regions correspond to water influxes resulting from sperm transfer, a feature previously reported in studies on *Chionoecetes opilio* (Fabricius, 1788) (Diesel, 1991; Sainte-Marie *et al.,* 2000) and *C. danae* (Assugeni *et al.,* 2021). The role of water in sperm transfer in crabs has been widely discussed (Assugeni *et al.,* 2021; Becker *et al.,* 2011; Beninger *et al.,* 1988; Brown 1966; Medina, 1992; Sainte-Marie *et al.,* 2000; Watson, 1972), and one possible function would be the dilution of seminal fluid during its passage through the gonopods during copulation (Assugeni *et al.,* 2021; Beninger *et al.,* 1988). However, it is still unclear how these water influxes occur in the SR of *J. lagostoma*, as this species exhibits a higher degree of terrestriality (Marin and Tiunov, 2023). This fact distinguishes this species from the aquatic ones previously mentioned, whose biological events are not limited by this resource. Terrestrial crabs face limitations in water access and, therefore, have developed adaptations that favour their acquisition and retention, ensuring greater water independence (see Pinheiro *et al.,* 2024 and references therein). In *J. lagostoma*, for example, there is a ventral tuft of hydrophilic setae located between the 5th pereiopod and the margins of the 1st–2nd pleonal somites, which aids in water retention (Bliss, 1963; Guinot *et al.,* 2018; Oliveira, 2014; Pinheiro *et al.,* 2024), facilitating the capture of water from the environment they inhabit.

In conclusion, for *J. lagostoma*, both the male and female reproductive systems follow the basic pattern previously described for Thoracotremata crabs. In males, the presence of coenospermic spermatophores immersed in seminal fluid is observed starting from the AVDd and extending along the other regions of the vas deferens, where different types of secretions are produced and surround the spermatophores. The accessory glands, located at the transition between the MVD and PVD, produce secretion type IV (SIV), characterized by basophilic granules strongly positive for neutral polysaccharides. Based on this profile, SIV may function as a matrix that spaces and stabilizes spermatophores, contributing to sperm protection and transfer (McLay and López-Greco, 2011).

In females, the seminal receptacle follows the traditional pattern described for Thoracotremata, with the dorsal mesodermal region composed of simple columnar epithelium with a merocrine secretion mechanism, without any evidence of apocrine secretion. There is no cellular shedding, i.e., no holocrine mechanism, as described for Heterotremata (Assugeni *et al.,* 2021; Becker *et al.,* 2011, 2013, 2012; Zara *et al.,* 2014). Spermatophore plugs and spermatophore packages were absent from the studied species. Additionally, no spermatophores with walls were observed inside the seminal receptacles, only free sperm or small clusters of sperm immersed in secretion. We speculate that the secretion produced by the females, derived from the mesodermal epithelium, and the secretions transferred by the males, may, in addition to maintaining the sperm, influence spermatophore dehiscence and facilitate the fertilization process.

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Competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethical standards. This work was carried out in accordance with the following ethical licenses: SISBIO #65446 For collecting specimens on Trindade Island and SisGen cadaster A4DA42C for histological and histochemical analyses.

Data availability. The data supporting the findings of this study will be available in the UNESP Institutional Repository upon publication. Until then, the datasets are available from the corresponding author (EEDM) upon reasonable request.

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