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Functional morphology of the female reproductive system of a crab with highly extensible seminal receptacles and extreme sperm storage capacity

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Abstract

We studied the functional morphology of the female reproductive system of the purple stone crab *Danielethus crenulatus*. The most remarkable feature is the relative storage capacity and extensibility of the seminal receptacles. These receptacles are a pair of simple sacs that lack internal structures dividing the internal lumen. Differences in seminal receptacle size and contents are accompanied by conspicuous changes in receptacle lining at a tissue level. Full seminal receptacles contain discrete sperm masses formed by hardened fluid and densely packed spermatophores. Different sperm masses are likely from different mates and their stratified disposition within the seminal receptacles is compatible with rival sperm displacement and last sperm precedence. Additionally, the anatomical structure of the vulva and vagina suggest active female control over copula. We discuss our results in the general context of sperm storage in brachyurans and the implications for the mating system of this species.

KEYWORDS
Danielethus crenulatus, Decapoda, *Platyxanthus*, reproduction, southwestern Atlantic

1 | INTRODUCTION

During mating, male true crabs (Eubrachyura) transfer sperm directly to the female seminal receptacles (SRs), a pair of elaborated organs derived from ectomesodermal extensions of the female reproductive system (Guinot & Quenette, 2005; Hartnoll, 1968). Females may retain sperm in the seminal receptacles during moult and may store sperm for extended periods. Therefore, the females do not need to re-mate between successive spawns. (Asakura, 2009; McLay & Becker, 2015; Sainte-Marie, 2007; Shuster, 2008). The storage of sperm from different mates is common among brachyuran crabs (Christy, 1987; Orensanz, Parma, Armstrong, Armstrong, & Wardrup, 1995) and hence the SRs become an arena for post-copulatory sexual selection, that is, sperm competition (Jensen & Bentzen, 2012) and female cryptic choice (Dennenmoser & Thiel, 2015). Morphological studies of the SRs are essential to understand the patterns of sperm use, the mating strategies and any ecological trait related to reproductive behavior (e.g., operational sex ratios, intraspecific competition, social structure, and distribution patterns). The knowledge of the functional morphology of the SRs is also of interest to test current hypothesis on the reproductive traits of brachyuran crabs within an evolutionary context (McLay & Becker, 2015; McLay & López Greco, 2011; Rodgers, Reaka, & Hines, 2011; Sainte-Marie, 2007).

A number of studies on the morphology of the SRs of brachyuran families show a generally conserved pattern that was the basis for the evolution of a variety of morphologies (Becker, Brandis, & Storch, 2011; Diesel, 1989; González-Pisani, Barón, & López Greco, 2011; Pardo, Riveros, Fuentes, & López Greco, 2013; Sal Moyano, Gavio, & Cuartas, 2010; Sal Moyano, Luppi, Gavio, Vallina, & McLay, 2012). However, this knowledge is uneven and scarce considering that the the Brachyura holds about 6600 species; nearly the half of the total species of decapod crustaceans (De Grave et al., 2009). Moreover, new
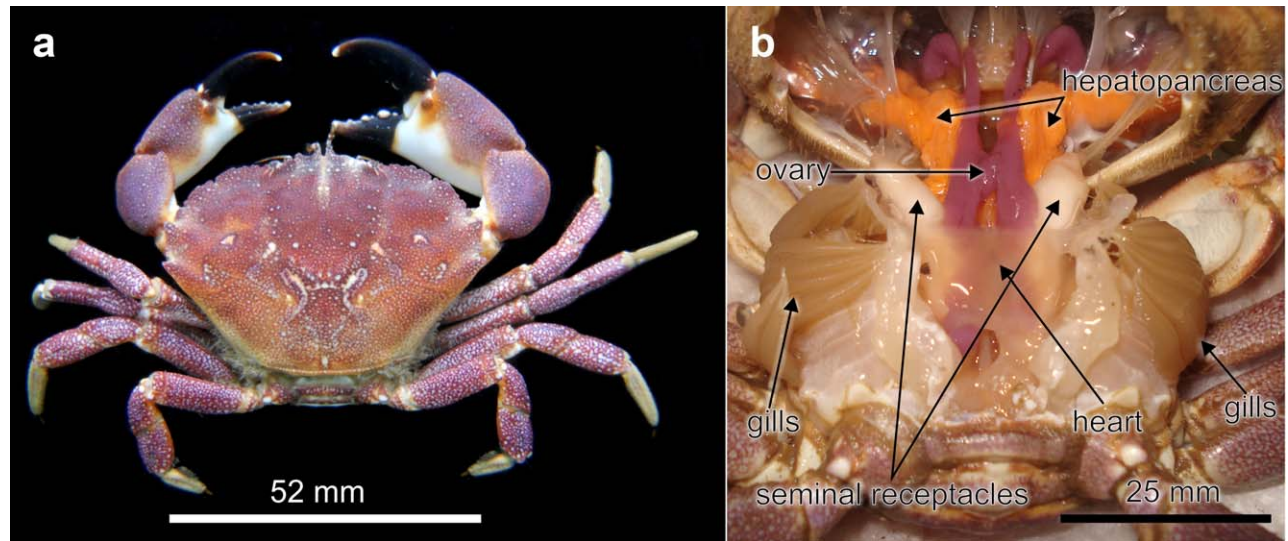
FIGURE 1 *Danielethus crenulatus*, external and gross anatomy of a female; (a) dorsal view; (b) situs dissection

studies confirm there is much more morphological variation than previously thought (Hayer, Köhnk, Boretius, & Brandis, 2016a,b; Hayer, Schubart, & Brandis, 2015). Typically the brachyuran SR has two distinct parts (Hartnoll, 1968; McLay & López Greco, 2011): (1) One of ectodermal origin, usually located ventrally and extending into a tube-like structure, the vagina, connected with the exterior via an opening of the sternum called the vulva. This part is lined with chitin so that is not distensible and is usually shed totally or partially at molting; and (2) a glandular, extensible and non-chitinous reservoir of mesodermal origin, often located dorsally and connecting with the oviduct. This part is not shed at molting, thus it has the potential for retaining viable sperm across molts (with the only known exception of *Dyspanopeus sayi*) (Swartz, 1978). Despite this general pattern, the SRs may differ widely in several important features, even at a genus level (e.g., Jensen, Orensanz, & Armstrong, 1996; Orensanz et al., 1995; Pardo et al., 2013). The main variables are the pattern of sperm delivery and storage, the ability for trans-molt sperm retention, the presence of internal structures and the position of the connection between the SRs and the ovaries relative to the vagina opening and the sperm masses inside (McLay & López Greco, 2011). Anatomically, the relative position of the seminal receptacle-ovary connection is classified as dorsal (opposite the vaginal entrance), ventral (adjacent to the vaginal entrance), or at an intermediate point between these extremes (McLay & López Greco, 2011). This is of major importance since the position of the oocyte entrance may determine which portion of the stored sperm mass has better chances (if any) to fertilize the oocytes passing through the SR (Diesel, 1989, 1991; Parker, 1970), and consequently, also affects the amount of sperm that is left as reserve to fertilize further broods.

The purple stone crab *Danielethus crenulatus* (Figure 1a), belongs to Platyxanthidae GUINOT 1977, a poorly known family of large marine edible crabs. The family is endemic of the Pacific and Atlantic South American coasts and includes only six species arranged into five genera (Thoma, Ng, & Felder, 2012). *Danielethus crenulatus* is restricted to the South-western Atlantic coast, from 23 to 44°S (Boschi, 2000;

Spivak, 1997) where it is mostly associated to rocky bottoms of intertidal and subtidal shallow waters (Farias, Luppi, & Spivak, 2014). The species have indeterminate growth (Farias, 2010); that is, that they continue molting indefinitely after reaching sexual maturity and the final size is determined by gradual decline of growth rates through the lifespan (Hartnoll, 2001). Egg-bearing females are present all the year round hence reproduction is not restricted to a particular season (Boschi, 1964; Farias, 2010; Farias et al., 2014). Both sexes present conspicuous heterochely related to molluscivorous habits (Laitano, Farias, & Cledón, 2013), but males develop larger chelae and reach larger body sizes than females (Farias et al., 2014). Here, we describe the morphology and internal anatomy of the female reproductive system in relation to the capability to deliver and store sperm by males and females, respectively. We discuss our results in the context of the current knowledge on sperm storage patterns of Brachyura and the implications for the mating system of this species.

2 | MATERIAL AND METHODS

Adult individuals of *Danielethus* (= *Platyxanthus*) *crenulatus* (A. Milne-Edwards, 1879) were collected by hand in large pools of intertidal flats located near San Antonio Oeste, Argentina (40°50'S, 64°41'W) during November 2007, January 2014, and February and September 2015; and by means of SCUBA diving up to 7 m depth in Mar del Plata harbor, Argentina (38°02'S, 57°31'30"W) and neighboring subtidal rocky outcrops, throughout the year, from 2008 to 2010. Sex, carapace width, maturity and the presence of eggs were registered in all captured specimens (following Farias, 2010). Individuals of both sexes were chosen opportunistically to be used either for morphological analysis or to assess the patterns of sperm storage and delivery. The reproductive system was dissected and subsequently observed and photographed without further treatment. After inspection of several individuals, the SRs of mature females were classified regarding its size as empty, intermediate or full. Females were grouped in three

categories on the basis of abdomen and vulvae morphology (Farias et al., 2014) and presence/absence of eggs as: immature (abdomen relatively narrow), ovigerous (mature females that carried egg masses), and non-ovigerous (mature females that did not carry egg masses). The study of the SRs was made with regard on these categories combined.

Images of the gross anatomy were taken using a stereomicroscope Olympus CZX7 or directly with a digital-SLR camera Nikon D5300 depending upon the size of the pieces. Histological sections were performed in 2008 and 2016. Individuals of each SR fullness category were dissected and fixed in Davidson's solution during 24 h, dehydrated through a series of graded alcohol (50–100% ethanol), cleared in xylene and embedded in paraffin; and 5 to 20 μm cross and longitudinal sections were cut with a Minot microtome. Sections were mounted on glass slides and most stained with Harris's hematoxylin and Eosin (H&E) according to standard protocols. We used an Olympus CH30 microscope for the examination of the slides and microscopic images were taken using a Lumenera's INFINITY1 digital camera mounted to the microscope. Pictures of large pieces (as sections of the whole SR) were made directly using a macro lens mounted to the digital-SLR camera aforementioned.

To assess the patterns of sperm delivery and storage, mature individuals were chosen so as to encompass evenly the size-range observed. The wet weight (WW) of the total body and the vasa deferentia or the whole SR (males and females, respectively), were registered using a digital scale (.01 g). Body weight always includes both chelae. For full and medium SR the contents and the receptacle itself were weighed separately.

To compare the sperm delivery and storage capacities of males and females respectively, the maximum deliverable sperm from a single male (MDS = WW of vas deferens) and the total sperm mass stored from an individual female (SMS = WW of female SR contents) were determined. The likelihood that the whole volume of sperm stored in the SR come from multiple mates was assessed by using a one-sided two-sample Kolmogorov–Smirnov test of SMS > MDS, under the null hypothesis of identity of the two distributions. For further interspecific comparisons the following indexes were calculated, following Sainte-Marie (2007): the male vasosomatic index (VSI % = 100 * MDS/WW body) and two female sperm reserve indexes using, respectively, the WW of the whole SR (SRI_{SR} = 100 x WW of the SR/WW of female body) and only the WW of the sperm contents (SRI_{cont} = 100 x WW of sperm contents/WW female body).

3 | RESULTS

3.1 | Overall morphology of the female reproductive system

The female reproductive system of *Danielethus crenulatus* is a paired and bilaterally symmetrical system of organs sequentially arranged from the ovaries to the vulvae. Each ovary is attached dorsally to its respective seminal receptacle. From this point the mature oocytes are transferred to the internal lumen where they meet the sperm stored from previous mating. Then, the oocytes pass to a narrow tube termed vagina, which connects ventrally the SR to the vulva at the sixth thoracic segment.

Both the ovaries and SRs varied in size and shape depending on the maturity of the oocytes and the volume of stored sperm, respectively. Ripe or spent ovaries were found with full or empty SRs suggesting that the size of both organs varies independently. Figure 1b shows the topography of the ovary and SRs.

3.2 | Seminal receptacles

3.2.1 | Gross anatomy

In immature females, the SRs are rudimentary and small, but readily visible. They appear as a pair of turgid and elongated sacs, lined by thin translucent epithelium and filled with a transparent liquid (Figure 2a). In mature females, the SRs are, in macroscopic anatomy, a paired structure with a uniform white and smooth external surface, independently of the volume of sperm stored. However, a ventral cuticle-lined part and a dorsal non-chitinous part were easily separated in dissection. The ventral part is a bowl-shaped expansion of the cuticle lined vagina, whose relative size and shape remains constant independently of the sperm load. In contrast, the dorsal part varied in size depending on the amount of sperm loaded. When empty, the dorsal part of the SR was barely visible under the ovary (Figure 2b). In full SRs, this part is conspicuous and extends forward, underneath the hepatopancreas, and curved continuously toward the anterolateral portion of the body so that the distal end points laterally outwards (Figure 2c). It occupies much of the internal cavity of the body, reaching volumes close to that of the mature ovaries. Once dissected, the empty SR resembled a Phrygian cap, having almost the same volume than the ventral cuticle-lined part below. When filled, the dorsal part was an elongated tube, with a white and smooth surface and a rounded distal end, closely resembling the finger of a latex glove (Figure 2d). Cross sections of fixed full seminal receptacles showed different sperm masses readily separable in dissection.

3.2.2 | Histology

The wall of the SR was composed by an external lining of muscle cells and loose connective tissue that encapsulates the two parts, giving these organs their uniform external appearance (Figure 3a).

In the dorsal portion of the SR the external capsule covers an inner multilayered glandular epithelium, differentiated into proliferative and innermost secretory strata (Figure 3b). Externally, the empty SRs looked as uniform, turgid organs with a smooth external surface but, internally, the lumen was collapsed and highly folded (Figure 3c). In non-ovigerous females with empty SRs the lumen was filled with a clear transparent fluid that stains light violet with H&E, and the internal lining had abundant multinucleated cells (Figure 3d–f). In ovigerous females with empty SR, the lumen of the dorsal part was lined by columnar cells of distorted shape, with elongated nuclei and apical secretory snouts. As in non-ovigerous females the lumen of the dorsal part also lacked sperm, but in this case some intact spermatophores were seen in the ventral chitinous part (Figure 4a–f).
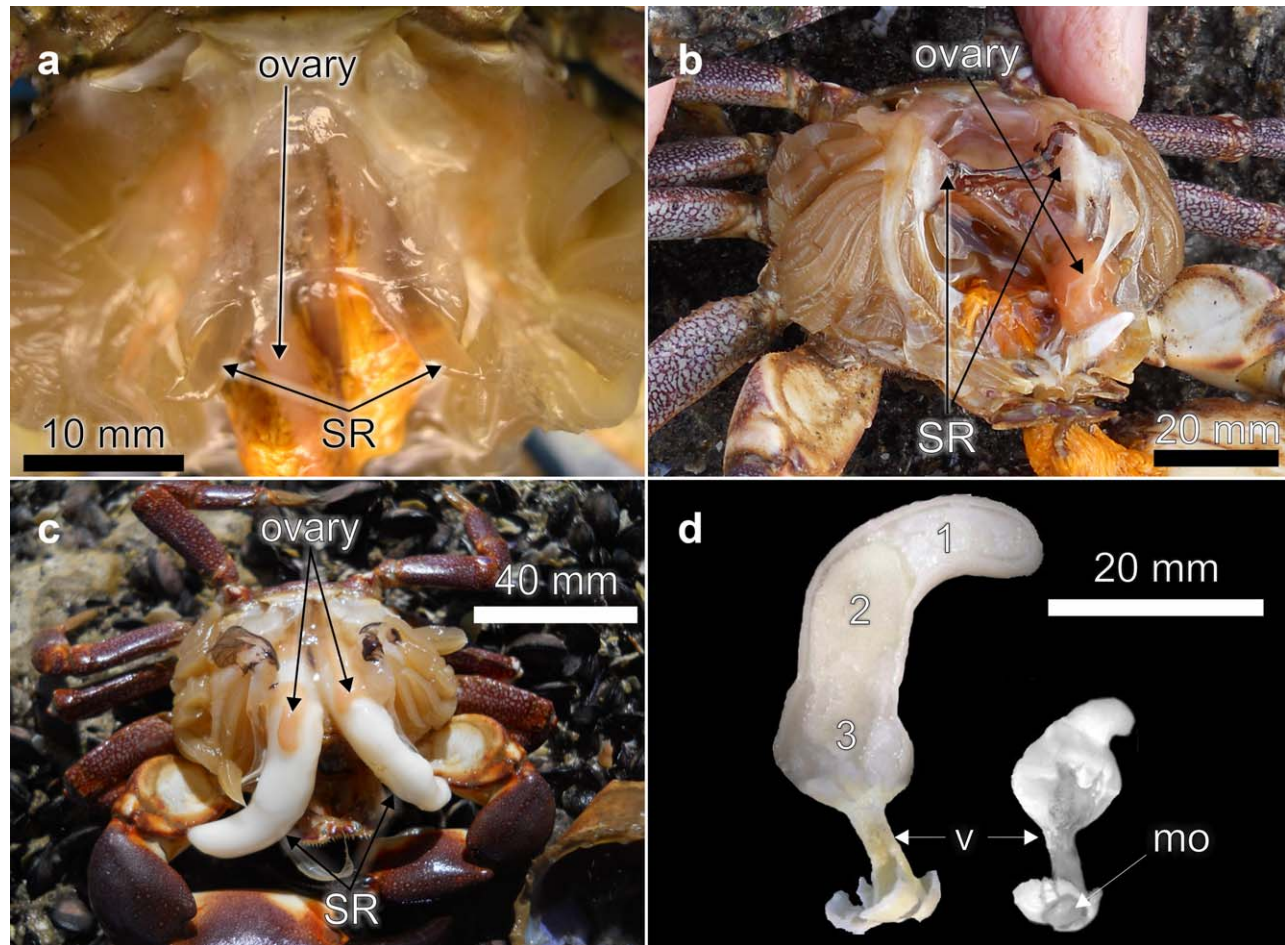
FIGURE 2 *Danielethus crenulatus*, gross anatomy and variation in size and shape of the female seminal receptacles; (a) immature female; (b) mature female with empty seminal receptacles; (c) mature female with full seminal receptacles; (d) shape and relative size of a full (left) and empty (rigth) seminal receptacles, both receptacles were dissected and are displayed on equal scale for comparison. Numbers in (d) are different sperm masses found in cross section

In SRs with large sperm loads (full and intermediate SRs) the secretory stratum is a stratified epithelium of very variable thickness (7 to 35 cell layers) depending on the organ or sites in the organ (Figure 5a). In the proliferative strata the cells were often flattened, progressively changing to a rounded or cuboidal shape toward the lumen. In most cases cells of the secretory strata were rounded or cuboidal without much variation in size or shape (Figure 5b). Holocrine activity was evident in the innermost layer of the secretory strata by the presence in the lumen border of many cells in disintegration, or sloughing from the epithelium and decaying thus forming flocculate secretions (Figure 5c,d).

In contrast to the dorsal part, the ventral part of the SRs did not show variation in relation to the amount of sperm stored. This part was characterized by a cuticle of chitin overlying a simple columnar epithelium surrounded externally by a layer of loose connective tissue which is actually a continuation of the capsule described above (Figure 6a). Several bundles of muscle run transversal to the surface of the ventral parts, with fibres anchored directly to the columnar lining and attached to the adjacent sternites at the opposite end (Figure 6b–d). In longitudinal sections the limit of the ventral and dorsal parts is marked by a groove that circles the SR transversely as a ring, denoted here as "annulus." The concave face of

this annulus is lined by densely grouped and slightly elongated cells, with large basal nuclei. Immediately ventral to the annulus the chitin lining of the dorsal part appears highly folded and projected dorsally, thus overlapping with the annulus. At this point, the cells that underlie the chitin layer are very elongated and distorted in shape, with stretched nuclei. Ventrally from the annulus these cells progressively change to the normal simple columnar form described above (Figure 6e,f).

3.3 | Ovary and oviduct

The two ovaries were connected at the cardiac region, each one having anterior and posterior branches, thus forming an X or H shaped structure (Figure 1b). Most of the ovary mass was in the anterior branches which extends anteriorly over the hepatopancreas and then laterally. The posterior branches were short and covered the SR attached to its surface by a small spot situated dorsally and medial to their longitudinal axis, with no visible oviduct connecting both organs. The relative position of the ovary-SR connection along the longitudinal axis of the receptacle changes according to the filling degree, from a more distal position in empty SRs (Figure 7a,b) to an intermediate point in full SRs
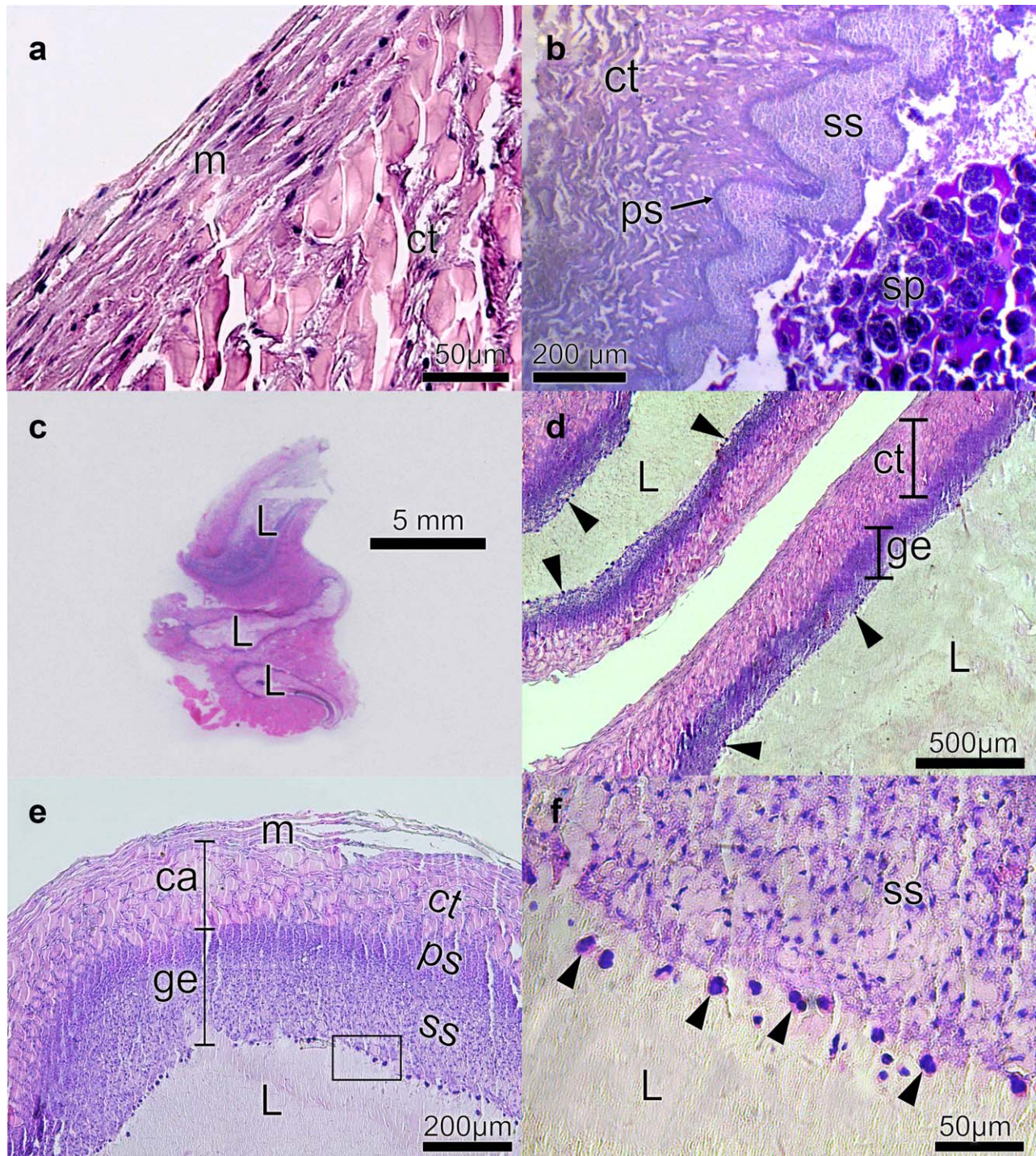
FIGURE 3 *Danielethus crenulatus*, histology of the wall of the dorsal part of empty seminal receptacles; (a) external capsule; (b) connective tissue surrounding the innermost multilayered glandular epithelium in a partially filled seminal receptacle; (c–f) progressive close-ups of the longitudinal section of an empty seminal receptacle showing histological features of the dorsal wall, the lumen filled with translucent fluids and large multinucleated cells lining the internal surface. Note that, internally, the lumen is collapsed and folded and therefore it appears repeatedly at the larger scales. Arrowheads point to some multinucleated cells as example; ca: external capsule; ct: connective tissue; ge: glandular epithelium of the dorsal part; L: internal lumen of the seminal receptacle; m: muscle fibers; ps: proliferative strata; sp: spermatophores; ss: secretory strata

(Figure 7c). However, regardless of the sperm loads, the relative position of the oviduct remained vertically opposite (along the dorso-ventral axis) to the vagina opening.

Although at the joining both organs were easily distinguishable by color, histological sections showed that the thin transparent epithelium of the ovaries forms a continuum with the external lining of the SR
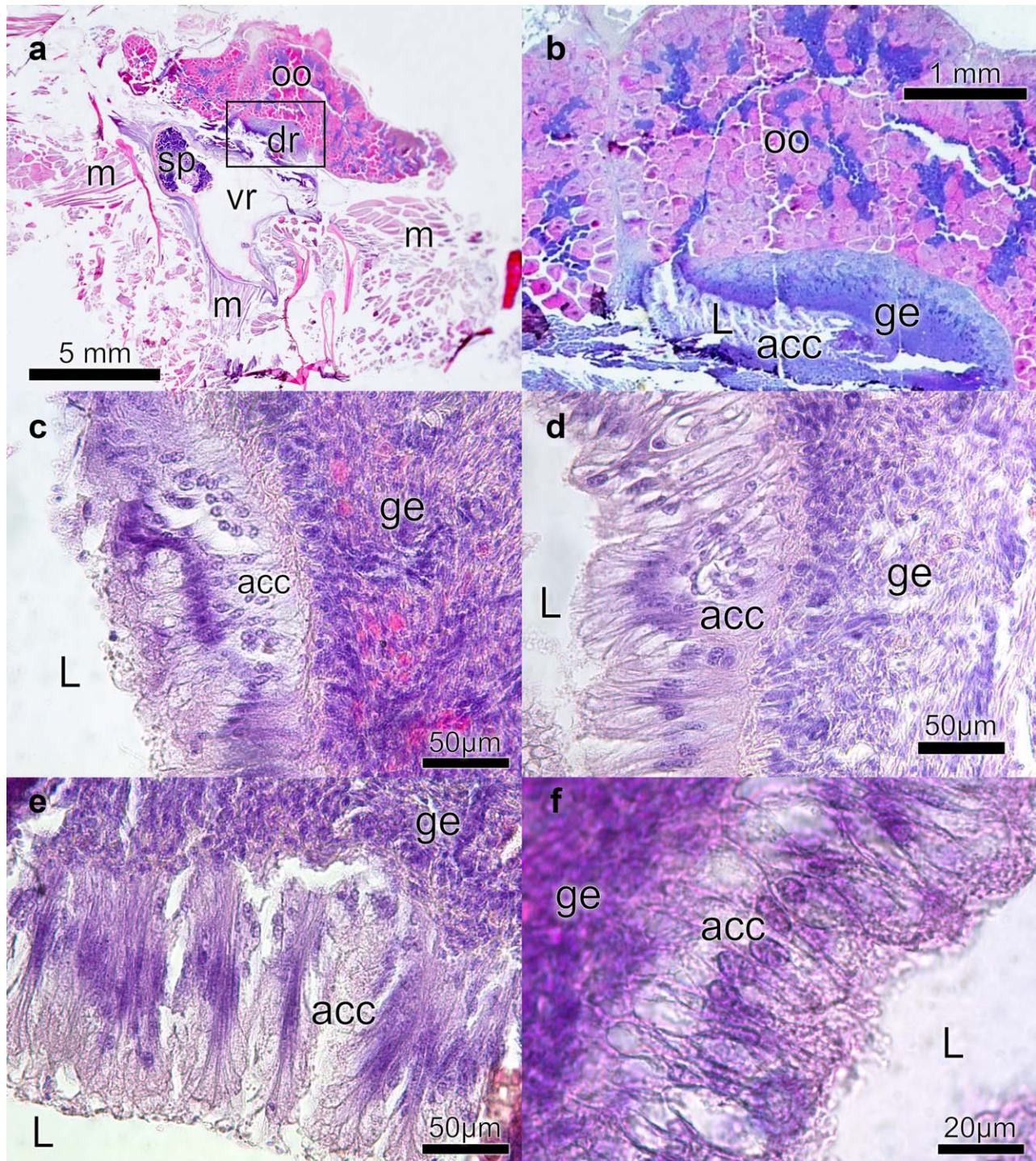
FIGURE 4 *Danielethus crenulatus*, histology of the wall of the dorsal part of empty seminal receptacles of egg-bearing females; (a) overall view of the sagittal section of a collapsed seminal receptacle of an ovigerous female; (b) close-up on the area framed in (a) showing the ovary and the collapsed lumen of the dorsal part of the seminal receptacle lined by atypical columnar ephitelium; (c–f) detail of the different forms of the atypical columnar cells lining the dorsal lumen of egg-bearing females. acc: atypical columnar cells; dr: dorsal part of the seminal receptacle; ge: glandular epithelium of the dorsal part; L: internal lumen of the dorsal part of the seminal receptacle; m: muscle fibers; oo: oocytes; sp: spermatophores; vr: ventral part of the seminal receptacle

(Figure 7d). Oocytes are transported from the distal lobes of the ovary to the SR wall via the oviduct (Figure 7e). At the point the ovary contacts the SR wall a small portion of it (some oocytes included) penetrates the external capsule and the oviduct fuses with the glandular epithelium of the receptacle (Figure 7f–i). From this point, oocytes seem to be transferred to the internal lumen by simply passing through the glandular epithelium of the SR since no evident duct or other type of passage was found in serial histological sections.
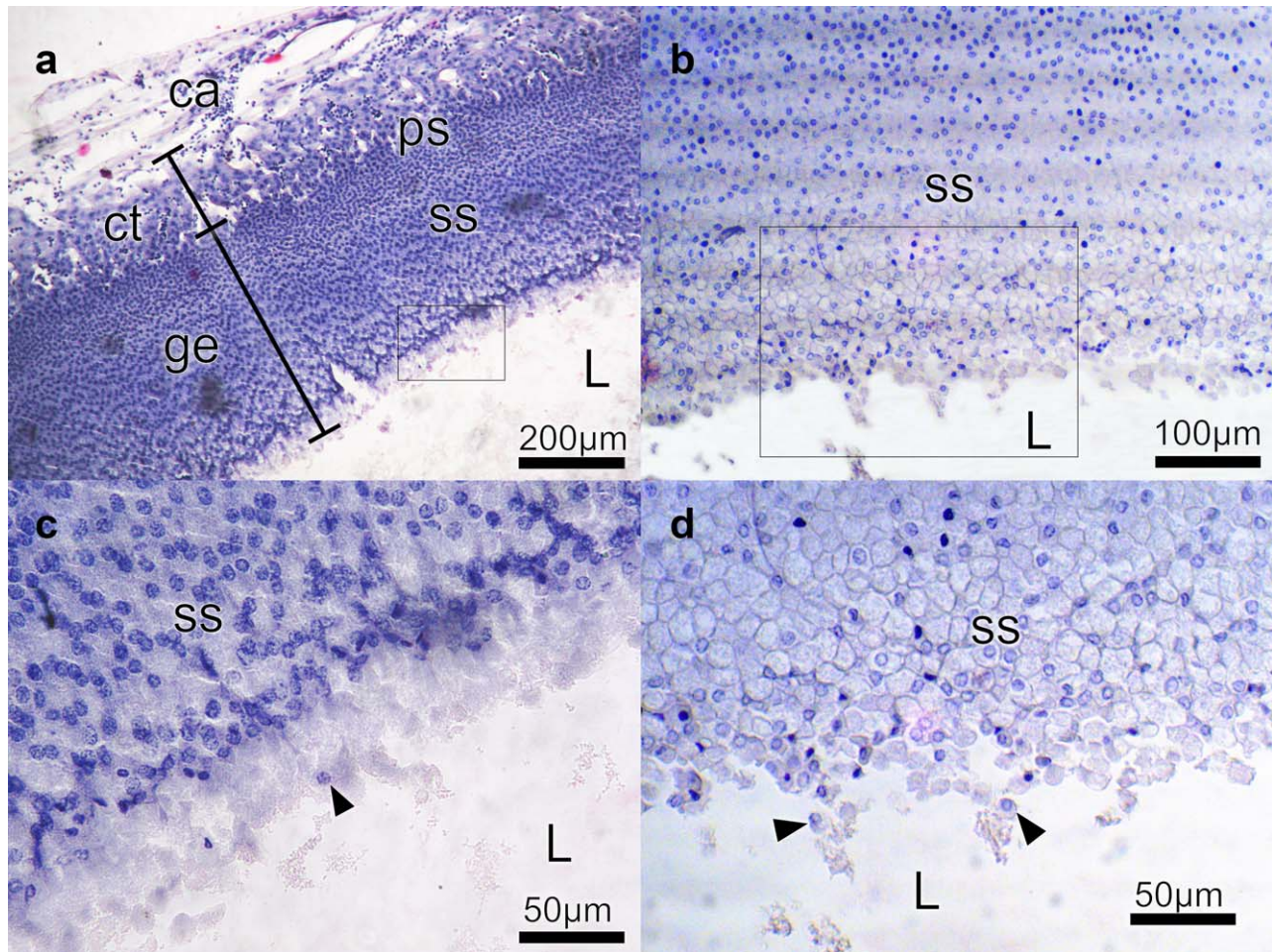
FIGURE 5 *Danielethus crenulatus*, histology of the wall of the dorsal part of the female full seminal receptacles; (a) cross section showing the external capsule and the different strata of the receptacle wall; (b) close-up view of A showing the secretory strata; (c and d) detail of the innermost cell layer showing holocrine secretion, note that (c) and (d) are closer views of the areas framed in (a) and (b), respectively. Arrowheads: still visible nuclei in some cells in disintegration that were sloughed from the epithelium; ca: external capsule; ct: connective tissue; ge: glandular epithelium of the dorsal part; L: internal lumen of the dorsal part; ps: proliferative stratum; ss: secretory strata

3.4 | Vulva and vagina

After removal of the muscle mass and the external capsule, it can be seen that the vulva and vagina are cuticle-lined organs that extend ventrally from the ventral part of the SR. A thickening of the cuticle layer becomes evident as a yellowish area at the external surface (Figure 8a), which forms a concavity internally; its structure is identical to the rest of the wall of the ventral chitinous part. In simulated penetrations using dissected male and female genitalia, the tip of the first gonopod invariably leaned on the concave surface of this structure (Figure 8b).

The vagina connects the ventral part of the SR with the vulva and is a straight and flattened U-shaped tube in cross section, which conforms to the "concave type" described in Hartnoll (1968). The concave outer wall of the vagina is flexible, and there are muscles attached longitudinally along it, running to the sternum and the internal wall of the vulva. The convex or internal wall of the vagina is thicker and has no muscles attached to it (Figure 8a,c).

The vulva of immature females of *D. crenulatus* is a small, partially calcified aperture that opens in the sixth thoracic sternite (Figure 8d).

Once sexual maturity is attained, it changes markedly in size and shape. The vulva of mature females (Figure 8e) is much larger and has a chitinous mobile operculum that seals off the opening, but remains flexible and folds inwards, allowing the penetration of the male gonopod, irrespective of the carapace hardness of the female. Some muscles are attached to the concave wall of the vagina and are anchored at the inner side of the vulva in a way that triggers the aperture of the vulva during their contraction.

3.5 | Sperm storage, delivery, and mating

Whenever two paired crabs were found, with the female positioned upside-down beneath the male and her abdomen flexed over the male's carapace, individuals were considered as mating (Figure 9a). All mating pairs observed in the field ($n = 16$) involved exclusively hard shelled-females. Histological sections and inspection of fresh male vasa deferentia (Figure 9b) showed that the sperm is delivered to the female packed into spermatophores jointly with ovoid gel structures or
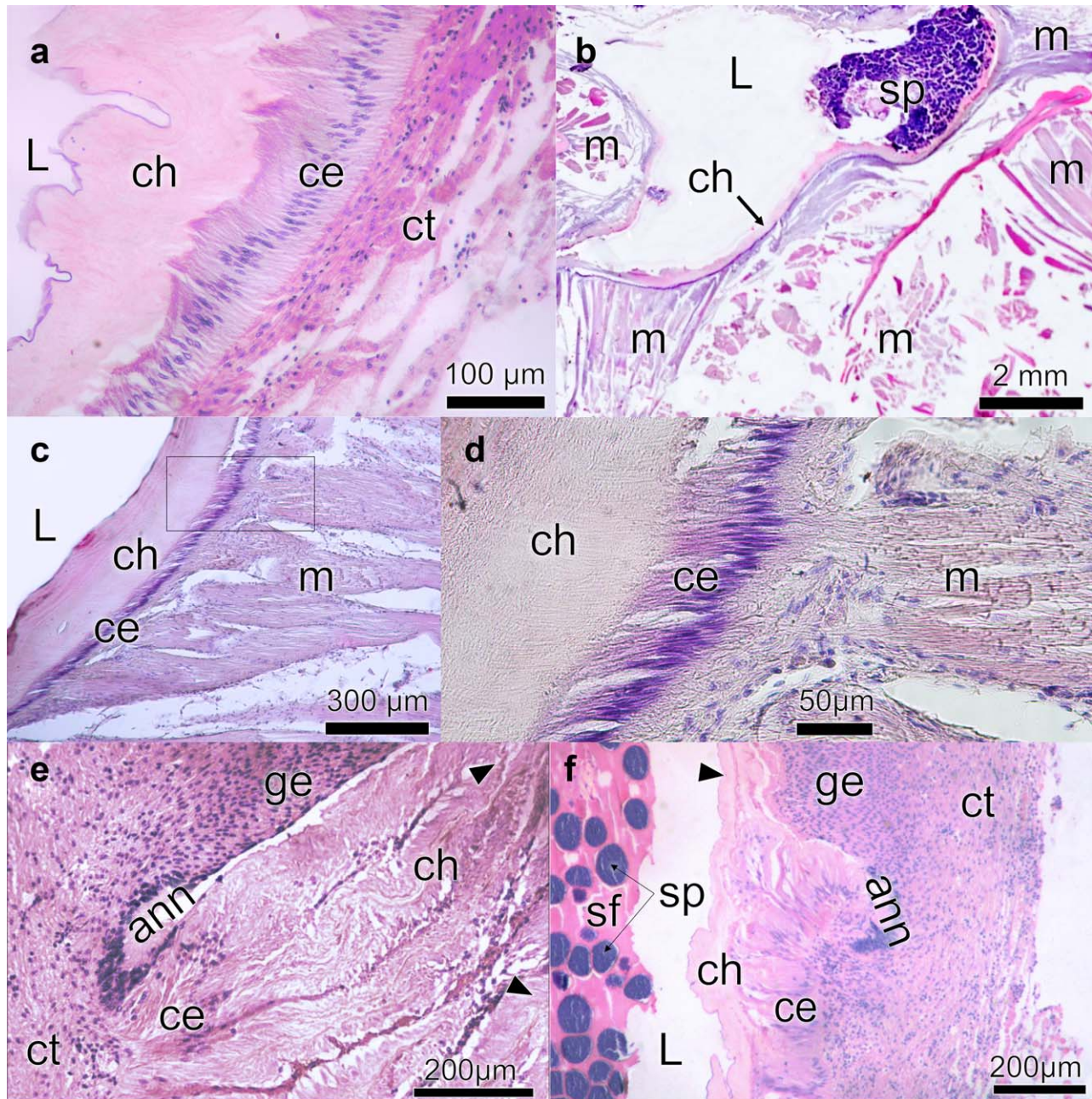
FIGURE 6 *Danielethus crenulatus*, histology of the wall of the ventral-chitinous part of the female seminal receptacles; (a) cross section showing the chitin lining and the different strata of the receptacle wall; (b) section of the dorsal part showing the muscle fibers attached transversely to the receptacle wall and anchored to the endosternites by the other end; (c) detail of the attachment of muscle fibers to the chitin secretory simple columnar epithelium lining the ventral part of the receptacles; (d) close-up view of the area framed in c; (e and f) cross section showing the transition from dorsal to ventral part of the seminal receptacles, the arrowheads marks the protruding chitin layer that project toward the dorsal part of the receptacle. ann: annulus; ce: columnar epithelium; ch: chitin lining; ct: connective tissue; ge: glandular epithelium of the dorsal part; L: internal lumen of the seminal receptacle; m: muscle fibers; sf: seminal fluids; sp: spermatophores

"capsules" (Figure 9c,d). Spermatophores in the mid and posterior vas deferens were usually embedded in these capsules. Within the SR, the sperm contents consisted of conglomerates of intact spermatophores forming distinct groups with no precise shape, but well separated by hardened fluids with no sign of the gel capsules (Figure 10a–f). Spermatophores were present in both dorsal and ventral parts. Dehisced spermatophores and free spermatozoa were detected, although scarce and mostly in areas neighboring the secretory epithelium of the dorsal part of the SR (not shown). A model summarizing the internal structure and sperm storage pattern of a full SR was derived by the combination of our macroscopic observations and histological sections (Figure 11).

Regarding the amount of sperm stored, the WW of a full SR (only one receptacle of the pair) was 2.6 ± 2.1 g and the Sperm Mass Stored (SMS) weighed 1.44 ± 1.47 g ($n = 22$). On the other hand, the WW of
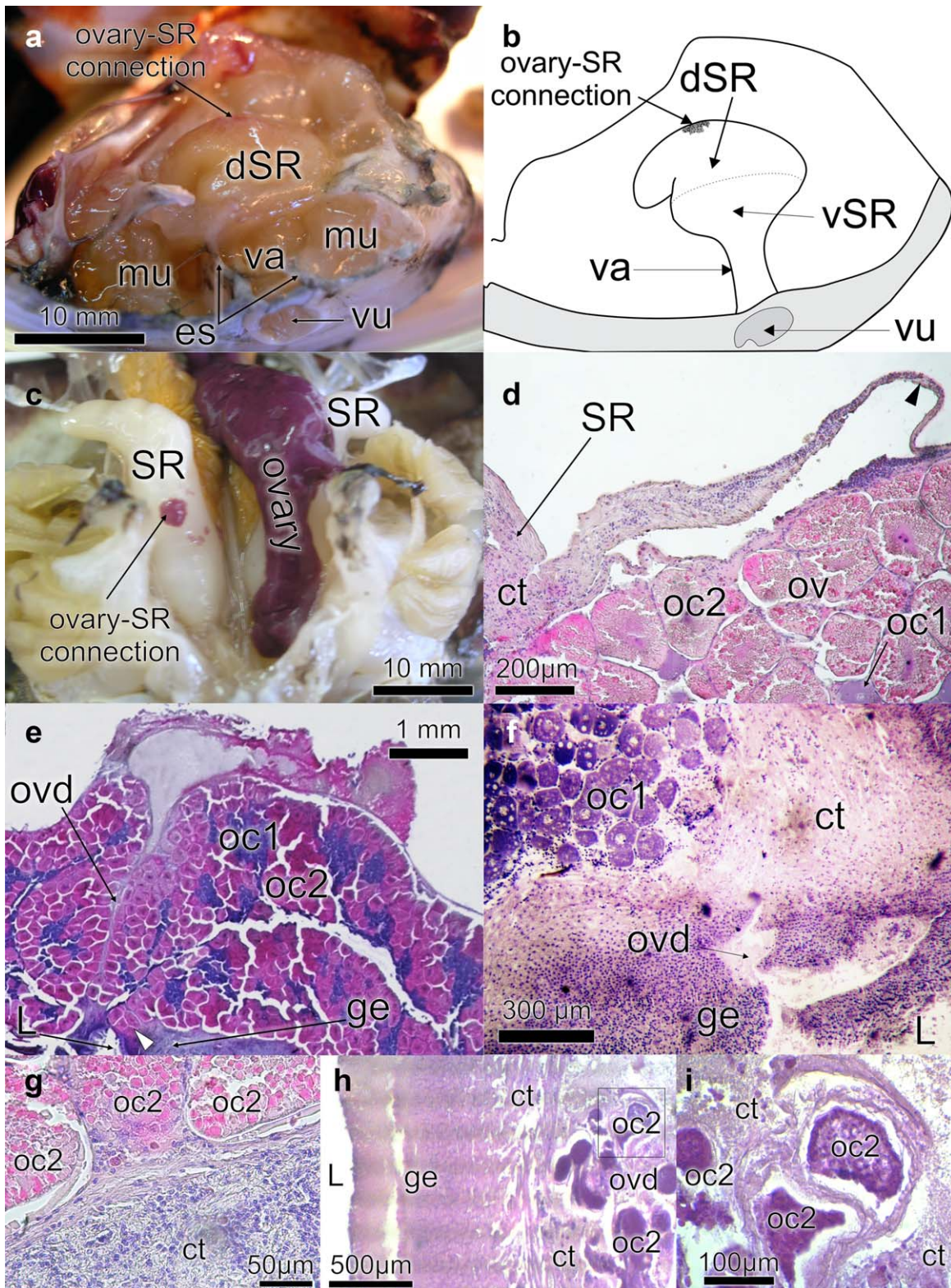
FIGURE 7 *Danielethus crenulatus*, ovary-seminal receptacle connection in mature females; (a) macroscopic longitudinal section of the body showing a right empty seminal receptacle and the position of the ovary connection; the reddish area at this point is a small portion of the ovary that remains attached to the receptacle wall after dissection, thus denoting the spot in which the oocytes enter to the seminal receptacle; (b) schematic view of b; (c) dorsal view of a left full seminal receptacle showing the point where the ovary is connected to the seminal receptacle via the oviduct; (d) transition between the ovary and the seminal receptacle showing the continuity of the external lining (black arrowhead) along both organs; (e) cross section of the ovary showing the oviduct that connects an already depleted lobe of the ovary to the dorsal wall of the empty seminal receptacle; the white arrowhead points a group of oocytes that are almost in contact with the internal lumen of the receptacle; (f) section of the ovary-receptacle connection of a full seminal receptacle showing part of the ovary included in its dorsal wall and the possible oviduct (torn tissue in the receptacle wall); (g) close-up view of the transition area between the ovary and the dorsal seminal receptacle wall; (h) Cross section of the oviduct showing the oocytes embedded into the connective tissue of the seminal receptacle; (i) Close-up view of the box area in h. ct: connective tissue; dSR: dorsal part of the seminal receptacle; es: endosternites; ge: glandular epithelium; l: internal lumen of the receptacle; mu: muscles; oc1: previtellogenic oocytes; oc2: vitellogenic oocytes; ov: ovary; ovd: oviduct; SR: seminal receptacle; va: vagina; vSR: ventral part of the seminal receptacle; vu: vulva
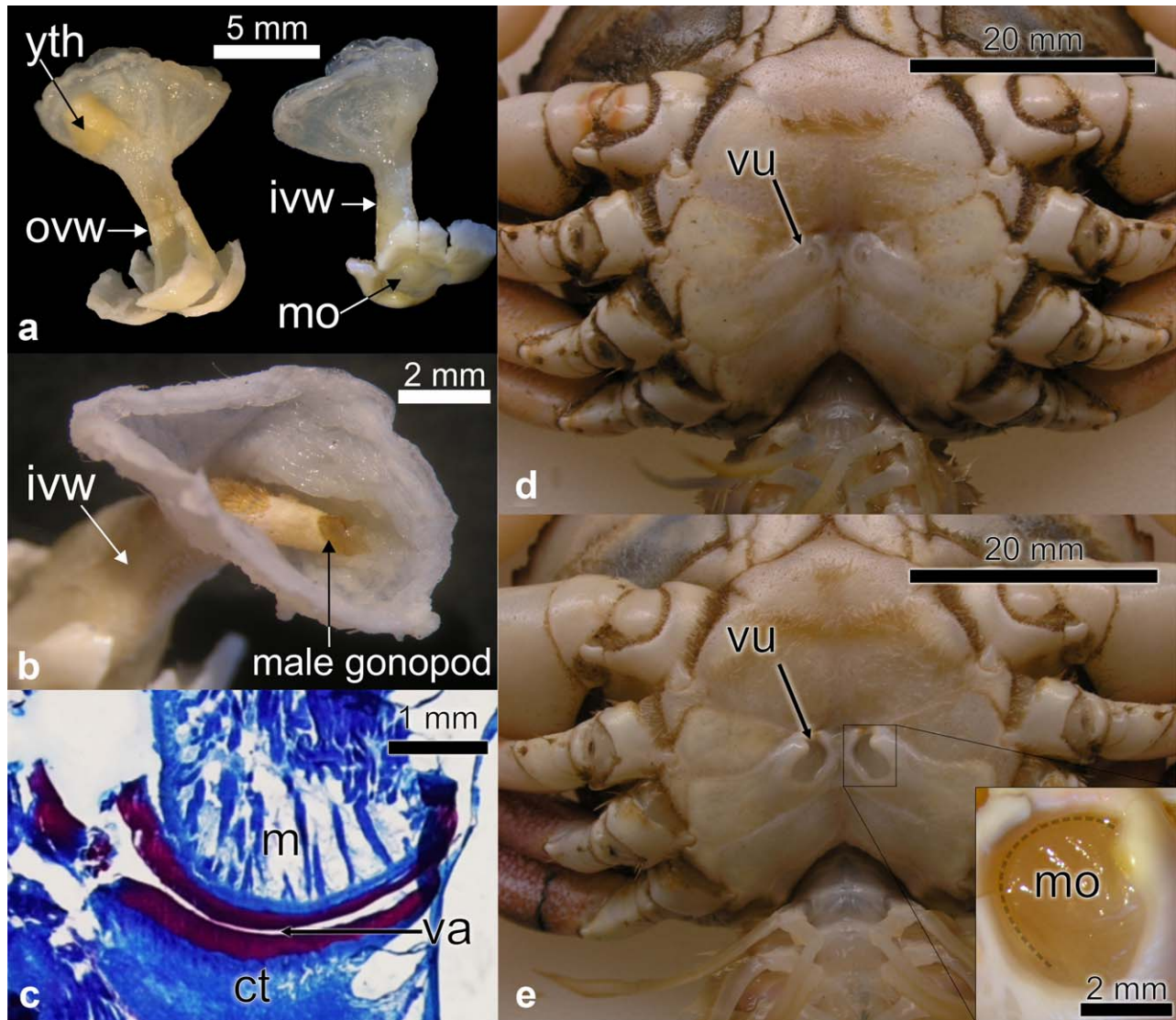
FIGURE 8 *Danielethus crenulatus*, morphology of vagina and vulva; (a) external (left) and internal (right) faces of the ventral part of seminal receptacle showing the concave outer vagina wall and the yellow thickening, the convex inner vagina wall and the mobile operculum of the mature vulvae; the dorsal part of the seminal receptacle and the muscle attached to the vagina were dissected; (b) simulated penetration using the dissected male and female genitalia; during copula the tip of the gonopod invariably rest over the internal- concave surface of the yellow thickening; (c) Gomori's one-step trichrome staining of a cross section of the vagina showing the chitin layer in red and the muscles fibres attached to the concave wall; (d and e) ventral view and close caption of two equal sized females showing the immature (top) and mature (bottom) forms of the vulvae; the close-up shows the mobile operculum of the vulva, its internal limit (dashed line) opens and folds inwards to allow gonopod penetration. ct: connective tissue; ivw: inner vagina wall; m: muscle fibres; mo: mobile operculum of the vulva; ovw: outer vagina wall; va: vagina; vu: vulvae; yth: yellow thickening of the ventral chitinous wall of the seminal receptacle

the male vas deferens (Maximum Deliverable Sperm, MDS) was 0.67 ± 0.55 g ($n = 71$) ranging from 0.33 to 1.08 g. The SMS was, on average, heavier than MDS (one-tailed two-sample K–S test; $D = 0.417$, $p < .05$). Only 3% of the males (two males of 75.2 and 85.2 mm CW, respectively) had vas deferens large enough to completely fill a SR, assuming their entire contents were transferred to the same female in one ejaculation.

Male vasosomatic index (VSI %) was calculated from 60 males ranging from 42.4 to 89.7 mm CW (65.8 ± 9.3 mm CW). Mean total body WW was 91.4 ± 38.8 g and mean WW of vas defer-

ens $= 0.64 \pm 0.56$ g, resulting in a VSI % $= 0.7$. In turn, two different values of sperm reserve index were calculated from 16 females having full SR. The SRI_{SR} % was 3.7 ± 2 and the SRI_{cont} % was 2.36 ± 1.84.

During the sampling period, ovigerous females from the different locations were routinely dissected to determine SR fullness. In all cases, the SRs of egg-bearing females were collapsed. The lack of ovigerous females with sperm reserves in their SR could not be explained by chance alone (two-tailed exact binomial test: $N =$ total number of observed ovigerous females $= 132$; $k = 0$; $q =$ females with empty SR/ total observed females $= 572/658 = 0.87$; $p < 1.59E\text{-}7$).
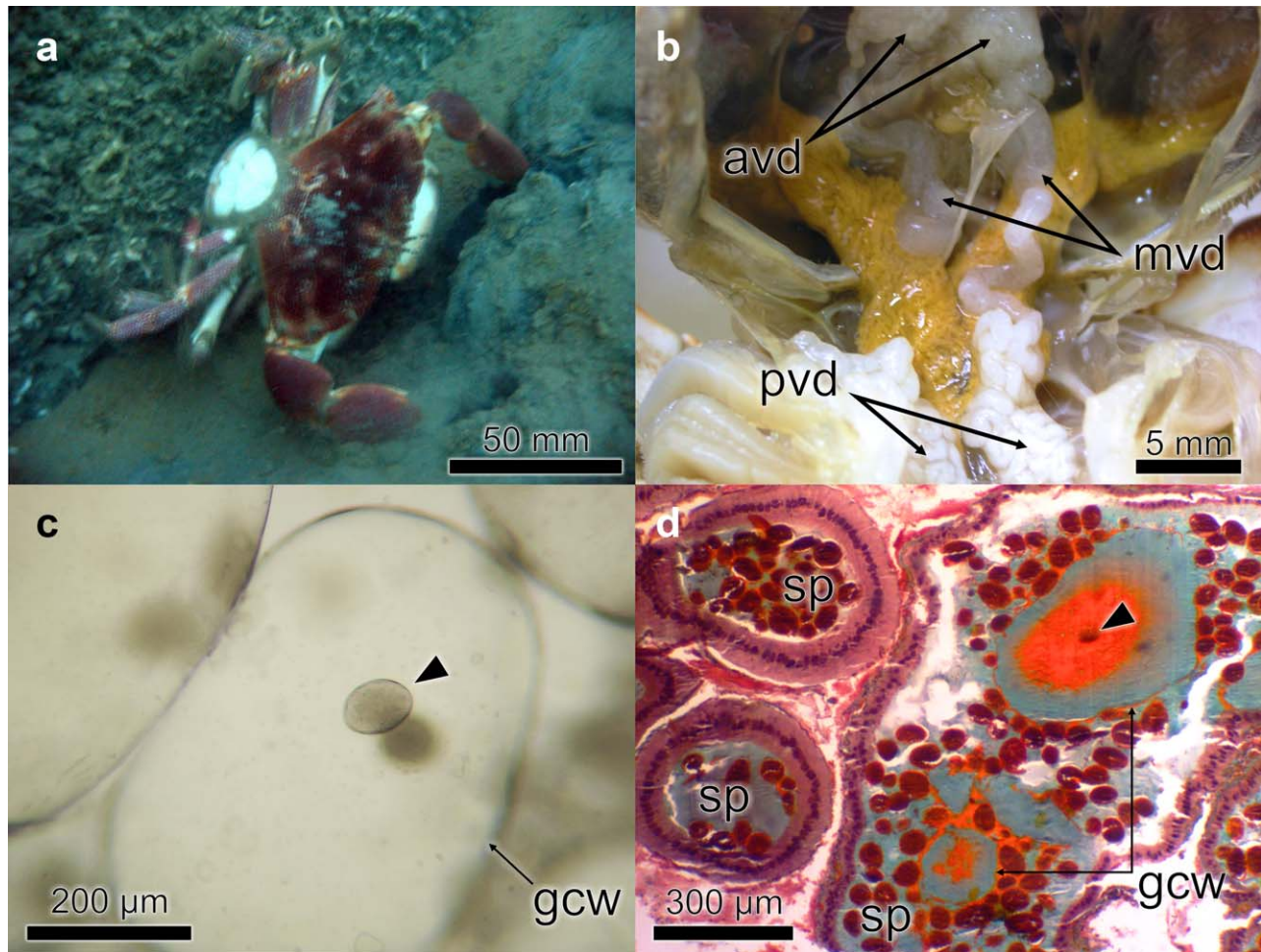
FIGURE 9 *Danielethus crenulatus*, male sperm delivery pattern; (a) mating pair in the field; female is upside-down, beneath the male, note the female's abdomen flexed over the male's carapace; (b) gross morphology of the male reproductive tract showing the highly coiled *vasa deferentia* (c) sperm structures present in the posterior vas deferens of a mature male; one spermatophore is embedded into a gel capsule (arrowhead); (d) Masson's trichrome staining of cross section of mid (left) and posterior (right) vas deferens showing that some spermatophores are included into a capsule (arrowhead) prior to be delivered into the female seminal receptacle. avd: anterior vas deferens; gcw: gel capsule wall; mvd: mid-vas deferens; pvd: posterior vas deferens; sp: spermatophores

4 | DISCUSSION

4.1 | Overall morphology

The female reproductive system of *D. crenulatus* follows the general pattern of Eubrachyura with paired ovaries, seminal receptacles, vaginae, and vulvae (Hartnoll, 1968; McLay & Becker, 2015; McLay & López Greco, 2011), with the particularity of having seminal receptacles capable of great variation in size and shape related to the amount of stored sperm.

4.2 | Seminal receptacles

4.2.1 | Gross anatomy

The SRs of *D. crenulatus* are a simple cul-de-sac lacking any of the internal structures described in other species, as for example the muscular diaphragm named "velum" that divides the receptacle of some spider crabs (Majoidea) in sperm storage and fertilization sites (Diesel,

1989, 1991; González-Pisani et al., 2011), or the "bursa" found in *Metacarcinus magister* (see Jensen and Bentzen, 2012; Jensen et al., 1996) and the pill box crab *Limnopilos naiyanetri* (see Klaus, Goh, Malkowsky, Becker, & Plath, 2014).

4.2.2 | Histology

In general terms, the histology of both the lined ventral cuticle and the dorsal extensible parts of the full SRs conform with previous descriptions in brachyuran crabs (Becker et al., 2011; Ewers-Saucedo, Hayer, & Brandis, 2015; González-Pisani et al., 2011; Hayer et al., 2015; McLay & Becker, 2015; Sal Moyano et al., 2010; Sant'Anna, Pinheiro, Mataqueiro, & Zara, 2007) with a soft secretory part, and a chitin lined part. In mature female *D. crenulatus*. The secretory part is responsible for the extreme size variation. The mechanism by which the enlargement and/or contraction takes place involves the collapsing of the lumen jointly with the folding of the secretory tissue wall, likely mediated by contraction of the muscle fibers of the external capsule, and possibly accompanied with changes at a cellular and tissue level.
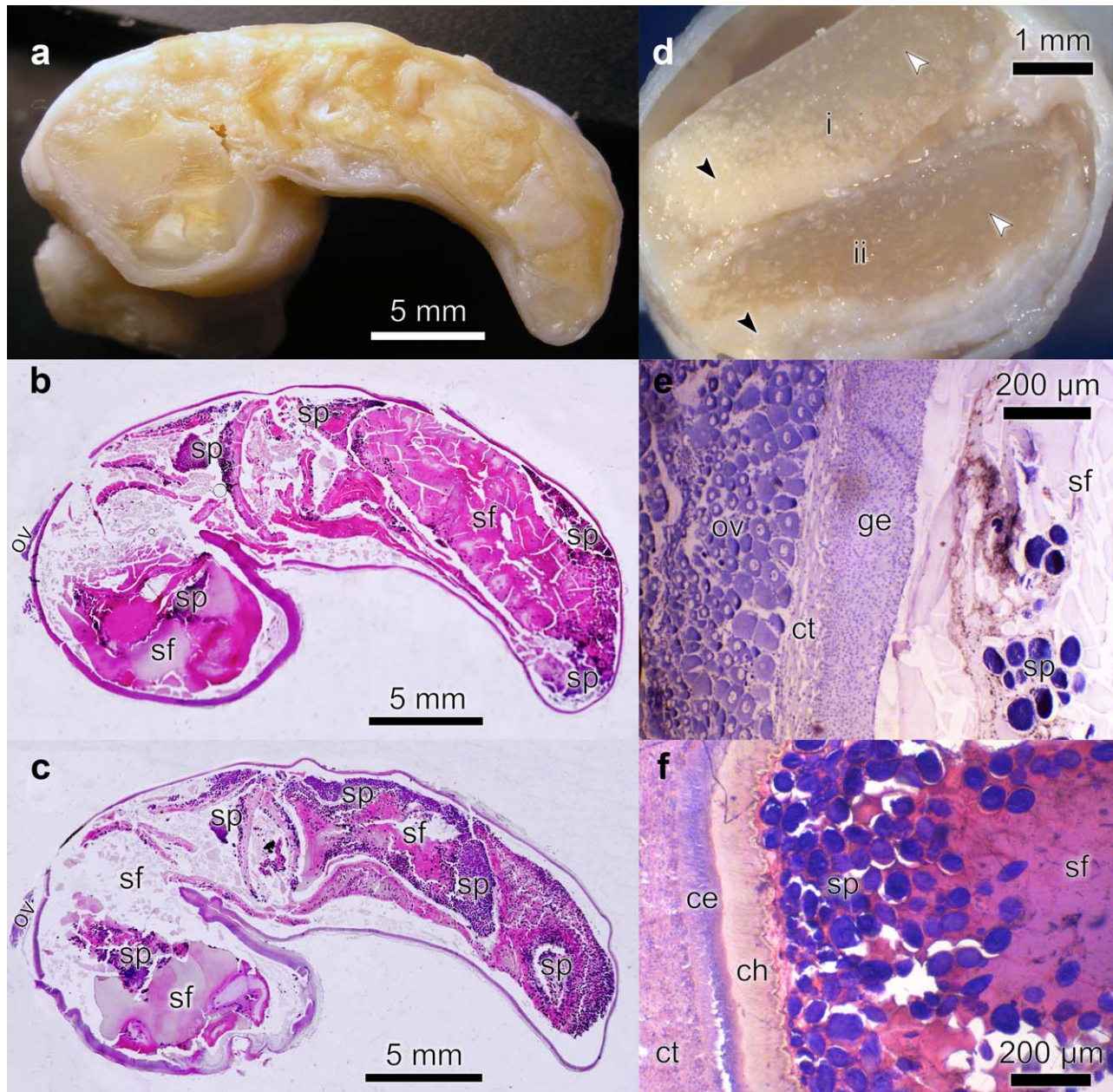
FIGURE 10 *Danielethus crenulatus*, female sperm storage pattern; (a) sagittal section of a full seminal receptacle showing the different sperm masses inside; (b and c) histologic sagittal sections of a full seminal receptacle at two different depths showing the pattern of the stored sperm masses formed by grouped spermatophores (dots in deep purple) and seminal fluids (homogeneous pink); (d) cross section of a fixed full seminal receptacle showing two different sperm masses inside (i and ii); the spermatophores appear as small whitish dots tightly aggregated at the lower-left area of each sperm mass (black arrowheads), leaving an area of translucent hardened fluid with few scattered spermatophores (white arrowheads); (e and f) cross sections of the receptacle showing spermatophores aggregations neighboring the wall of the dorsal and ventral parts, respectively. ce: columnar epithelium; ch: chitin lining; ct: connective tissue; ge: glandular epithelium of the dorsal part; ov: ovary; sf: seminal fluids; sp: spermatophores

Additionally, the chitinous ventral part might be involved in the extrusion of oocytes and mobilization of the sperm contents by contraction of the muscle fibers attached transversally to the ventral part (Figures 4a and 6b).

Secretory activity of the dorsal SR wall of brachyurans has been routinely addressed, but the role of these secretions remains unclear in almost all species studied to date (McLay & Becker, 2015; Zara, Raggi Pereira, & Sant'Anna, 2014). The usually proposed functions are sperm plug dissolution (Jennings, McLay, & Brockerhoff, 2000), sperm plug formation and maintenance of bacterial populations within the SRs (Jensen et al., 1996), and dehiscence of spermatophores and sperm maintenance (Anilkumar, Sudha, & Subramoniam, 1999; Diesel, 1989; Sal Moyano, Gavio, & Luppi, 2012). Depending on the species, the spermatophores are stored intact for several months (Diesel, 1989,
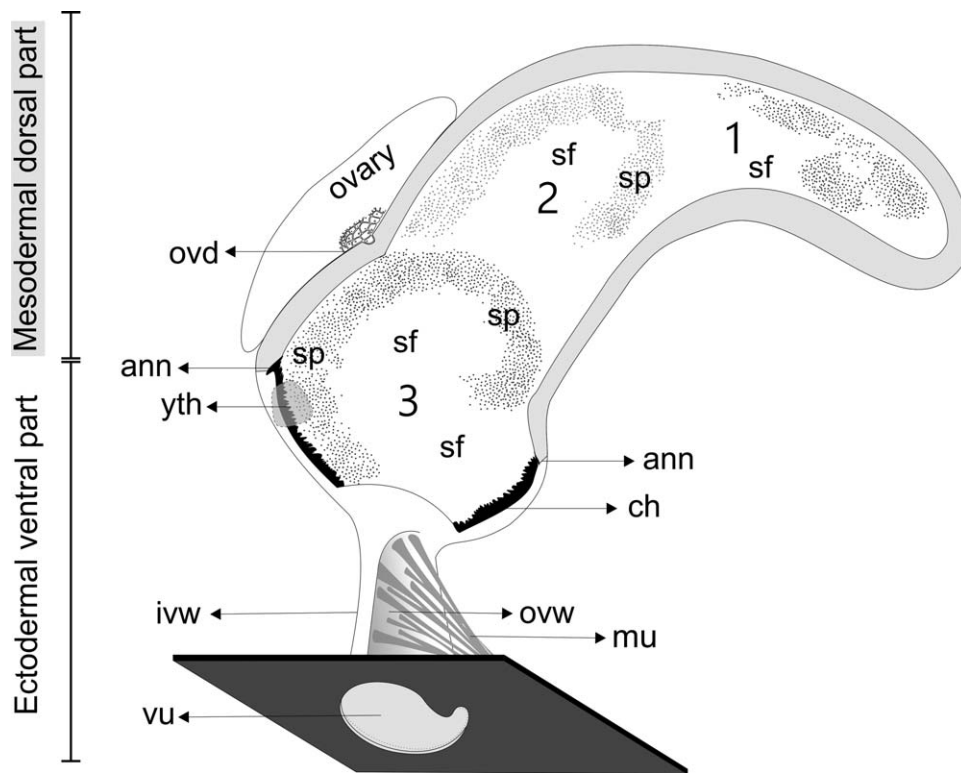
FIGURE 11 *Danielethus crenulatus*, scheme of a partial transverse section of a filled right seminal receptacle showing the internal arrangement of sperm masses and the distribution of spermatophores and seminal fluid within; a small portion of the ovary at mature stage is also shown. ann: annulus; ch: chitin layer; ivw: inner vagina wall; mu: muscle fibers; ovd: oviduct; ovw: outer vagina wall; sf: seminal fluids; sp: spermatophores; vu: vulva with mobile operculum; yth: yellow thickening of the ventral wall. 1, 2, and 3 are different sperm masses

1991) or dissolved shortly after copulation (Anilkumar et al., 1999). In any case, at the time of spawning the spermatophores must be dissolved and the spermatozoa set free for fertilization. With the only exception of ovigerous females (discussed below), the SRs of *D. crenulatus* contained almost exclusively intact spermatophores and hardened seminal fluids, with no free spermatozoa regardless of the season and the ovary or molt stages. This fact implies that most of the dissolution of the spermatophores and the sperm gel must occur shortly before spawning and suggests the existence of a mechanism linking sperm dissolution with ovarian maturity. Thus, the dissolution of the stored sperm is a likely function for the secretory tissue of the dorsal part found in full SRs of *D. crenulatus*.

The holocrine tissue found to occur in the internal lumen of the dorsal part of full SRs agrees well with that already described for heterotreme crabs (McLay & Becker, 2015). However, the lining of the dorsal lumen of the SRs shows marked differences according to the distinct reproductive stages, suggesting that changes in this secretory tissue have functional significance for the reproductive cycle. The columnar cells lining the dorsal part of the SRs of ovigerous females found in this study are comparable with the columnar epithelium described in immature stages of *Hyas coarctatus*, *Chionoecetes opilio* (see Lanteigne, Beninger, & Gionet, 1996) and *Paratelphusa hydromous* (see Adiyodi & Adiyodi, 1975). Particularly, the atypical elongated cells found in *Danielethus crenulatus* resemble the atypical cells found in tis-

sues with abnormal (neoplastic) growth involving mucsin-secretory epithelia and suggests rapid proliferative and secretory activities. A possible function of this lining might be the provision of lubrication and the necessary substances to facilitate the passage and fertilization of the oocytes and/or the expulsion/absorption of the sperm that was not used for oocyte fertilization. In turn, the abundant binucleated cells found lining the dorsal lumen of the empty SRs in non-ovigerous females may have a role in the secretion of nutrients for receiving new sperm, similarly to that described in the male reproductive accessory glands and ejaculatory duct (Radhakrishnan, Marchini, & Taylor, 2009) and female follicle cells (Szklarzewicz, Jankowska, Łukasiewicz, & Szymańska, 2007) of some insects.

4.3 | Ovary and oviduct

In the empty SRs of *D. crenulatus*, the ovary enters dorsally in close proximity to the distal end of the receptacle. This implies that an oocyte entering the SR from this point has to cross most of the sperm mass to reach the vagina opening; a situation that supposedly favors the fertilization by the first male to mate, and is associated to the dorsal receptacle type. However, as the SRs are filled up, the dorsal part elongates and extends forward and outwards, so that the SR-ovary connection is still dorsally located, but in a relative closer position to the vagina opening than to the distal end of the receptacles. From the

point of view of the chances of fertilization of the sperm masses, the full SR resembles more the situation posed by the "intermediate" or "ventral" types described in McLay and López Greco (2011). This is particularly interesting, because it links the type of SR (dorsal, ventral, or intermediate) with the amount of sperm stored, with implications for the individual mating strategies. McLay and López Greco (2011, table 2) placed platyxanthid crabs with others (such as panopeids and xanthids) showing hard-shell mating and a dorsal seminal receptacle types, but our present work suggests that *D. crenulatus* may be grouped with crabs which have hard shell mating and intermediate SRs, or even with those having ventral SR types (such as grapsoids and ocypodoids). This would suggest that platyxanthids have a last-male precedence rather than first male precedence.

4.4 | Vulva and vagina

A peculiarity of the SRs of *D. crenulatus* is the yellowish thickening of the ventral cuticle wall which, due to the asymmetry of the copulatory organs, supports the tip of the male gonopod during penetration. The function of this thickening in the cuticle wall remains unknown. It may be simply a structure to withstand the pressure exerted by the rigid tip of the first gonopod during mating. Alternatively, given that in male *D. crenulatus* the second gonopod is slightly longer than the first (as described for some brachyuran families including Platyxanthidae) (Ewers-Saucedo et al., 2015; Thoma et al., 2012), this structure may play a role during copulation by directing the tip of gonopod 2 and therefore the ejaculates within the SRs, similar to that suggested for box crabs (Calappidae; Ewers-Saucedo et al., 2015) and for *Ethusa mascarone* (see Hayer et al., 2016a).

4.5 | Sperm storage, delivery, and mating

The most outstanding feature of the SRs of *D. crenulatus* is their relative size when full, which reflects a large sperm storage capacity. The sperm storage capacity (1.4 ± 1.47 g) and the WW of a single full SR (2.6 ± 2.1 g) ranks among the largest in absolute terms reported to date. Other crabs of similar size have much smaller and lighter SRs (Jensen et al., 1996; López Greco & Rodríguez, 1999; Rodgers, 2010; Sainte-Marie, 2007; Sant'Anna et al., 2007), and even species that attain larger body size than *D. crenulatus* and are considered to have highly extensible SRs as *Chionoecetes opilio* and *Callinectes sapidus* (see Sainte-Marie, 2007) have receptacles of similar or smaller weight, reaching 1.8 g and 1.5 g, respectively (Hines et al., 2003; Sainte-Marie & Sainte-Marie, 1998). Except the work of Sainte-Marie (2007), which includes only six brachyuran species, studies dealing with interspecific comparisons of the female sperm storage capacity are lacking. Data on individual species appear sporadically in the literature (often anecdotal) and mostly report the weight of the contents or the whole SR and the body size of the respective female instead of its body mass, thus preventing a wider comparison using only SRI. A singular compilation of the wet weight of the SRs related to the size of the females can be found in Rodgers (2010). Because this data set is compatible with our data, we used it to compare the sperm storage capacity of *D. crenulatus*

with that of other Brachyura available and confirmed that D. crenulathus has the largest female sperm storage capacity known in Brachyuran crabs up to date (Supporting Information).

Unfortunately, there are no detailed descriptions of the SRs in other platyxanthids, but at least the extreme sperm storage capacity and thereby a high SRI % might be a feature shared by the family since *D. patagonicus* also has highly extensible SR that attain relative size close to that reported here (see figures in Leal, Dima, Dellatorre, & Barón, 2008). Moreover, for *Homalaspis plana* a WW of 0.80 ± 0.22 g per SR was reported by Rodgers (2010), ranking it as the heaviest among more than 30 species included in that study. An antagonistic relation between sperm storage and ovary development has been observed to occur in some species (Sainte-Marie, 2007; Zara et al., 2014) and hypothesized to be the result of the competition of these largely extensible organs for space within the female body (Sainte-Marie, 2007). Given the extreme size of the full SRs and the large volume occupied by the mature ovaries of female *D. crenulatus*, such competition for space seems likely. However, the alleged potential antagonism is not supported here since the variation in size of both organs was found to be independent.

Interestingly, the unusually large sperm storage capacity of female *D. crenulatus* was not accompanied by a particularly large maximum male deliverable sperm as might be expected from what appears to be the general pattern of Eubrachyura, where the mass of the female seminal receptacles is positively correlated with that of male testis and vasa deferentia (Rodgers, 2010). The VSI % in males was 0.7, much closer to that of other large crabs with comparatively smaller SRs (as *Cancer irroratus*, VSI % = 0.74 and SRI % = 0.19) (Sainte-Marie, 2007) than that of the species with the largest SRs (*Chionoecetes opilio* and *Callinectes sapidus*; VSI % = 2.03 and 3.27, respectively) (Sainte-Marie, 2007). Moreover, most males did not have enough sperm available to supply the average sperm stored in a full SR at once, with exception of the largest males that are rare in natural populations (Farias et al., 2014). Such difference between the weights of available sperm and female sperm storage capacities suggest that full SRs contain sperm from multiple mates, which in that case point to a role of sperm competition to explain the extreme extensibility of the SRs of *D. crenulatus*.

The spermatozoa of brachyuran crabs are nonmotile and therefore their chances to fertilize eggs depend largely on their location with respect to the place where the mature oocytes enter the SR. The arrangement of the sperm masses inside the full SRs of *D. crenulatus* suggests that new ejaculates displace the previously stored sperm distally, confining it to the blind end of the receptacle, far from the oviduct opening. The result of this configuration is that in full SRs the last ejaculate would have better chances of fertilizing eggs due to the favored location of its spermatophores in relation to the oviduct opening. This corresponds to the scenario of "last sperm precedence" which has been typically associated to the "ventral type" (Diesel, 1991; McLay & López Greco, 2011). In contrast, if the volume of sperm stored is small, then the spermatozoa from the first ejaculate would be the only one present or would be not far from the oviduct, thus enhancing their fertilization chances. Similar mechanisms for the displacement of

previous sperm were reported in other brachyurans as some portunids (Hartnoll, 1969; Spalding, 1942) and the majid *Inachus phalangium* (see Diesel, 1991). Hence, a more detailed study on the sperm dynamics of *D. crenulatus* may provide a new variant to the possible patterns of sperm use in Brachyura.

Large sperm storage capacity and the presence of intact spermatophores within the SR are both traits traditionally related to long-term sperm storage. Hence, the lack of trans-spawn sperm retention in females *D. crenulatus* is puzzling. None of the dissected egg-bearing females had sperm reserves in their SRs, and this fact could not be explained by chance alone. This implies that most of the residual sperm is discarded somehow, maybe absorbed by the glandular dorsal tissues of the SRs or expelled during or just after spawning as discussed above. The fact that the SRs of *D. crenulatus* lacks internal structures that could be used for dividing the sperm contents into two portions, one for the immediate use and the other for fertilization of future broods, is also coherent with the lack of trans-spawn sperm retention observed. Lacking or insufficient trans-spawn sperm retention is rare among the Brachyura studied up to date. It was observed in females of *Dyspanopeus sayi*, which also lack trans-molt sperm retention (Swartz, 1978) (under the former name *Neopanope sayi*). Swartz (1978) reported that some females did not have enough spermatozoa to fertilize a second egg mass, even after mating more than once between two spawns. Here, we did not follow the individual females but derive our conclusion from populational observations. Hence, a study performing experimental matings would be needed to definitely confirm this point.

Mating pairs found in the field confirm that female *D. crenulatus* copulate in a hard-shelled condition, as reported for the closely related stone crab *Homolaspis plana* (see Carvacho, Tapia, & Vidal, 1995). Hard shelled mating in *D. crenulatus* is possible due to mature females having flexible vaginas and vulvae with hinged opercula that allow gonopod penetration independently of the carapace hardness. Such decoupling between mating and moulting provides the female *D. crenulatus* the chance to resist actively the male mating attempts. Indeed, the strong muscles attached to the vulva and vagina walls suggest that the females may have additional control over mating by voluntarily opening the duct to facilitate the intromission of male gonopods as described in other species (Hartnoll, 1968; Klaus et al., 2014; McLay & López Greco, 2011).

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