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The ontogeny of the female reproductive system in the parasitic castrator pea crab *Calyptraeotheres garthi*: Implications for its mating system

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Abstract

The knowledge of the mating system of pea crabs is still fragmentary as it remains dubious whether females copulate in the juvenile and free-living 'hard' or in the obligatory symbiotic stages (adult stage 'V' or intermediate stages II to IV). To discriminate between these two possibilities, we analysed the female seminal receptacles, vagina and opercula, and the sperm content in different stages of the pea crab *Calyptraeotheres garthi*. Our histology and scanning electron microscopy results revealed that in the hard stage the seminal receptacle is simple without secretory epithelia, and vagina and opercula are not controlled by musculature. In stages II to IV, the seminal receptacles, vagina, and opercula are under development and these structures reach maturity in stage V. These results suggest that females become receptive in stage V and not during predating stages. We found no spermatozoa in SR of 'hard' and stage II to IV females while these structures were loaded of sperm in most stage V, indicating that females start to mate in stage V. Our results support the notion that males of *C. garthi* roam among hosts in search for sedentary stage V females, as predicted by Baeza and Thiel's (2007) model of mating systems for symbiotic crustaceans. Nevertheless, we failed to reveal whether females mate repeatedly: the accumulation of sperm in larger females might indicate occurrence of multiple copula or a high variability in male sperm transfer.

KEYWORDS
mating behaviour, parasite, Pinnotheridae, reproductive anatomy, seminal receptacle, spermatozoa

1 | INTRODUCTION

A species' mating system (i.e., the number of mates a male or female acquire during life-time and the circumstances in which mating occurs) and its sexual strategy profoundly influences the genetic structure of its populations and can therefore constitute a main driver of its evolution (Greenwood, 1980; Wright, 1965). The temporal and spatial distribution of receptive females appears to be the key element affecting the mating system in many taxa (Emlen & Oring, 1977). In symbiotic species (*sensu* de Bary, 1879), however, the evolution of mating systems in the smaller (= symbiont) partner mainly depends on host traits. The host constitutes the 'environment' in which most processes in the life of the symbiont occur, such as growth, feeding and reproduction (Baeza & Thiel, 2007). Therefore, host characteristics impose selection on the symbiont and determine the evolution of its mating system and sexual behaviour (Shuster, 2007; Thiel & Baeza, 2001).

For symbiotic crustaceans, Baeza and Thiel (2007) proposed a conceptual model in which the host's relative size, its complexity and abundance, and the predation risk off the host determine different mating strategies: monogamy, pure-search polygynandry with either mobile or sedentary females, female-centred polygyny, and host-defence polygyny (Baeza & Thiel, 2007). This is because the cost and benefits the male symbiotic crustacean experiences by host switching, roaming among hosts, host-monopolization, or by host guarding vary according to the host environmental factors (Baeza & Thiel, 2007). For instances, if host size is large enough as to harbour more than one female, the male benefits by monopolizing the host because it can access to copulate with all hosted females. However, if the host is too large the

energy resources the male spend defending the host against other potential male competitors could be too high. Then, host switching is more beneficial. The same holds when host abundance increases (because a densely distributed host reduce the costs associated to roam among them) or host complexity is high (because a complex host conformed by substructures is difficult to defend and monopolize). This theoretical framework received considerable support from descriptive and experimental studies (Baeza, 2008; Fernandes Rodrigues Alves, Hirose, Barros-Alves, & Baeza, 2017; Hernández et al., 2012; Jossart et al., 2014; Prakash, Kumar, Subramoniam, & Baeza, 2017). Nevertheless, in most symbiotic crustaceans there is little evidence on the exact timing (stage of ontogeny) of copulation and whether or not individuals mate several times.

Among symbiotic crustaceans, those pertaining to the Brachyuran family Pinnotheridae (pea crabs) engage in symbiotic associations with diverse macro-invertebrate hosts, such as bivalves, gastropods, polychaetes, ascideans, and echinoderms (Castro, 2015; Schmitt, McCain, & Davidson, 1973). The notable variety of sizes, abundances, and body plans that these host species exhibit makes pea crabs an interesting group for studying the influence of the host environmental factors on the symbiont's mating system. Some pea crab species (e.g., *Zaops ostreum*, *Fabia subquadrata*, *Nepinnotheres novaezelandiae*, *Calyptraeotheres garthi*) deserve special attention because of their peculiar life history. The invasive stage (i.e., the first crab stage after the larval cycle) colonizes the host and moults into the juvenile soft-shelled stage (Møller Christensen & McDermott, 1958). Later, both sexes moult into a well-calcified 'hard' stage that exhibits free-living adaptations facilitating to leave the host and move in the external environment (Hartnoll, 1972). Males reach sexual maturity in this stage (Becker, Klaus, & Tudge, 2013) but the ovary appears to be not yet developed in hard stage females (Becker, Brandis, & Storch, 2011). Hard-stage females recolonize the host and once inside they moult into the 'post-hard' stages (Atkins, 1926). In some species, four post-hard stages were identified (named II, III, IV, and V) and these stages are characterized by a soft exoskeleton and the progressive increment of the abdominal width (Jones, 1977; Ocampo, Spivak, Baeza, & Luppi, 2017). The ovary matures and females start to breed eggs soon after they moult into the stage V (Becker et al., 2011). The major enigma around the life history of these small symbiotic crustaceans is at which stage of female ontogeny copulation takes place. Mating was never observed in pea crabs but sperm masses were detected in the seminal receptacles (i.e., female internal organs storing sperm) of hard females in some pea crab species (*Pinnotheres pisum* – Atkins, 1926; *P. ostreum* – Møller Christensen & McDermott, 1958; *Fabia subquadrata* – Pearce, 1966; *P. bidentatus* – Hsueh, 2001). This suggests that females mate precociously, at least in those species. Whether or not post-hard females copulate or if mating is restricted to the hard stage is presently unknown. Nevertheless, post-hard mating appears to be possible in few species: Møller Christensen and McDermott (1958) found a hard male of *Zaops ostreum* with extended sexual appendages enclosed under the abdomen of a female of stage V, and Trottier and Jeffs (2015) observed males of *Nepinnotheres novaezelandiae* strongly attracted by females stage V in laboratory experiments. Until this has been resolved, the pea crabs' life history will remain dubious, and their possible mating systems will be incompletely understood.

In species where the small size and cryptic mode of life impose operative difficulties to collect direct observations, such as in pea crabs, the analysis of the anatomy of sexual structures during the ontogeny could help to reveal details of the mating behaviour. The development of some structures including the seminal receptacles, vaginae, and opercula (i.e., the structures that externally occlude the vaginal lumen) would correlate with the timing of copulation (Hartnoll, 1968). For instances, the seminal receptacles of some brachyuran species exhibit specialized secretory tissues associated with maturity (e.g., the apocrine glandular epithelium in some pea crabs—Becker et al., 2011). The physiological role of those secretory epithelia is not completely clear. Nevertheless, they likely interact with the spermatozoa, either to protect the sperm against infections, maintain it viable over time, or elicit it maturity, among others (Becker et al., 2011; McLay & Becker, 2015). Whatever the function, these epithelia should be developed at the moment of copulation. Furthermore, in some brachyuran crabs the operculum and vagina are mobile (muscle operated) playing two principal roles: to control egg-laying during extrusion and to permit females to choose which male to copulate with (McLay & Becker, 2015). Therefore, it is possible that operculum and vagina should be developed by the time female crabs start mating. Finally, the absence or presence of the sperm in the seminal receptacles can permit verification that female crabs have already copulated or not (filled vs empty receptacles).

The pea crab *Calyptraeotheres garthi* inhabits different gastropod species of slipper limpet (family Calyptraeidae) in the South-western Atlantic Ocean (Ocampo, Nuñez, Cledón, & Baeza, 2012). The limpet species colonized by *C. garthi* (e.g., *Crepidula cachimilla* and *Bostrycapulus odites*) are structurally simple and exhibit small body sizes in comparison to the crab body size (see Figures 1a and 4a). In the field, *C. cachimilla* and *B. odites* form dense aggregations. Baeza and Thiel (2007) stated that under condition of moderate or high host abundance and small host sizes, the evolution of a 'pure-search polygynandry of sedentary females' is expected in symbiotic crustaceans. In this mating category, females are solitaries and mate with males that abandon the host soon after copulation to find other sexual partners (Baeza & Thiel, 2007). Mature (stage V) females of *C. garthi* lead a solitary and sedentary lifestyle inside their hosts while adult males likely switch between different limpet individuals (Ocampo et al., 2012). This host-use pattern appears to support that this pea crab species displays pure-search polygynandry of sedentary females. Nevertheless, to confirm that this mating strategy has evolved in *C. garthi* it is necessary to determine the timing of copulation and specifically whether or not post-hard sedentary females copulate.

Here, we analyse the anatomy of the seminal receptacles, vagina and operculum in different stages of females of *C. garthi*. Considering the potential roles of the apocrine glandular epithelium (sperm maintenance, protection, maturity, among others, see Becker et al., 2011), we expected to find this tissues developed (or under development) from hard stage if the onset of mating in females occur at this stage. We also analyse the presence/absence of sperm to determine when females of *C. garthi* start to mate. Finally, as the amount of sperm in

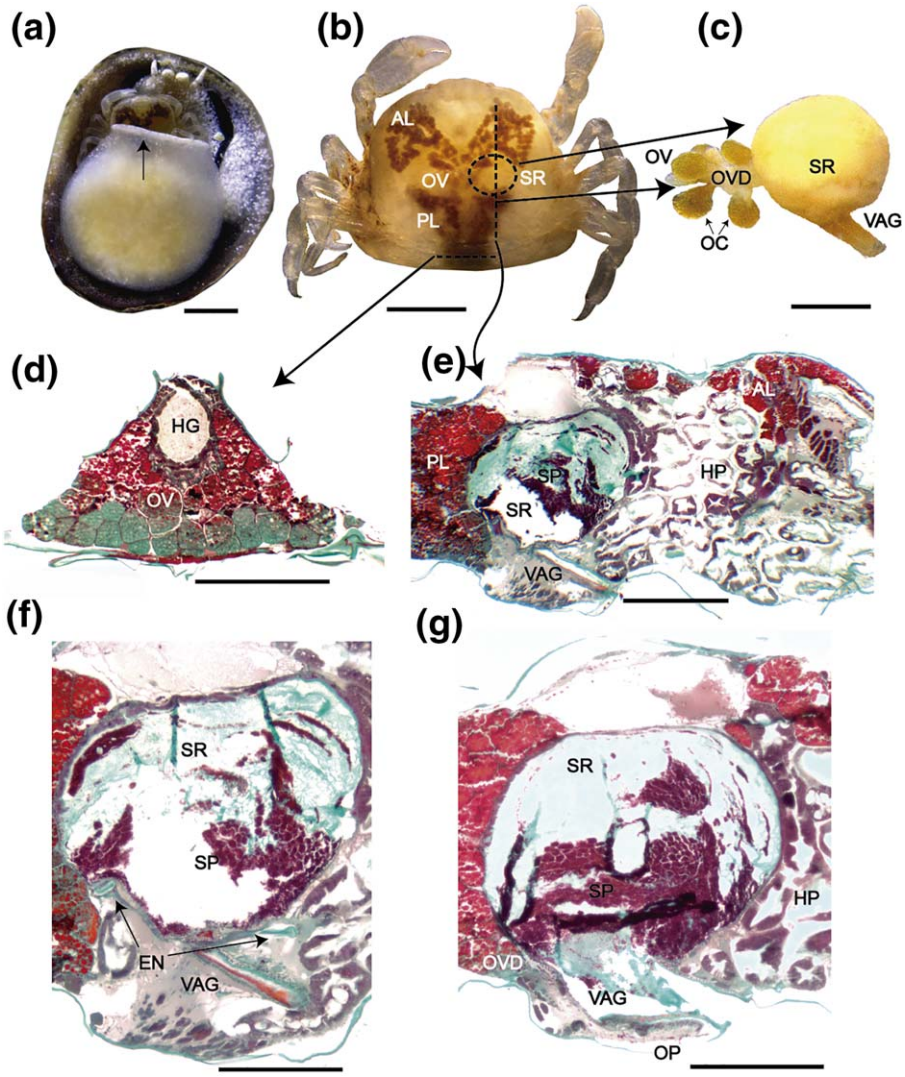

FIGURE 1 (a) *Calyptraeotheres garthi*, ventral view of the limpet *Bostrycapulus odites* harbouring a stage V female (arrow). (b) Dorsal view of a stage V female crab retrieved from its host. The ovary and the seminal receptacles (dotted circle) are observed through the thin and translucent tegument. Right and left ovary subunits are divided into anterior and smaller posterior lobes. Horizontal and vertical dotted lines indicate the sectioning planes in (d) and (e), respectively. (c) Lateral view of a fresh seminal receptacle, vagina and ovary containing oocytes (arrows). (d) Transversal section of the pleon with the ovary and hindgut. (e) Sagittal section of the carapace showing the seminal receptacle containing sperm, vagina, the anterior and posterior ovary lobes and the hepatopancreas. (f, g) Sagittal sections of the seminal receptacle. AL = anterior lobe of the ovary, EN = endophragmal system, HG = hindgut, HP = hepatopancreas, OC = oocytes, OP = operculum, OV = ovary, OVD = oviduct, PL = posterior lobe of the ovary, SP = sperm, SR = seminal receptacles, VAG = vagina. Scale bars: (a) 5 mm, (b) 2 mm, (c–e) 500 μm, (f, g) 200 μm

the seminal receptacles has never been analysed in Pinnotheridae we also measured the sperm content to determine whether or not this parameter vary during ontogeny of females.

2 | MATERIAL AND METHODS

2.1 | Collection of crabs

The symbiotic pea crab *Calyptraeotheres garthi* (Fenucci, 1975) was collected from two geographically segregated limpet hosts, *Crepidula cachimilla* at El Sótano (S 40°57′, W 65°06′) and *Bostrycapulus odites* at the intertidal zone of San Antonio Oeste (S 40°43′, W 64°55′). Both sample sites are located in San Matías Gulf, North Patagonia, Argentina. *Crepidula cachimilla* was sampled during September 2010 (end of the austral winter) using dredges deployed from a fishing boat (approx. 30 m depth). *Bostrycapulus odites* was sampled during November 2014 and February 2015 (austral spring and summer, respectively) by hand (approx. 0.5–1 m depth). *Crepidula cachimilla* is found attached to the mussel *Mytilus edulis* and other bivalve species while *B. odites* occurs mainly attached to small-sized rocks. After collection, host specimens were detached from its substrate and inspected for pea crabs, which are found between the ventral side of the neck and an anterior fold of the limpet's foot (see Figures 1a, 4a, and 5b). Female crabs retrieved from limpets were fixed 24 hr in Davidson solution or 4%

formaldehyde for histological and sperm counts procedures, respectively. Then, these crabs were individually stored in 70% ethanol. For scanning electron microscopy (SEM), female crabs were fixed 24 hr in 2.5% glutaraldehyde and then preserved in cacodylate buffer (Na 0.1 M, pH = 7.2). The carapace width (CW, as the distance across the carapace at the widest point) of sampled females was measured using a stereo microscope (Olympus SZX7) equipped with a calibrated ocular micrometre (precision = 0.01 mm). Each female crab was classified as pre-hard, hard, stage II, III, IV, or V according to its external shape and morphology of pleopods (Ocampo et al., 2017). Based on the appearance through the translucent dorsal carapace (see Figure 1b), the ovaries of stage V females were classified as 0—no apparent ovary tissue or non-developed yellowish ovary, I—red ovary not fully developed, II—deep red ovary, entirely full of oocytes.

2.2 | Histology and electron microscopy

A total of 12 female crabs were used for histology: three hard stage, one stage II, one stage III, one stage IV, and six stage V females. The carapace of each specimen was carefully detached at the posterior side and all pereopods removed at the coxa level allowing the fixatives and paraffin to enter into the tissues. Samples were dehydrated by transfer through a graded series of ethanol solutions with increasing concentration. Then, samples were embedded in paraffin and sectioned with a microtome (Leica RM2165, Leica Microsystems, Nussloch, Germany) at 5–8 μm. The trichromatic Masson-Goldner staining light green (Romeis, 1989) was used for tissue differentiation. Photographs were taken using a Leica MC 120 HD camera attached to the microscope. Contrast and/or brightness of some images were slightly adjusted using the software LAS 4.3.0 (Leica Microsystems Switzerland Ltd.). Histological work was conducted at the Department of Ecology and Evolution, J.W. Goethe-Universität, Frankfurt am Main, Germany.

A total of 15 female crabs were used for SEM-preparation (i.e., three female crabs from each stage). Samples were dehydrated through a series of ethanol solutions as above. Then samples were immersed in hexamethyldisilazane (HMDS) solution and dried at room temperature (23-25° C) for 12 hours. Samples were then coated with gold-palladium in a Denton Vacuum Desk II metallizer (Denton Vacuum, LLC, NJ, USA). Vulvae of female crabs from different stages were viewed and photographed with a Jeol JSM 6460LV (JEOL Ltd., Tokyo, Japan) microscope in the Electron Microscopy Laboratory at the Universidad Nacional de Mar del Plata, Argentina.

2.3 | Spermatozoa counts

We determined the sperm number in different female crab stages of *C. garthi* collected from the limpet *C. cachimilla* during the non-reproductive season (i.e., September 2010 during the austral winter, when no ovigerous stage V females are observed) and from *B. odites* during the reproductive season (November 2014 and February 2015 – austral spring and summer, respectively, when ovigerous females are found). A total of 13 hard, six stages II to IV (two specimens in each stage), and 45 stage V female individuals were dissected. In females of stage V, the thin and translucent cuticle of the carapace allows to easily localize from dorsal both seminal receptacles (Figure 1B). The excision of the seminal receptacles was conducted using a stereo microscope by first removing the pleon and localizing the sternal vulvae. Then, the cuticle surrounding the vulva was cut and the part of the cephalothorax containing the seminal receptacle, including the partial ovary, and the vagina were dissected out with forceps. Each piece was gently scraped to remove any remaining piece of muscles and/or ovary covering the seminal receptacle and vagina. The diameter (as the distance across the widest point) of each seminal receptacle was measured under the stereo microscope equipped with a calibrated ocular micrometre (precision = 0.01 mm). Each piece (i.e., seminal receptacles and vagina) was individually placed in a tube with 0.1 ml of distilled water and then homogenized with a manual homogenizer consisting of a plastic piston fitting into the tube. After homogenization, spermatozoa were stained by addition of 5 μl of methylene blue and then counted with the microscope using a glass hemocytometer. Prior to counting, we conducted a control experiment to determine whether or not sperm remained attached to the piston after sample processing, potentially leading to underestimation of sperm numbers. For this purpose, immediately after we used the piston it was placed in another tube containing 0.1 ml distilled water and strongly vortexed. Then, the water was stained and observed in a glass hemocytometer as explained before. This control was repeated three times using three different seminal receptacles. In these three replicates, a considerable low number of spermatozoa were found (less than 0.5% of the number of spermatozoa found in the seminal receptacle).

Ten microliter samples from each homogenate were used for sperm counting in the hemocytometer. The sperm number estimates were based on three sub-samples from each homogenate. The volume of each seminal receptacle was calculated using the formula: seminal receptacle vol. (μl) = $(4/3) \times \pi \times (\text{diameter}/2)^3$. The spermatozoa number and volume of right and left seminal receptacles were averaged in each individual. Pearson correlation tests were used to detect significant relationships between sperm number and body size, seminal receptacle volume and body size, and sperm number and seminal receptacle volume (Sokal & Rohlf, 1981). A Wilcoxon signed-rank test was used to determine whether sperm content was equally distributed between the right and left seminal receptacles in the analysed female crabs (Sokal & Rohlf, 1981).

3 | RESULTS

3.1 | The reproductive system of females in *calyptraeotheres garthi*

3.1.1 | Stage V females

Ovary

The ovary in stage V females of *C. garthi* exhibits an X-shape which is visible from dorsal through the thin and translucent cuticle (Figure 1a, b). The ovary consists of right and left strands or subunits, interconnected by a central bridge; each subunit is divided into the small posterior and large anterior lobes (Figure 1b,e). The posterior lobes of each

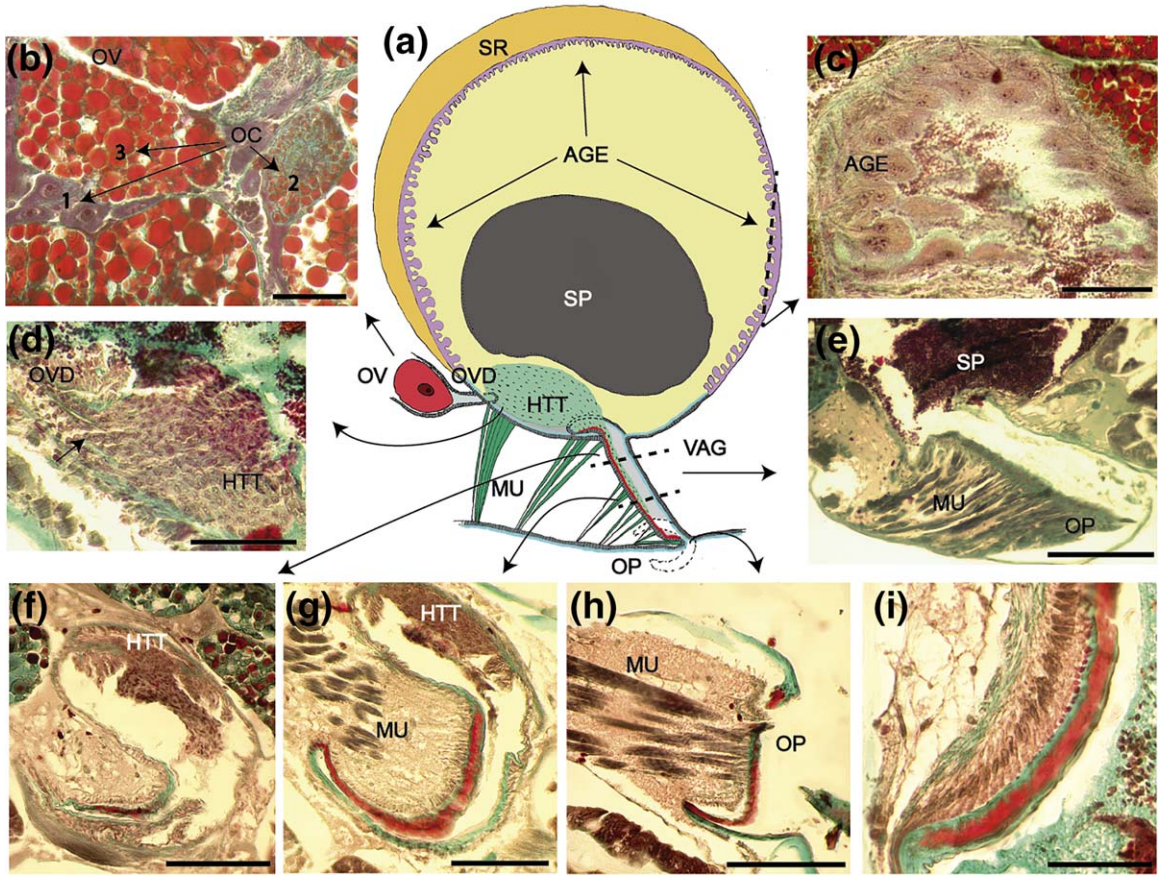

FIGURE 2 (a) *Calyptraeotheres garthi*, schematic overview (lateral perspective) of the seminal receptacle of stage V female. Sperm masses are typically located at the ventral area. The dorsal side of the seminal receptacle is lined by an apocrine glandular epithelium. The ventral side of the seminal receptacle is covered by cuticle and contains the holocrine transfer tissue. The seminal receptacle connects to the vagina and to the ovary through the oviduct. Muscles are externally attached to the seminal receptacle, vagina, and mobile operculum. Dotted lines indicate the sectioning planes in (e), (f), and (g). (b) Sagittal section of the ovary with oocytes in different stages of vitellogenesis and yolk drop accumulation (arrows 1, 2, 3). (c) Sagittal section trough the dorsal area of the seminal receptacle wall with the apocrine glandular epithelium surrounding free sperm. (d) Sagittal section trough the ventral part of the seminal receptacle wall. The arrow points to the oviduct connecting the ovary with the seminal receptacle by the holocrine transfer tissue. (e) Sagittal section through the ventral seminal receptacle, vagina and the muscular operculum, showing muscles. (F-G) Cross sections of the vagina. The holocrine transfer tissue runs through the vaginal lumen. Cuticle lining the mobile part of the vagina stains red. (h) Cross section of the muscular mobile operculum. (i) Closer view of the flexible part of the operculum. AGE = apocrine glandular epithelium, HTT = holocrine transfer tissue, MU = muscles, OC = oocytes, OP = operculum, OV = ovary, OVD = oviduct, SP = sperm masses, SR = seminal receptacles, VAG = vagina. Scale bars: (b) 50 μm, (c) 200 μm, (d–h) 100 μm, (i) 20 μm

subunit extend into the pleon (Figure 1d) and connect anteriorly with the seminal receptacle (Figure 1c,e,g). Histological sections show that this posterior lobe of the ovary extends from dorsal to ventral sides nearly filling this part of cephalothorax while the anterior lobe extends close to the dorsal tegument overlying the hepatopancreas (Figure 1e). We could identify oocytes in different vitellogenetic stages characterized by differences in size and amount of red and/or green-stained vitellogenic drops (Figure 2b). The oviduct consists of a single-layered epithelium that encloses the ovary and opens into the holocrine transfer tissue (see below) inside the seminal receptacle (Figure 2e).

Seminal receptacle

The seminal receptacles are spherical structures (Figures 1C,E–G and 2a). In the six sectioned specimens of stage V, the seminal receptacles store masses of sperm mainly located free in the ventral part of the receptacles (Figure 1f,g). In two individuals, spermatozoa were observed in aggregations, likely spermatophores (Figure 3f,g). Neither mucous layers separating portion of sperm nor sperm plugs were observed. A thin layer of connective tissue coats the external surface of the seminal receptacle and some muscle packages connect the ventral part of this receptacle with the sternal tegument. Few thin and small pieces of the endophragmal skeleton could be recognized that closely surround oviduct and vagina (Figure 1f). Vagina and oviduct are positioned close to each other; thus, the seminal receptacle follows the ventral type (*sensu* Diesel, 1989). The seminal receptacle is divided into ventral and dorsal areas. The ventral part connects to the vagina, and to the ovary via the oviduct (Figure 2a,d). The ventral part is internally lined by

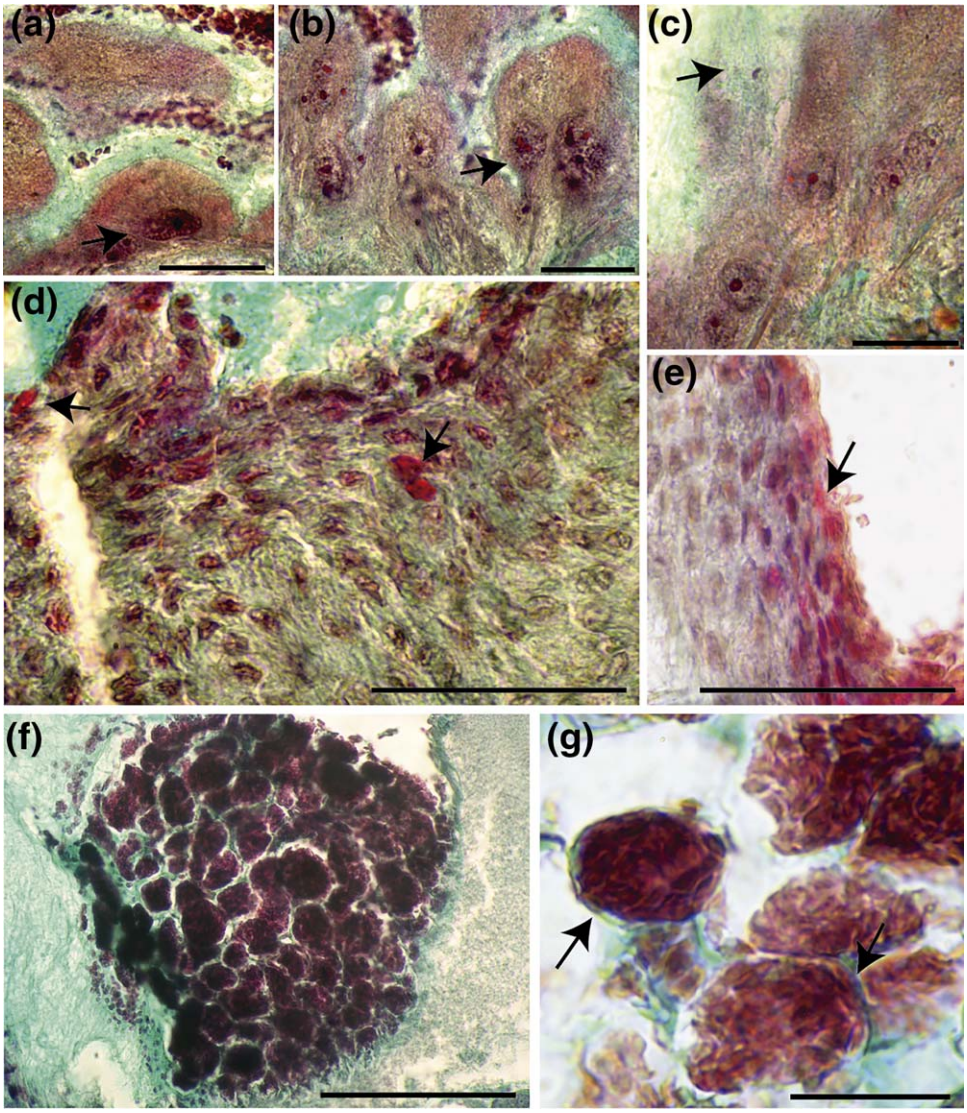

FIGURE 3 *Calyptraeotheres garthi*, (a, b, c) Apocrine glandular epithelium. Arrows indicate basal nuclei of cells (a, b) or the disintegrating apical part of the cells (c). (d, e) Holocrine transfer tissue. Cells facing the lumen take deep violet coloration; secretions staining light red (arrows) are observed in this zone. (f) Sperm aggregations inside the seminal receptacle. (e) Closer view of sperm aggregations, likely spermatophores. Arrows indicate the spermatophores' membrane (= pellicle). Scale bars: (a–f) 50 μm, (g) 10 μm

cuticle except for the area where it connects to the ovary. A secretory tissue, the holocrine transfer tissue (HTT, *sensu* Becker et al., 2011), is observed at the junction between the oviduct and the seminal receptacle. This tissue consists of a multi-layered mass of comparatively small cells with oval nuclei (Figure 2d). Transversal sections revealed the HTT extends into the vaginal lumen (Figure 2f,g). The HTT-cells facing the lumen of the receptacle appear to be dissolved, transforming into a light red stained secretion (Figure 3d,e). The dorsal part of the seminal receptacle is internally lined by a second specialized tissue, the so-called apocrine glandular epithelium (AGE, *sensu* Becker et al., 2011; Figure 2c). This secretory epithelium is more pronounced at the dorso-lateral walls than in the dorsal top of the receptacle (Figure 2a). The cells of the AGE are characterized by their comparatively large, basal nuclei, and lobed apical edges projecting into the receptacle lumen (Figure 3a,b). The apical part of the cells dissolves into a turquoise stained secretion (Figure 3c).

Vagina

The vagina, a short duct consisting of columnar epithelium, is lined by cuticle. It connects through a diagonal trajectory the most ventral wall of the seminal receptacle with the sternal vulva (Figure 1c,e). Cross sections show that the vagina is concave (*sensu* Hartnoll, 1968) with two distinct parts; the flexible 'inner vagina wall' and the non-flexible 'outer vagina wall' (*sensu* Diesel, 1989, see Figure 2e–i). The inner vagina wall is attached to the sternum by strong longitudinal muscle packets (Figure 2e) that control the opening of the vaginal lumen. The cuticle of the two walls stains differently; the flexible wall stains red while that overlaying the outer wall stains turquoise (Figure 2a,g–i). Cross sections revealed that the lumen of the vagina is asymmetric; at one side of the inner vagina the lumen is large and contains part of the HTT, at the other side the lumen is narrow (Figure 2f,g). The vagina entrance is occluded by a well-developed mobile operculum (Figure 6a). Sections reveal musculature inserting at the operculum indicating that this

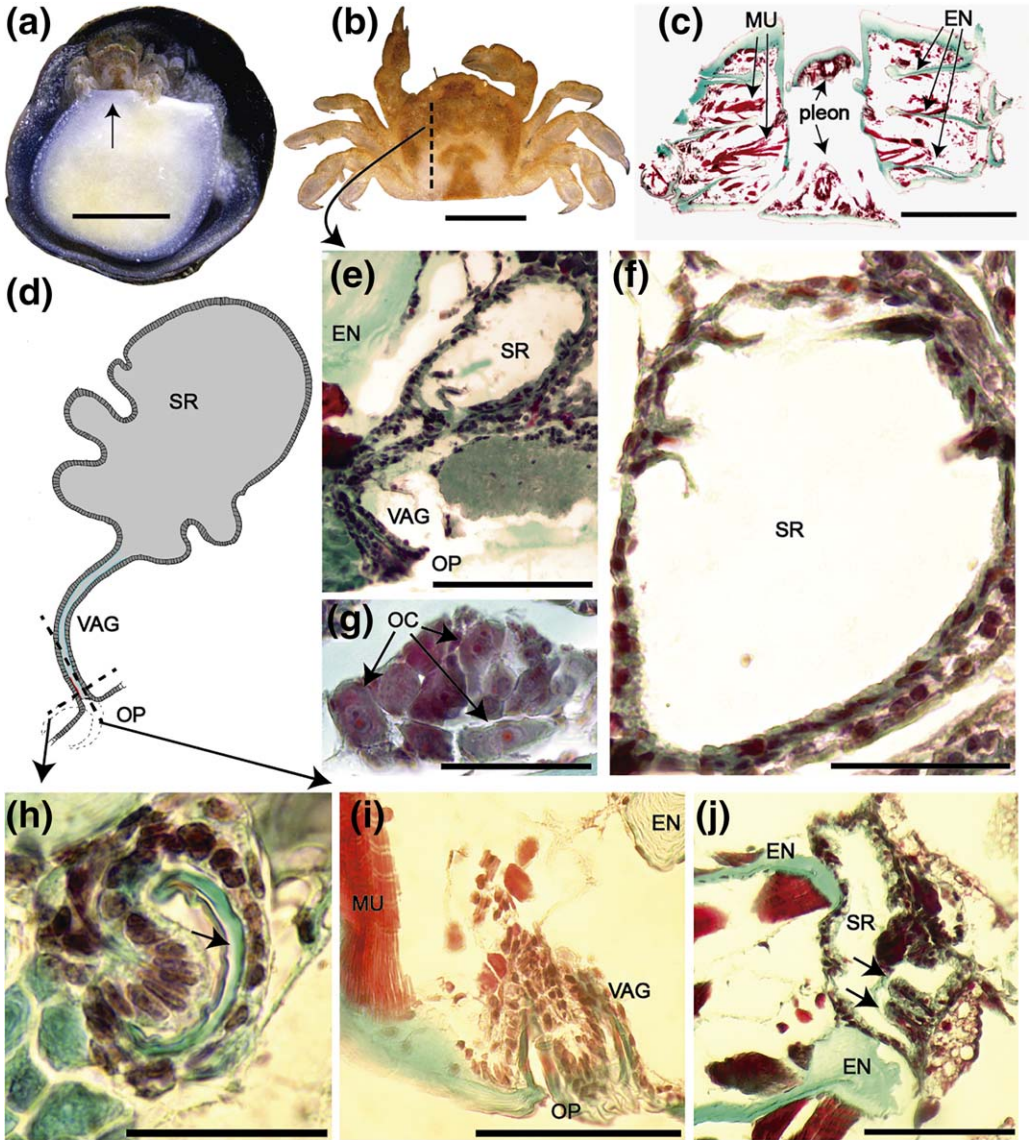

FIGURE 4 (a) *Calyptraeotheres garthi*, ventral view of the limpet *Bostrycapulus odites* harbouring a hard stage female (arrow). (b) Dorsal view of a hard stage female crab retrieved from its host. Vertical dotted line indicates the section plane in (e). (c) Section through the ventral cephalothorax showing the endophragmal skeleton system (turquoise) and muscles (red). (d) Schematic overview of the seminal receptacle from a lateral perspective. The wall of the seminal receptacle consists of one layer of simple epithelium. The vagina is lined by cuticle. (e) Sagittal section of the empty seminal receptacle, vagina and operculum. (f) Closer view on the seminal receptacle. (g) Transversal section of the pleon with previtellogenic oocytes. (h) Cross section of the vagina (section planes indicated with dotted lines in d). The arrow indicates the narrow gap between inner and outer walls of the vagina. (i) Sagittal section of the operculum (section planes indicated with dotted lines in D; flexible part stained turquoise and red) without muscles attaching this structure to the ventral tegument. (j) Section through the dorsal cephalothorax showing the empty and folded seminal receptacles surrounded by endophragmal skeleton (arrows: folds). EN = endophragmal system, MU = muscles, OC = oocytes, OP = operculum, SR = seminal receptacles, VAG = vagina. Scale bars: (a) 5 mm, (b, c) 2 mm, (e, j) 100 μm, (f, g–i) 50 μm

structure is muscle-operated (*sensu* Guinot, Tavares & Castro, 2013, see Figure 2e). As in the vagina, the flexible wall of the operculum stains red while the non-flexible wall stains turquoise (Figure 2h,i).

3.1.2 | Hard stage females

Ovary

Sections through the cephalothorax show transversal and longitudinal muscles attached to the exoskeleton and parts of the strong endophragmal skeleton system (Figure 4c). The ovary and oviduct are not identified in the cephalothorax. Some pre-vitellogenic oocytes are nevertheless detected close to the hindgut in cross sections of the pleon (Figure 4g).

Seminal receptacle

The seminal receptacles are small structures lined by a single-layered epithelium without cuticle (Figure 4d). In the three sectioned hard stage specimens, no spermatozoa are observed. Both sagittal and transversal sections failed to reveal any specialized secretory tissue similar to those observed in stage V females (Figure 4e). Seminal receptacles are found

closely surrounded by, and in some sections resting on, pieces of the endophragmal skeleton (Figure 4e,j). In some sections the empty seminal receptacles appear strongly folded (Figure 4j).

Vagina

The vagina is a long duct consisting of a single-layered epithelium apically lined by a thin cuticle, its inner wall staining greenish with parts red and the outer one greenish (Figure 4h,i). Cross sections reveal that the inner wall is not attached to muscles (Figure 4h). The inner wall extends into the outer wall which results in a narrow vaginal lumen (Figure 4h). The vulva consists of a small and non-developed operculum (Figure 6b). No musculature is observed attached to this structure (Figure 4i).

3.1.3 | Stage II, III, and IV (post-hard) females

Ovary

Previtellogenic oocytes are observed in the pleon of the post-hard stages II and III. Oocytes in different vitellogenic phases are identified in pleon and cephalothorax of stage IV females.

Seminal receptacle

In the three female post-hard stages II–IV (i.e., one specimen from each stage), the seminal receptacles are smaller than those of stage V females and they all exhibit a spherical or oval shape (Figure 5d). In the three specimens analysed histologically, the seminal receptacles do not contain spermatozoa. Externally (i.e., the outer side of the SR), connective tissue and, muscles as parts of the endophragmal system are observed similarly to stage V females (Figure 5d). The thickness of this endophragmal system decreases from stages II to IV. The cell morphology of HTT and AGE are similar to that previously described for stage V, and as in this last stage both tissues divide the receptacle in two areas, dorsal and ventral, in stages II to IV (Figure 5d–f).

Vagina

The vagina is similar to that described in stage V (Figure 5h). The size of the operculum increases with transition from stage II to IV (Figure 6c,d,e).

3.2 | Spermatozoa counts

No sperm was observed in the seminal receptacles of the 13 hard specimens dissected to evaluate the spermatozoa content sampled in three different seasons (austral spring, summer, and winter). Similarly, no sperm was found among the six (two in each stage II, III, and IV) post hard individuals. By contrast, 41 out of the 45 (91%) analysed stage V females contained spermatozoa inside their right and left seminal receptacles.

The sperm-bearing stage V crabs varied in body size from 3.2 to 7.7 mm CW (average $\pm$ *SD* = 5.61 $\pm$ 1.43; those retrieved from *C. cachimilla*) and from 3.9 to 6.5 mm CW (5.13 $\pm$ 0.83; those retrieved from *B. odites*). No ovigerous individuals were found among sperm-bearing stage V females retrieved from the host *C. cachimilla*, and 32%, 47%, and 21% of these crabs exhibited ovaries classified as 0, I, and II, respectively. In turn, 70% of the sperm-bearing stage V females retrieved from the host *B. odites* were ovigerous females, and 44%, 50%, and 6% of these individuals exhibited ovaries classified as 0, I, and II, respectively. The four stage V female individuals that had empty seminal receptacles were relatively small and non-ovigerous, two were retrieved from *C. cachimilla* (2.75 and 4.2 mm CW) and two were extracted from within *B. odites* (3.3 and 3.6 mm CW).

The sperm number per seminal receptacle varied from 6×10^4 to 4.6×10^6 cells ($1.95 \times 10^6 \pm 1.39 \times 10^6$) and 18×10^3 to 6.4×10^6 cells ($1.45 \times 10^6 \pm 1.69 \times 10^6$) in crabs retrieved from *C. cachimilla* and *B. odites*, respectively. A positive and significant relationship between the sperm number and body size was observed in stage V females retrieved from *C. cachimilla* (Pearson correlation test: $R = .661$, $p = .0015$, Figure 7a). By contrast, the correlation of sperm number and body size was not significant in those stage V females dwelling in *B. odites* (Pearson correlation test: $R = .154$, $p = .506$, Figure 7a). The volume of seminal receptacles ranged from 0.06 to 0.70 μL (average $\pm$ *SD* = 0.33 $\pm$ 0.19) in stage V females inhabiting *C. cachimilla* and 0.05 to 0.86 μL (0.28 $\pm$ 0.20) in those females retrieved from *B. odites*. A positive and significant relationship between seminal receptacle volume and body size was found in stage V females retrieved from *C. cachimilla* (Pearson correlation test: $R = .513$, $p = .0207$, Figure 7b). By contrast, the correlation of seminal receptacle volume and body size was not significant in those stage V females extracted from *B. odites* (Pearson correlation test: $R = .0596$, $p = .798$, Figure 7b). Finally, a positive and statistically significant relationship between sperm number and seminal receptacle volume was observed for stage V females inhabiting the two limpet species (Pearson correlation test, *C. cachimilla*: $R = .80$, $p < .0001$; *B. odites*: $R = .727$, $p < .0001$, Figure 7c).

Differences in sperm load between right and left seminal receptacles were detected in 10 out of 21 and 12 out of 20 individuals inhabiting *C. cachimilla* and *B. odites*, respectively. In those female crabs, differences in sperm number varied from 10% to 67%. Nevertheless, there was no bias in favour of right or left seminal receptacle for the spermatozoa number in females inhabiting *C. cachimilla* (Wilcoxon signed-rank test: $N = 21$, $p = .198$) and *B. odites* (Wilcoxon signed-rank test: $N = 21$, $p = .460$).

4 | DISCUSSION

The gross morphology of the reproductive system in adult stage V females of *C. garthi* is similar to that previously described in the European pea crabs *Pinnotheres pisum*, *Pinnotheres pectunculi* and *Nepinnotheres pinnotheres* (see Becker et al., 2011), especially the general shape of the ovary and shape and tissue composition of vagina and seminal receptacle. Also, the cell morphology of the glandular epithelia lining the seminal receptacles of stage V females appears to be similar to that reported previously in pea crabs (Becker et al., 2011). However, in the European pea crabs the HTT lines the seminal receptacle close to the oviduct while it extends into the vaginal lumen in *C. garthi*. Also, no pattern was observed in distribution of sizes in the cells of the AGE in European species while in *C. garthi* the portion of the AGE lining the lateral walls has larger cells than that covering the dorsal surface. Considering that the function of these secretory epithelia in brachyurans is

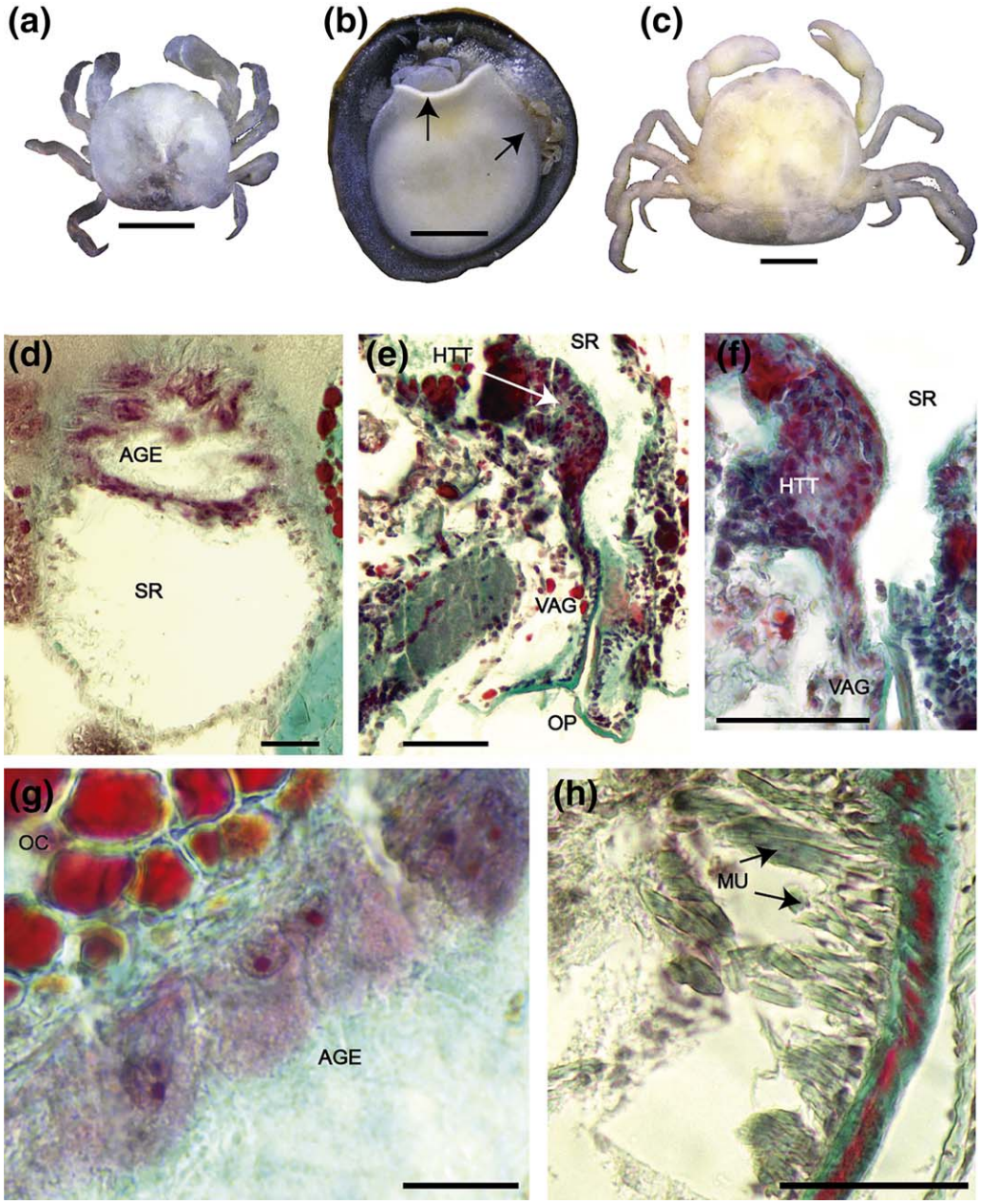

FIGURE 5 (a) *Calyptraeotheres garthi*, dorsal view of a stage II female. (b) Ventral view of the limpet host *Bostrycapulus odites* harbouring a stage III female (left arrow). Right arrow points to a second crab (sex and stage undetermined) inside the host. (c) Dorsal view of a stage IV female retrieved from its host. (d) Sagittal section through the empty seminal receptacle in a stage IV specimen. The apocrine glandular epithelium lines the internal dorsoventral walls. (e) Sagittal section through the ventral seminal receptacle and vagina of a stage III individual. The arrow points to the holocrine transfer epithelium. (f) Closer view of the holocrine transfer epithelium in the same individual. (g) Sagittal section through the dorsal wall of the seminal receptacle in a stage IV crab showing cells of the apocrine glandular epithelium. (h) Sagittal section of the vagina in a stage II individual. The mobile part of the vagina is attached to muscles. AGE = apocrine glandular epithelium, HTT = holocrine transfer tissue, MU = muscles, OC = oocytes, OP = operculum, SR = seminal receptacles, VAG = vagina. Scale bars: (a, c) 1 mm, (b) 5 mm, (d–h) 50 μm

incompletely understood yet, it is not possible to explain such differences among species depicted above. The anatomy of the reproductive system of female pea crabs in the ontogenetic stages predating stage V is described here for the first time. In the following, we discuss the anatomy of these structures and the number of spermatozoa within the seminal receptacles throughout post-larval ontogeny, its relation to the timing of copulation, and the mating system in *C. garthi*.

4.1 | Anatomy of the reproductive system, sperm content and timing of mating in females of *C. garthi*

During post-larval life, most pea crab species pass through a hard stage which exhibits morphological adaptations to live in open waters (e.g., plumose natatory setae on the legs, compressed body shape, and strong carapace hardness; Hartnoll, 1972; Ocampo et al., 2017). These characteristics can facilitate hard crabs to roam in the external

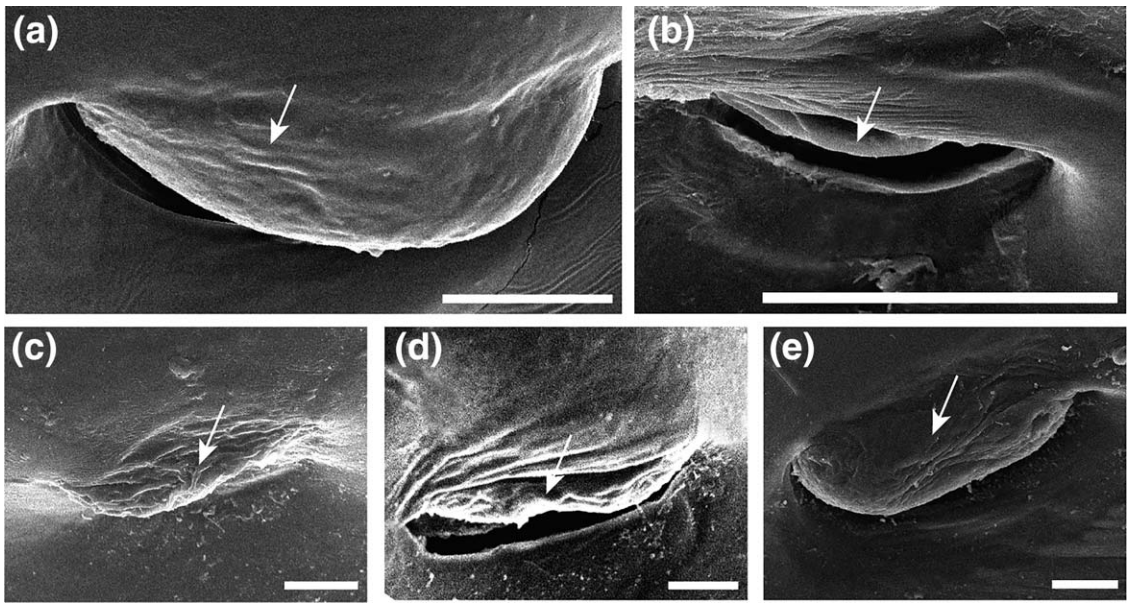

FIGURE 6 *Calyptraeotheres garthi*, scanning electron micrographs of the external appearance of the gonopore of females in different ontogenetic stages. (a) In stage V, the mobile operculum (arrow) has developed its maximum size. (b) Poor-developed operculum in hard stage. The operculum grows and develops while transitioning from II (c), III (d), and IV (e) stage female crabs. Scale bars: 50 μm

environment and switch among hosts while actively searching for sexual partners (Orton, 1920; Stauber, 1945). In some species inhabiting bivalves (e.g., *Pinnotheres bidentatus* – Hsueh, 2001), sperm masses were recognized inside the seminal receptacles of hard females indicating that copulation occurs during this stage (Atkins, 1926; Hsueh, 2001; Pearce, 1966). The hard stage displays the same free-living adaptations in *C. garthi* than in other bivalve-inhabiting pea crabs and hard crabs in this species apparently live part of their life outside their hosts (Ocampo et al., 2012, 2017). Therefore, we expected that copulation in females of *C. garthi* started in the hard stage. Surprisingly, we did not find any support for copulation in hard females of *C. garthi*. Neither hard females dissected to count sperm ($n = 13$) nor those sectioned during histological procedures ($n = 3$) collected during three seasons (austral winter, summer and spring) had sperm inside their seminal receptacles. Empty seminal receptacles were also found in the analysed females of stages II, III, and IV ($n = 9$). Instead, the seminal receptacles of most (91%, $n = 45$) stage V females of *C. garthi* were filled with sperm masses. Therefore, our data indicate that copulation in *C. garthi* takes place after females moult into the last post-hard stage and not earlier, during hard stage or post-hard stages II to IV.

In some brachyuran crabs (most thoracotremes, including members of Pinnotheroidea - Becker et al., 2011, and some heterotreme species —Hayer, Schubart, & Brandis, 2015), females exhibit a concave-type vagina with muscle-operated opercula (McLay & Becker, 2015), permitting the intromission of the male sexual organs upon contraction. The seminal receptacles of both thoracotreme and heterotreme brachyurans are internally lined by secretory epithelia. Secretions of those epithelia likely interact with the stored sperm, supposedly helping to maintain its viability, protect it from infections, elicit dehiscence of spermatophores, or a combination of some of these functions (Diesel, 1989; Jensen, Orensanz, & Armstrong, 1996; McLay & Becker, 2015). The ontogeny of the above organs (operculum, vagina, and seminal receptacle) has been little studied (but see Lanteigne, Beninger, & Gionet, 1996). However, considering they play essential roles either during the copulation (opening of the vaginal lumen) or after copulation (storing sperm) it is expected these structures would develop by the time females start mating. For instance, in some varunid species the operculum is not mobile until females pass the puberty moult and become sexually mature (McLay & Sal Moyano, 2016). Similarly, the secretory epithelia of the seminal receptacles in the swimming crab *Arenaeus cribarius* develop when females attain sexual maturity and start finding sexual partner (Zara, Pereira, & Sant'Anna, 2014). Whether or not these structures are already developed in the hard stage of pea crabs species that copulate in that stage (i.e., *Pinnotheres pisum*—Atkins, 1926; *P. ostreum*—Møller Christensen, & McDermott, 1958; *Fabia subquadrata*—Pearce, 1966; *P. bidentatus*—Hsueh, 2001) is presently unknown. In *C. garthi*, however, hard stage females appear not to be ready-to-mate, as they exhibit poorly developed opercula, vagina, and seminal receptacles. By contrast, all these structures seem to be under development and they become completely developed in stage V.

Altogether, the results from histology and SEM of the reproductive system as well as the absence of sperm within the receptacles of hard and II to IV stages indicate that females of *C. garthi* become ready-to-mate and start to copulate only in the last (V) post-hard stage. Earlier, during the transition from stages II to IV, the reproductive system is likely under development in preparation for the subsequent copulation. Muscle packages in vaginae and opercula control the process of egg-laying but also these structures could enable to select or reject sexual partners. In the hard stage, the absence of muscle packages might leave

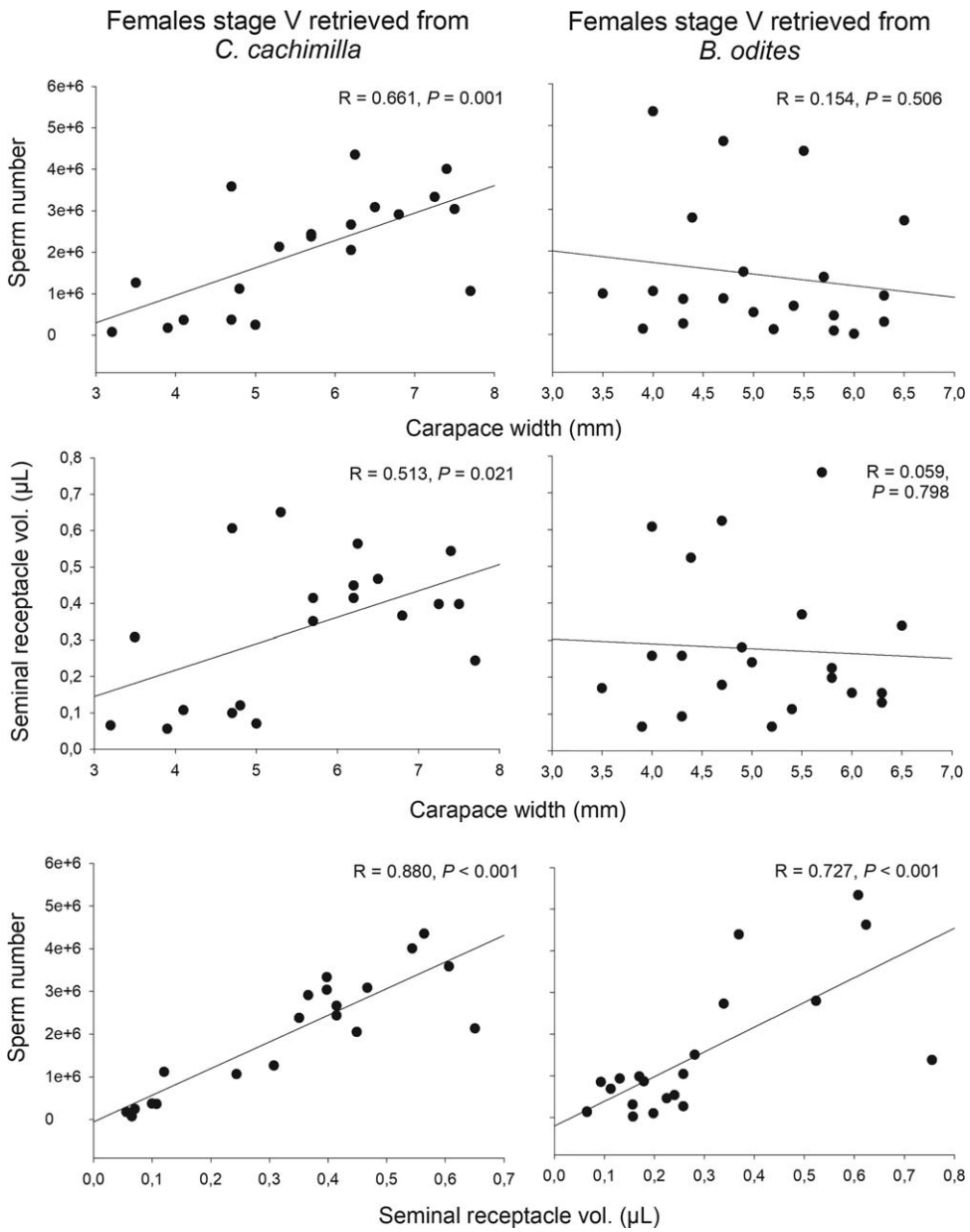

FIGURE 7 Relationship between sperm number and carapace width (above), seminal receptacle volume and carapace width (middle), and sperm number and seminal receptacle volume (below) in stage V females of *Calyptraeotheres garthi* retrieved from *C. cachimilla* during austral winter and *B. odites* during austral spring and summer (pooled data). *R* = coefficient of determination

females unable to control the movements of opercula and vagina, making it unlikely that this stage is engaged in copulation. Also, storing sperm masses appears to be impossible in the seminal receptacles of this stage. In addition to the simple structure, the seminal receptacles in the hard stage are closely surrounded by the rigid endophragmal skeleton which would not permit its enlargement after insemination.

4.2 | The mating system in *Calyptraeotheres garthi*

The Baeza and Thiel's model (2007) stated that the evolution of a 'pure-search polygynandry of sedentary females' mating system in a crustacean symbiont is favoured when its invertebrate host is abundant and small, such as the limpet hosts of *C. garthi*. In this mating system, symbiotic males maximize their reproductive success by roaming among hosts searching for immobile females which in turn maximize their success by choosing the 'better male' among the visitants. Accordingly, a previous study reported that females stage V of *C. garthi* lead a solitary lifestyle (one individual per host) living inside the same host for long time periods while hard males often move among host individuals (Ocampo et al., 2012). However, whether sedentary females in stage V copulate or not has been unknown in pea crabs before the present study. Nevertheless, in *C. garthi*, we herein provide the first conclusive evidence that females of stage V do copulate: (a) hard and stage II–IV post-hard females showed empty seminal receptacles and non-developed reproductive tracts; (b) sperm filled seminal receptacles and developed genital tract (including seminal receptacles) were found in

stage V females; (c) some small stage V females were found with empty seminal receptacles which also support that the onset of copulation occurs in this stage; (d) in two out of the six sectioned stage V individuals sperm aggregations of variable size and shape were observed. A thin membrane (= pellicle) lined some of these sperm clumps, which suggests these structures are indeed spermatophores. In Brachyura, the spermatophores dissolve shortly after their arrival in the seminal receptacle (Sainte-Marie, 2007; but see Beninger, Lanteigne, & Elner, 1993). Therefore, the presence of these structures in the seminal receptacles of females of *C. garthi* suggests mate have occurred recently, during the stage V. Overall, our present results indicate sedentary females stage V copulate and, together with a previous study (see Ocampo et al., 2012), suggest this species displays a 'pure-search polygynandry of sedentary females' mating system.

In *C. garthi*, a variable number of sperm was found in the analysed seminal receptacles of stage V females collected from *C. cachimilla* and *B. odites*. Seminal receptacle size in *C. garthi* increases proportionally to sperm number, as previously observed in other brachyurans (Rondeau & Sainte-Marie, 2001). This result indicates that the seminal receptacle size can be used as a proxy of sperm content in this species. Furthermore, in those females extracted from *C. cachimilla*, the sperm number correlates well with body size, indicating larger females accumulate more spermatozoa than smaller ones. This could be an evidence of multiple mating in stage V females. If females mate repeatedly we would expect that the sperm number increases over time as older crabs acquire more sperm from repeated copulation events than younger ones, as reported in other brachyuran species (e.g., the snow crab *Chionoecetes opilio*—Sainte-Marie, Sévigny, & Carpentier, 2003). Multiple mating has been discovered in most studied brachyurans (see examples and exceptions in Sainte-Marie, 2007), including one pea crab species: the ecto-parasite *Dissodactylus primitivus* (Jossart et al., 2014). However, the amount of sperm stored in seminal receptacles of brachyurans will depend not only on the frequency of mating but also on the quantity of sperm transferred in each copula (Rodgers, Reaka, & Hines, 2011; Sainte-Marie, 2007). Therefore, the increase in sperm number with body size herein observed may have to do with males ejaculating different sperm volume. In some brachyuran species (e.g., *Hemigrapsus sexdentatus* – Brockerhoff & McLay, 2005; *Eurypanopeus depressus* and *Rhithropanopeus harrisii* – Rodgers et al., 2011), males modulate the ejaculate according to female size, transferring larger quantities of sperm when mating with larger females. Our present data do not allow us to discriminate between the above hypotheses. The analysis of the sperm content in the vas deferens of males as well as controlled experiments using virgin females might help to unmask these unknown aspects of the mating behaviour of *C. garthi*.

We cannot conclusively explain the differences in sperm charge within seminal receptacles between females of *C. garthi* collected from *C. cachimilla* and *B. odites*. While sperm number positively scale with body size in females retrieved from *C. cachimilla*, in those extracted from within *B. odites* sperm number does not show significant correlation with body size. In brachyurans, both quantity and rate of sperm transfer by males might vary according to the distribution of male and female in space and time (Sainte-Marie, 2007). In *C. garthi*, pronounced differences in the frequency of male and female crabs have been found between the two limpet hosts (Ocampo et al., 2012). In *C. cachimilla*, the prevalence of pea crabs reaches up to 70% with a burden (= number of crabs per host) of up to four crabs per limpet while pea crabs are rare in *B. odites* reaching prevalence of 5% and two crabs as maximum observed burden (Ocampo et al., 2012). In the pea crab population that dwells *C. cachimilla* males would easily find females whilst male-female sexual encounters would be infrequent for pea crabs associated to *B. odites*. Differences in those crab frequencies could result in the present sperm storage pattern. In the case of females from *C. cachimilla*, the higher probability of sexual encounters would result in a progressive accumulation of sperm (either because older females copulate more times than younger or because males modulate the ejaculate volume). In contrast, the lower probability of mates in the pea crabs inhabiting *B. odites* would results in males transferring as much sperm as possible in each infrequent mating. This would explain why some small females of stage V found in *B. odites* stored high quantities of sperm in their seminal receptacles.

5 | CONCLUSIONS

Our results indicate that females of *C. garthi* became receptive and start to mate during the last post-hard stage. Male pea crabs likely mate with different sedentary females while roaming among host individuals, in consistency with theoretical predictions (Baeza & Thiel, 2007). Stage V females of *C. garthi* may mate repeatedly, thought this possibility needs to be further investigated. Classical literature considers that the Pinnotheridae exhibit a unique life history (Atkins, 1926; Orton, 1920; Pearce, 1966; Stauber, 1945). In contrast, recent studies indicate that these symbiotic crustaceans display a wide range of host-use patterns (De Bruyn, Rigaud, David, & De Ridder, 2009; Hamel, Ng, & Mercier, 1999; Ocampo et al., 2012), host exploitation modes (i.e., commensalism—Grove, Finelli, Wethey, & Woodin, 2000, mutualism—Campbell, 1993, parasitism—Ocampo, Nuñez, Cledón, & Baeza, 2014), and different mating strategies (Jossart et al., 2014; present study). Considering the vast disparity of hosts that members of this family inhabits, a challenge in the future will be to analyse whether different pea crab species have evolved toward different mating systems as suggested by Baeza and Thiel's (2007) model.

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AUTHOR CONTRIBUTIONS

E. O., T. L., E. S., and S. K. designed the study; E. O. and T. L. sampled specimens; E. O. conducted the laboratory work (SEM, histology, sperm count) and performed the statistical analyses; E. O. and S. K. wrote the manuscript.

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