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Ontogenetic changes in the external anatomy of the parasitic castrator crab *Calyptraeotheres garthi*: implications for the timing of host colonization and sexual behaviour

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Pea-crabs are symbiotic crustaceans that live in association with a diverse array of macro-invertebrate hosts. Some pea-crabs exhibit an unusual and incompletely known post-larval cycle characterized by the alternation of free-life and symbiotic forms. We analyzed post-larval morphology, the allometry of various body parts, and sexual dimorphism in *Calyptraeotheres garthi*, an endosymbiotic pea-crab infesting the brooding chamber of limpets in the southwestern Atlantic. In *C. garthi*, the smallest invasive crab moults into a male or female pre-hard stage, which is immediately followed by a hard stage. Then, hard-stage females, but not hard-stage males, pass through four post-hard stages before attaining a fifth terminal stage. The invasive and hard stages exhibit morphological traits (plumose natatory setae on the legs, compressed body shape, and moderate or strong carapace hardness) that likely permit them to swim efficiency while outside of hosts and entering and/or leaving host individuals. In contrast, pre- and post-hard crabs are well endowed for an endosymbiotic lifestyle featuring a soft and rounded carapace, and slender appendages. The allometry of selected traits suggests that males attain sexual maturity during the hard stage and likely roam among host individuals in search of mating opportunities. It remains unclear at which moment females become sexually active and whether hard females abandon host individuals in search of sexual partners. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000–000.

KEYWORDS: crustacean – dimorphism – mating system – pea-crab – post-larval cycle – symbiosis.

INTRODUCTION

Phenotypic traits result from the interaction between genes and the environment acting over the ontogeny of individuals (Schmalhausen, 1949; Mayr, 1997). The environment consists of a complex set of abiotic (e.g. temperature) and biotic conditions (e.g. preys and predators, parasites, competitors). In species that have adopted a symbiotic lifestyle (symbiosis defined as two or more dissimilar species living together – de Bary, 1879), however, a smaller partner (the symbiont) acquires food, grows, and also reproduces in or on a second larger partner (the host) (Ross, 1983). Consequently, hosts represent in many aspects the whole 'environment' in which symbionts live, partially or entirely, their lifespan (Renaud, Clayton & De Meeüs, 1996). Considering the above, any anatomical, physiological and/or reproductive modification exhibited by the symbiont is understood as an adaptive response to the selective pressures imposed by the 'host environment' during their co-evolutionary history (Renaud *et al.*,

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1996; Sotka, 2005). Symbiotic associations constitute excellent biological systems to explore the role that the environment (the host) plays in favouring or constraining particular anatomical and/or behavioral features in symbionts.

Symbiotic associations are widespread in marine and terrestrial environments (Margulis & Fester, 1991). In the marine realm, crustaceans may act both as hosts and as symbionts and develop associations with other invertebrates belonging to many different phyla. As symbionts, they participate in relationships characterized for their unrivalled diversity, ecological, and economical importance (Ross, 1983; Castro, 2015). In some taxa, symbiotic crustaceans exhibit a high degree of specialization and associate only with a restricted number of hosts. For instance, rhizocephalan barnacles have only been recorded as parasitic castrators of crustaceans (mainly decapods and stomatopods) (Høeg, 1995). Other taxa of symbiotic crustaceans, however, associate with a vast diversity of hosts, such as corals, mollusks, sea anemones, sea urchins, ascidians, and other crustaceans (Baeza, 2015; Castro, 2015). The latter group includes, among others, symbiotic crabs in the brachyuran family Pinnotheridae. Crabs from this family, also known as pea-crabs, comprise species that live in association with benthic invertebrate hosts (Schmitt, McCain & Davidson, 1973; Palacios Theil, Cuesta & Felder, 2016). Pea-crabs typically live inside of the mantle cavity of gastropods and bivalves (e.g. the pea-crab *Tumidotheres maculatus* inside of *Mytilus edulis* – Bierbaum & Shumway, 1988), in the atrial chamber of ascidians (e.g. *Tunicotheres moseri* in *Styela plicata* – Ambrosio & Baeza, 2016), on the surface of irregular echinoids (e.g. *Dissodactylus primitivus* in *Meoma ventricosa* – Pohle & Telford, 1982), within the cloaca of sea urchins (e.g. *Pinaxodes chilensis* in *Loxechinus albus* –Vásquez Rojas & Bay-Schmith, 2010), as well as in galleries constructed by worms and other burrowing crustaceans (e.g. *Austinixa aidae* in burrows of *Callichirus major* – Peiró, Pezzuto & Mantelatto, 2011). The species richness attained by the family Pinnotheridae and the disparity of macro-invertebrate taxa used as hosts by these species suggest that pea-crabs represent a model system to study which conditions (i.e. host traits) favour or constraint the evolution of morphological adaptations in marine symbiotic species.

Perhaps one of the most remarkable, and still incompletely known, aspects about the life history of pea-crabs is the morphological changes that these crabs experience throughout the progression of post-larval stages. After settlement and metamorphosis, the post-larval development in pea-crabs deviates substantially from the classical pattern exhibited by free-living brachyuran species. Pea-crabs inhabiting bivalves, for instance, typically exhibit a complex post-larval life cycle characterized by the alternation of free-living and symbiotic stages and concomitant shifts in morphology (Hartnoll, 1972). In some species, the first crab instar that develops immediately after the megalopa (last larval stage) is considered the invasive stage, capable of colonizing host individuals for the first time (Møller Christensen & McDermott, 1958; Pearce, 1966). This invasive first crab exhibits morphological traits that allow efficient swimming in the open water as well as host invasion; well developed setae on their ambulatory legs (pereopods), a rather flat profile with a dorso-ventrally compressed cephalothorax, and a well calcified exoskeleton (Møller Christensen & McDermott, 1958; Pearce, 1966). After host colonization, the invasive stage moults into a soft-shelled 'pre-hard' crab stage which, in turn, moults again into a second hard-shelled form named the hard-stage (Pearce, 1966; Jones, 1977a). Hard-stage crabs are typically described as free-living although they can also be found living within body cavities of host individuals (Pearce, 1966). Hard-stage crabs are similar in morphology to invasive first crab stages, also being capable of move along the external environment and also swimming efficiently in open water (Hartnoll, 1972). Male and/or female hard-stage crabs leave their host individuals in search of mating partners (Pearce, 1966). In some pea-crabs, mating between male and female hard-stage crabs appears to take place within host individuals (e.g. *Zaops ostreoum* that lives inside of the oyster *Crassostrea virginica* – Møller Christensen & McDermott, 1958) while in other species, mating seems to occur during 'copulatory swarmings' in the open water (e.g. *Fabia subquadrata* inhabiting the mussel *Modiolus modiolus* – Pearce, 1966). The hard-stage appears to be the terminal stage in males (Pearce, 1964, 1966). By contrast, females continue growing and developing after mating, passing through other four well defined stages that differ in external morphology (i.e. stages II–V) that are invariably found in body cavities of host individuals. Females store sperm in seminal receptacles during the II–IV post-hard stages (Becker, Brandis & Storch, 2011). All female post-hard stages have a decalcified, soft, and membranous cuticle similar to that found in pre-hard-stage crabs (Stauber, 1945). The ovary becomes mature when females achieve the fifth stage, and at that point in time, they start spawning and brooding eggs during the rest of their life (Møller Christensen & McDermott, 1958).

The post-larval cycle depicted above is often assumed to occur in most pea-crab species (e.g. Bierbaum & Shumway, 1988; Becker *et al.*, 2011). Nonetheless, the post-larval behaviour and morphology of only a few species in this taxon have been

properly described. To date, detailed studies on the post-larval life of pea-crabs are those by Møller Christensen & McDermott (1958), Pearce (1966), and Jones (1977a), in the bivalve-inhabiting pea-crabs *Zaops ostreum*, *Fabia subquadrata*, and *Nepinnotheres novaezelandiae*, respectively. Importantly, in *Zaops ostreum* and *Fabia subquadrata*, neither drawings nor pictures of the different post-larval stages were provided by the authors (Møller Christensen & McDermott, 1958; Pearce, 1966). In turn, in *N. novaezelandiae*, not all crab stages were dissected and features described; the first crab was not found. Other published studies report detailed but partial descriptions of a few stages (Atkins, 1926; Stauber, 1945; Hartnoll, 1972; Watanabe & Henmi, 2009). Conversly, a few other ecological studies have demonstrated that pea-crabs that inhabit invertebrate taxa other than bivalves do not exhibit the post-larval life cycle described above (Pohle & Telford, 1982). For instance, species of *Dissodactylus* that spend their entire life on the external surface of irregular echinoids do not have soft-shelled stages throughout its post-larval life (Telford, 1978; Pohle & Telford, 1982). Thus, different species of Pinnotheridae associated with different hosts might exhibit notable morphological disparity throughout the post-larval cycle. Certainly, there is a need for studies on the morphology of post-larval stages in pea-crabs that inhabit host taxa other than bivalves.

Understanding ontogenetic changes exhibited by pea-crabs after settlement and recruitment to the benthic population may shed light into poorly known life history events happening later during the life of symbiotic crabs. For instance, several landmarks in the post-larval life of pea-crabs, including the timing of host initial colonization, maturity, and sexual activity are expected to correlate well with particular morphological changes (Møller Christensen & McDermott, 1958; Pearce, 1966). Furthermore, drastic differences in shape among different life stages within a species are known to produce taxonomic problems (Campos, 1989). For instance, the pea-crab *Pinnotheres reticulatus* was erected as a new species based on the description of a single pre-hard individual which later, after more detailed morphological studies, was demonstrated to belong to the already described *Juxtafabia moliniarum* (Campos, 1993). Therefore, studies focusing in describing changes in morphology during the post-larval life of pea-crabs can improve our ability to accurately identify different species within this group. In the present study we focused in describing the external morphology and changes in body shape occurring after settlement in the pea-crab *Calyptraeotheres garthi*.

Calyptraeotheres garthi belongs to the subfamily Pinnotherinae, a taxon that most often infest bivalve hosts (Manning, 1993). Interestingly, *C. garthi* exclusively inhabits gastropods pertaining to the family Calyptraeidae (limpet snails), including *Crepidula cachimilla*, *C. argentina*, and *Bostrycapulus odites*, among others (Ocampo *et al.*, 2014). A previous study has described the larval development of this species that comprises five zoeae larvae and one megalopa (Ocampo *et al.*, 2011). After settlement, crabs of *C. garthi* inhabit the brooding chamber of limpets and feed on plankton-rich mucus strings produced by their host individuals (Ocampo *et al.*, 2012, 2014). *Calyptraeotheres garthi* is considered a 'parasitic castrator' given that large female crabs physically impede host reproduction (see Ocampo *et al.*, 2014). *Calyptraeotheres garthi* also exhibits behavioral similarities with bivalve-dwelling pea-crabs. For instances, large females lead a solitary and sedentary life in limpets while males likely roam among different host individuals (Ocampo *et al.*, 2012), as reported in *Pinnotheres pisum*, *Z. ostreum*, and *F. subquadrata* (Orton, 1920; Møller Christensen & McDermott, 1958; Pearce, 1966). Whether *C. garthi* exhibits a sequence of 'metamorphic' morphological changes similar to that reported in some pea-crabs that dwell in bivalves, however, needs to be investigated. Herein, we described the post-larval morphology of both male and female crabs. We have focused on describing those morphological features that correlate well with the timing of host colonization, mating, and maturity (e.g. exoskeleton hardness, development of swimming setae). We also examined the allometry of various body parts in *C. garthi*, including male gonopods and female pleon (= abdomen), considering that the allometric status of the traits above most often evidence important ontogenetic landmarks, including sexual maturity in crustaceans (Hartnoll, 1982).

MATERIAL AND METHODS

Collection of hosts and crabs

Individuals of the limpet snail *Crepidula cachimilla*, host to the symbiotic pea-crab *Calyptraeotheres garthi*, were collected during August and September, 2010 in San Matías Gulf (40°57′S 65°06′W), North Patagonia, Argentina. *Crepidula cachimilla* were sampled from the subtidal (~30 m depth) using dredges deployed from a fishing boat. Immediately after collection, host specimens were detached from its natural substrate, the mussel *Mytilus edulis*, 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 Ocampo *et al.*, 2014). Then, all limpets and crabs retrieved from them were fixed (4% formaldehyde), stored

individually in 50 mL flasks, and transported to the laboratory. There, the following dimensions were measured under the stereomicroscope (Olympus SZX7) equipped with a calibrated ocular micrometre (precision = 0.01 mm): carapace width (CW, as the distance across the carapace at the widest point), carapace height (CH, as the distance across the cephalothorax at the maximum height), pleon (abdomen) width (AW), at the junction between the second and third segment of the pleon in pre-hard, hard, and stage II crabs; at the fourth segment in stage III and stage IV crabs; and at the junction between the fourth and fifth segment in stage V female crabs), length of the propodus of the right claw (ClwL), and claw height (ClwH, across the widest portion of the right propodus). The length of the right gonopod (GL; the maximum length of the second pleopod) was measured under the microscope (Olympus CH30) equipped with a calibrated ocular micrometre (precision = 0.001 mm). For all dimensions, values are expressed as mean ± standard deviation.

Descriptions of the post-larval stages in *Calyptraeotheres garthi*

We first drew whole specimens when examining them under the stereomicroscope equipped with a *camera lucida* using at least three individuals per stage. Then, these individuals were carefully dissected and particular body parts were drawn using either a stereomicroscope or microscope equipped with a *camera lucida*. Crab body parts that vary widely in shape and/or setation pattern among individuals from the same stage [e.g. shape of female pleopods or setation on walking legs (WL)] were described using an 'average' representative individual (see Results). Crab ontogenetic stage denomination and morphological terminology follows that of Møller Christensen & McDermott (1958) and Jones (1977a). Herein we use 'stage' to designate 'one or more moults in which a crab does not change its morphology' while 'instar' is used to designate 'each moult within a specific stage', as these terms are employed in the classical literature (Møller Christensen & McDermott, 1958; Pearce, 1966).

Allometric growth in *Calyptraeotheres garthi*

We explored whether selected body dimensions (i.e. AW, CH, ClwL, ClwH, GL) increased linearly with body size (CW) in females and males from different ontogenetic stages. For this purpose, the relationship between each of the dimensions above and CW was examined using the allometric model $y = ax^b$ (Hartnoll, 1978, 1982). The slope b of the log–log least-squares linear regression represents the rate of exponential change of each dimension with a unit of increase in CW. The relationship between each studied body dimension and CW was considered isometric when $b = 1$, positively allometric when $b > 1$ and negatively allometric when $b < 1$ (Hartnoll, 1978). Departures from isometry were tested using independent Student's *t*-tests (Sokal & Rohlf, 1981). Prior to allometric analyses, we investigated whether each body dimension above differ between particular stages using different analyses of covariance (ANCOVA) (Sokal & Rohlf, 1981). Specifically, we evaluated the effect of crab ontogenetic stage in AW, CH, ClwL, ClwH, GL among pre-hard and hard males, pre-hard and hard females, hard females and females in stages II–IV, and female in stages II–IV and V. In each independent ANCOVA we used CW as the covariate, ontogenetic stage as the main independent (categorical) factor, and the different body dimensions as dependent variables. Homogeneity of slopes between ontogenetic stages was tested by exploring whether there were significant interactions between the categorical factor (ontogenetic stage) and the covariate (CW) in each ANCOVA (Sokal & Rohlf, 1981). If the ANCOVAs did not detect a significant effect of ontogenetic stage in a particular body part, we concluded that a dataset might be described by a unique regression and data from different stages (e.g. AW of pre-hard and hard females) were pooled together to calculate the allometric status of each studied body dimension.

RESULTS

The post-larval stages in *Calyptraeotheres garthi*

The smallest, putatively 'invasive', crab

Whitish or colourless; exoskeleton soft or hardness intermediate between pre-hard and hard forms, i.e. somewhat calcified and does not yield to touch but it is not as firm as the hard-shelled stage (see below).

Carapace (Fig. 1A): (CW = 0.752 ± 0.105 mm, $N = 3$) sub-pentagonal, longer than broad, two smooth, dorsoventrally flattened; eyes prominent projecting beyond the front.

Chelipeds (Fig. 2A): Finger tips crossing when closed; dactylus slightly curved, with proximal tooth inserted in notch of propodus. Propodus ventrally with six long plumose setae similar to, but shorter than, those swimming long plumose setae of second and third walking legs (WL).

Walking legs (WL) (Fig. 2A): Slender, flattened in cross-section, bearing short simple and slightly

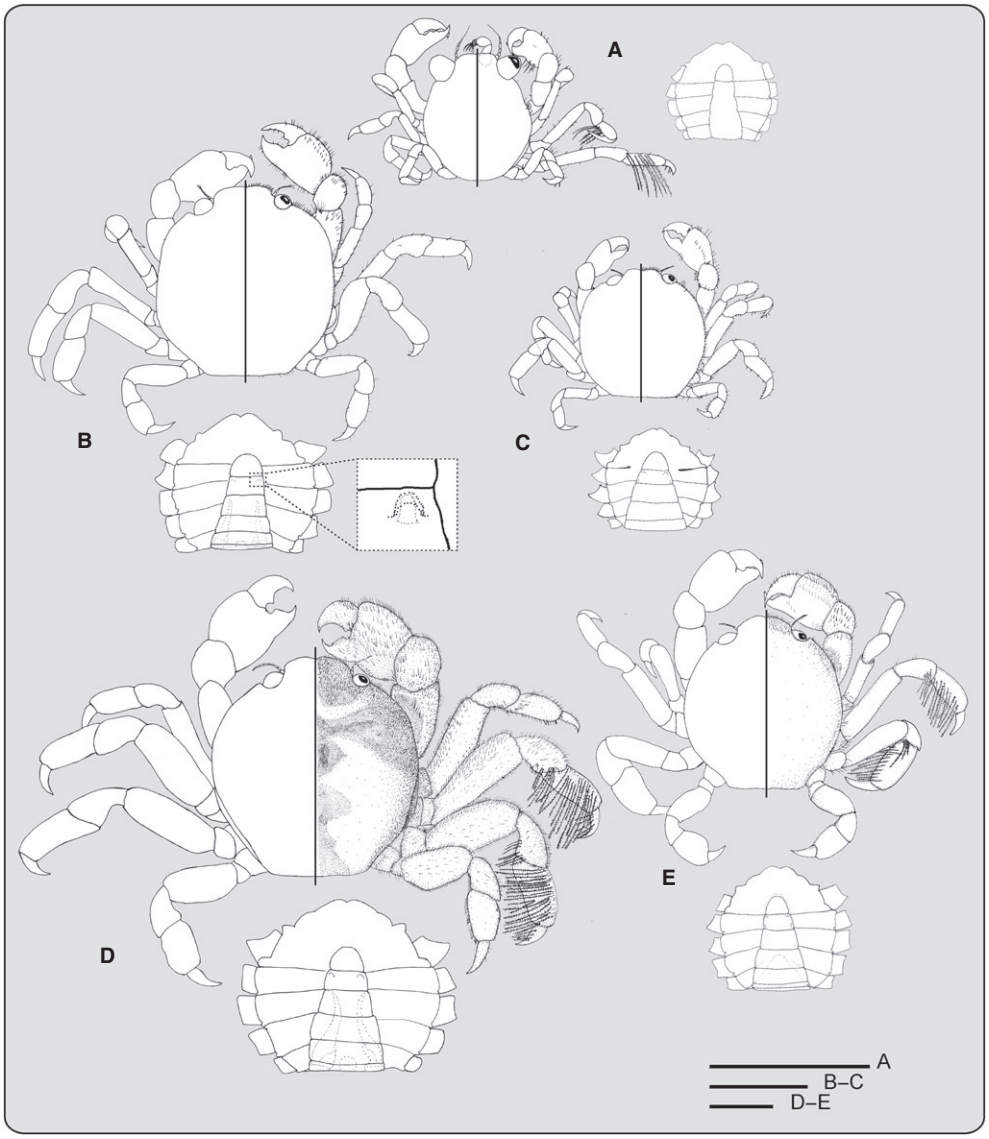

Figure 1. *Calyptraeotheres garthi*, dorsal view of whole animals (right side show the natural aspect) and ventral view of cephalothoraxes and abdomens in (A) the smallest, putatively 'invasive', crab, (B) male pre-hard, the dotted line box shows a detail of the locking mechanism (see Results), (C) female pre-hard, (D) male hard, (E) female hard. Scale bars: 1 mm.

longer plumose setae. All WL similar in shape, relative length in decreasing order 3:2:1:4. Dactyli falcate, dactylus of WL 4 the smallest; the propodus of both WL 2 and 3 with five swimming long plumose setae, carpus of the WL 3 distally with two swimming long plumose setae.

Pleon (Fig. 1A): (AW = 0.250 ± 0.061 mm, $N = 3$) narrow, fitting into sternal groove; telson (seventh segment) visible but the other six are not well delimited. Pleopods are not developed, and genital openings are not distinguishable; thus distinction of the sexes is not possible in this putatively 'invasive' stage.

Male and female pre-hard stages

Membranous soft-shelled exoskeleton; whitish or colourless.

Sexual dimorphism: Female and male pre-hard are indistinguishable except for the presence of genital openings on the sixth sternite (sternite of WL 2) in the female, and for differences in number and shape of the pleopods. The smallest (CW = 1.06 mm) pre-hard crab found did not have secondary sexual traits, thus the sex of this crab was not determined.

Carapace (Fig. 1B, C): (male CW = 1.99 ± 0.48 mm, $N = 15$; female CW = 1.34 ± 0.19 mm, $N = 7$), sub-orbicular, slightly longer than broad;

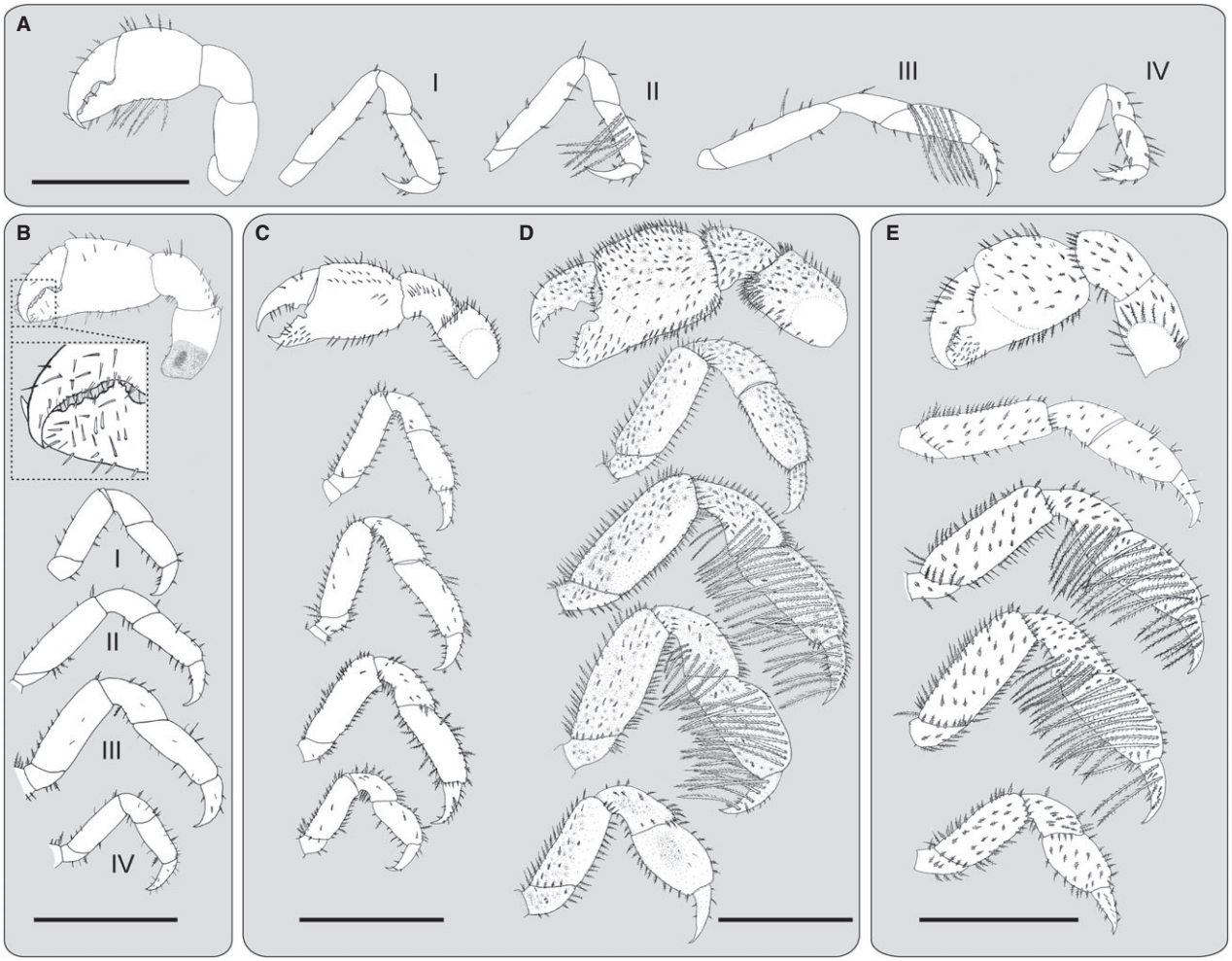

Figure 2. *Calyptraeotheres garthi*, cheliped and first (I), second (II), third (III), and fourth (IV) walking legs in (A) the smallest, putatively 'invasive', crab, (B) female pre-hard, the dotted line box details the spines and setae on the inner surface of the cutting edges of fingers and propodus of the cheliped, (C) male pre-hard, (D) male hard, (E) female hard. Scale bars: (A) 0.5 mm, (B–E) 1 mm.

smooth with fringe of simple setae on margins; front projected, medial sulcus dividing front at the middle; eyes prominent and visible from the dorsal surface.

Chelipeds (Fig. 2B, C): With simple setae as shown in Fig. 2B, C; finger tips crossing when closed; dactylus slightly curved, with proximal tooth inserted in notch of propodus, cutting edges of both fingers with minute teeth, simple setae and spines (Fig. 2B) but without long plumose setae; inner surface of the propodus distally with a group of simple setae.

Walking legs (WL) (Fig. 2B, C): Slender, similar in shape than those of the invasive stage but now cylindrical in cross-section; short simple and slightly longer plumose setae also present but without long natatory setae.

Pleon (Figs 1B, C, 3A): (female AW = 0.56 ± 0.14 mm, N = 7; male AW = 0.80 ± 0.19 mm, N = 15) seven-segmented, narrow, fitting closely into sternal groove; slightly broader in bigger pre-hard females than those of males and smaller females. Lateral margin of male's pleon is straight or slightly concave, while it is slightly convex in female. Edges with small simple setae (Fig. 3A). A pair of chitinous knobs on fifth thoracic segment conform part of the locking apparatus that held close the pleon (Fig. 1B). These knobs are antero-ventrally directed and hook under pocket-like shelves found on the ventral surface of the sixth segment of the pleon (Figs 1B, 3A).

Pleopods (Figs 3A, 4A, B): Female with four pairs of pleopods whose morphology varies according to size and/or moult instar (Fig. 3A). A small pre-hard female (e.g. CW = 1.12 mm) bears minute and rudimentary pleopods, only the second pair is bilobed (Fig. 3A above). Pleopods are bigger and partially

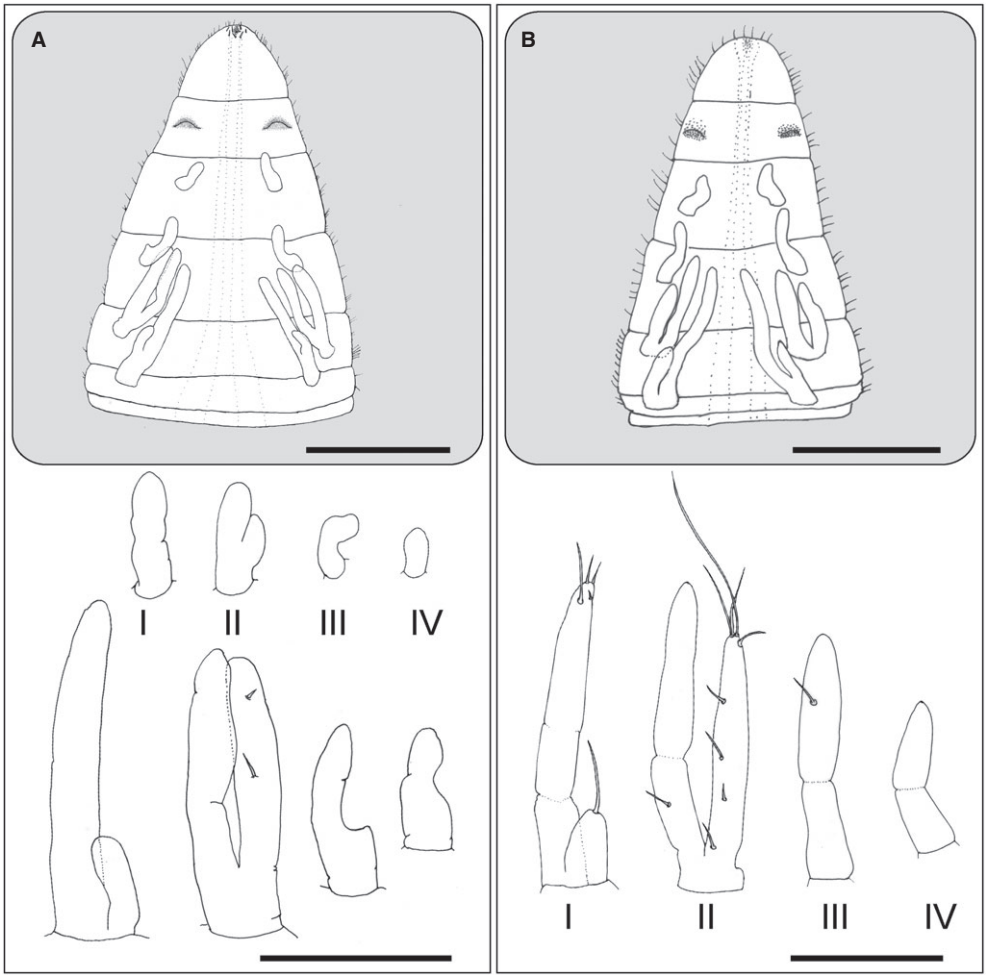

Figure 3. *Calyptraeotheres garthi*, ventral view of the abdomen (above), first (I), second (II), third (III), and fourth (IV) pleopods (below) in (A) female pre-hard, (B) female hard. In (A) pleopods shown above correspond to a small individual, while pleopods shown below were dissected from a large individual. Scale bars: (above) 0.5 mm, (below) 0.2 mm.

segmented in those late pre-hard female (e.g. CW = 1.57 mm; Fig. 3A below), in this case the first pair is biramous with a small exopod and large endopod, the second pair is biramous, the exopod with two simple setae and it is similar in length to the endopod, the third and fourth pairs are uniramous and typically naked. Male with two pairs of pleopods whose morphology also varies according to size and/or moult instar (Fig. 4A, B). Early (small) pre-hard male (e.g. CW = 1.13 mm) only with first rudimentary and small pair of pleopods (Fig. 4A); late pre-hard male (e.g. CW = 2.60 mm) with first pair long and slender, slightly curved outwards, tapering distally, ending in a pore, covered by simple setae (Fig. 4B) and second pair that consists of rudimentary knobs under the base of first pair (not shown).

Male and female hard stages

Both sexes with well calcified exoskeletons.

Sexual dimorphism: Both sexes are similar in overall shape. Males are light brown in colour, with a conspicuous pattern of yellowish orange or brown marks in carapace. These marks are greater and more intense in larger males. Females are lighter than males, and rarely contain yellowish orange tints; when present these marks are diffuse and not well defined as in males. A reliable sex determination, however, is only possible by inspecting secondary sexual organs.

Carapace (Fig. 1D, E): (male CW = 2.23 ± 0.53 mm, $N = 62$; female CW = 1.73 ± 0.28 mm, $N = 22$) suborbicular or sub-pentagonal, slightly longer than broad; dorsally flattened, thought in larger specimens carapace that it tends to be a little swollen

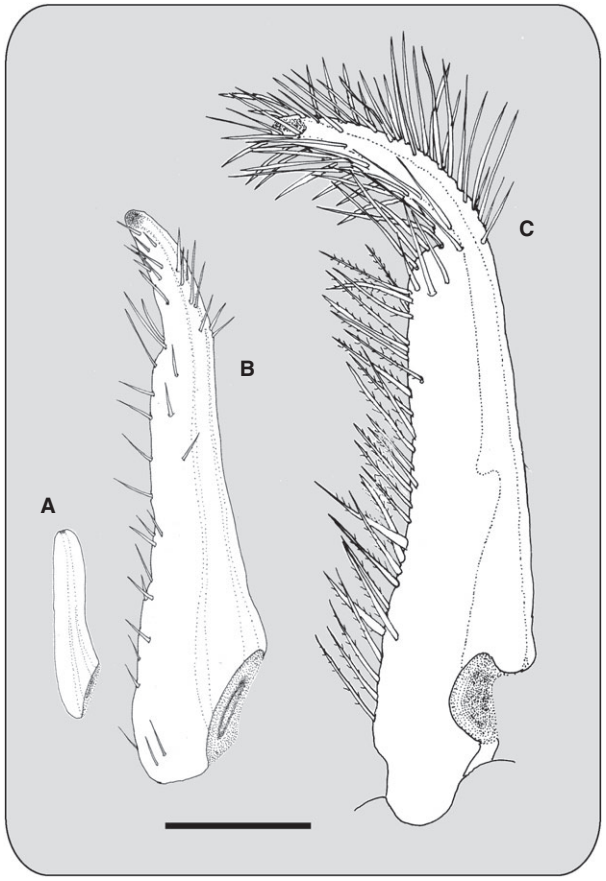

Figure 4. *Calyptraeotheres garthi*, gonopods (= first pairs of male pleopods) in (A) early (small) pre-hard male, (B) late (large) pre-hard male, (C) male hard. Scale bar: 0.2 mm.

forward; front projected with a medial sulcus as in the previous stage; surface of frontal, gastric, and orbital regions setose; lateral margins setose, thin and rather sharply bent from the dorsal side. Higher density of setae in males than in females.

Chelipeds (Fig. 2D, E): Stouter than in the pre- and post-hard stages; propodus, merus and carpus swollen; fingers strong; dactylus more curved than in the pre- and post-hard stages; short plumose setae scattered on surfaces; margins covered with plumose setae. Setation is more abundant in males than females. Inner surface of the fixed finger and cutting edge of both fingers with simple setae and spines as in previous stage.

Walking legs (Fig. 2D, E): Stouter than either the pre- or post-hard stages, flattened in cross-section; surfaces covered by short plumose setae, margins with short simple and slightly longer plumose setae. This setation is denser in males than in females. Swimming (long plumose) setae are now present in WL 2 and 3. The arrangement of the setae also varies according to body size and sex. Setae are more numerous in large than in small individuals. The arrangement of the swimming setae can be described in a male (CW = 2.8 mm) and female (CW = 1.92 mm) as follows (Fig. 2D, E): *WL 2*: carpus with two ventral and five antero-dorsal in the male and two ventral and four antero-dorsal in the female long plumose setae; propodus with a row of 12 dorsal in the male and three antero-ventral and a row of ten dorsal in the female long plumose setae; dactylus with six in the male and five in the female long plumose setae. *WL 3*: carpus with a row of eight in the male and seven in the female long plumose setae; propodus with a row of ten dorsal and five ventral in the male and a row of nine dorsal and three ventral in the female long plumose setae; dactylus with five in the male and two in the female long plumose setae.

Pleon (Figs 1D, E, 3B): (female AW = 0.71 ± 0.13 mm, N = 22; male AW = 0.91 ± 0.24 mm, N = 62), similar in form to that of male pre-hard-stage. Edges with short simple and plumose setae (Fig. 3B). Locking mechanism similar to that of pre-hard stage.

Pleopods (Figs 3B, 4C): Females: morphology varies according to size and/or moult instar. In small hard females (e.g. CW = 1.07 and 1.27 mm), pleopods are rudimentary appendages (not shown) similar to those of smallest pre-hard crabs, although the first pair is now bilobed. Pleopods are segmented and more complex (Fig. 3B) in late and/or large hard females (e.g. CW: 1.92 mm). First pair: exopod short, distally with one long simple seta; endopod two-segmented, elongated, distally with four simple setae. Second pair: exopod unsegmented with one proximal, three medial, four (three short, one long) distally simple setae; endopod two-segmented, first segment with one simple seta, second naked. Third pair uniramous, two-segmented with one simple seta in the second segment. Fourth pair uniramous, two-segmented, naked. Males (Fig. 4C): first pair longer than in previous stage, curved outwards, tapering distally, ending in a pore, outer margin covered by simple and plumodenticulate setae; this setation is denser in large individuals. Second pair (not shown) is a small, naked, and rod-like appendage, commonly observed within the groove of the first pair.

Female post-hard stages

Stages II, III and IV–V are easily differentiated by morphology of pleopods and sizes of carapace and pleon. The pleon size, carapace size, and pleopod complexity in stage IV, however, are similar to those observed in the smallest individuals of stage V. The colour of the ovary might help to differentiate between these two stages: whitish or colourless in

stage IV while yellow or red in stage V. The stage IV females, however, could be considered small stage V females with still non-mature ovary. Whether stages IV moult and then develop the ovary becoming stage V or stages IV are stage V with immature ovaries remains unclear.

Stage II: Morphologically, stage II female resemble late pre-hard forms; exoskeleton smooth and thin. As in the subsequent III and IV, stage II is whitish or colourless.

Carapace (Fig. 5A). (CW = 1.84 ± 0.27 mm, $N = 6$) sub-orbicular, slightly broad than longer, convex from the front backward; lateral margins rounded; front not projected forward as far as in the previous stage; antero-lateral margins with a row of simple setae.

Chelipeds (Fig. 6A). Similar to that of pre-hard forms.

Walking legs (Fig. 6A). Slender, semi-cylindrical in cross-section. Margins with shorter simple and longer plumose setae, as shown in Fig. 6A. Dactylus similar than that of pre-hard forms, dactylus of WL 4 the smallest.

Pleon (Figs 5A, 7A). (AW = 0.97 ± 0.35 mm, $N = 6$) slightly wider than that of late pre-hard female. Margins convex, extending beyond edge of sternal groove; edges covered with plumose setae. Locking mechanism vestigial.

Pleopods (Fig. 7A). Setation of pleopods may vary among individuals. In a stage II female (CW = 2.25 mm) the setation arrangement may be described as follows: first pair with exopod short, with three (two short, 1 min) medial simple setae, and endopod elongated and four-segmented, second and third segment with 1 min seta each one, first and fourth segments naked. Exopod of the second pair two-thirds of the endopod, with two medial minute setae, and endopod three-segmented, first segment with 1 min seta, second and third segments naked. In four out of the six analyzed stage II individuals, the third and fourth pleopods were uniramous, naked, and three- and two-segmented, respectively (Fig. 7A). In the remaining two individuals, both third and fourth pleopods presented small exopod buds (not shown).

Stage III: Carapace (Fig. 5B). (CW = 2.28 ± 0.40 mm, $N = 4$) similar in shape, although bigger, than that of stage II.

Chelipeds (Fig. 6B). Similar to those of stage II.

Walking legs (Fig. 6B). Similar to those of stage II, WL 4 more slender than the others WL in the same stage.

Pleon (Figs 5B, 7B). (AW = 1.55 ± 0.18 mm, $N = 4$) broader than that of stage II, edges with plumose setae; third segment partially covers the basis of WL 4; fourth segment widest; telson twice as wide as long. Locking mechanisms absent.

Pleopods (Fig. 7B). Although morphology of pleopods is conserved among stage III individuals, the number and positioning of setae notably varies. Therefore, the follow description is based on a representative stage III female (CW = 2.75 mm). First pair: exopod short unsegmented, with two distal simple setae; endopod four-segmented, with (proximal to distal) two (one short, 1 min), two (one short, one long), three (two long, one short) and six (two long, 1 short basal and two long, 1 short distal) simple setae respectively. Second pair: exopod two-thirds of the endopod, with five medial and four (one long, three short) distal simple setae; endopod four-segmented with one short distal, one short proximal, five (three long, two short) medial and two (one long, one short) distal simple setae, respectively. Third pair uniramous, three-segmented, first and second segments naked, third with two short distal simple setae. Fourth pair uniramous, two-segmented, naked.

Stage IV: Carapace (Fig. 5C). (CW = 3.08 ± 0.32 mm, $N = 4$) sub-orbicular, slightly broad than longer; bigger than that of previous stages; dorsally translucent, trace of non-developed ovary seen through carapace; longitudinal cervical depressions arising from orbital region now visible.

Chelipeds (Fig. 6C). With simple and plumose setae on merus and carpus. Setation more abundant than in stage II and stage III. Similarly, spines and simple setae on the inner surface of the fixed finger and on cutting edges of fingers are now more abundant than in previous stages.

Walking legs (Fig. 6C). Dactylus of WL 4 two-thirds of the propodus, similar in length to those of WL 2–3, bearing distally a tuft of simple setae.

Pleon (Figs 5C, 8A). (AW = 2.85 ± 0.21 mm, $N = 4$) sub-circular, nearly as wide as the carapace, more concave than in previous instars, telson more than three times as wide as long; pleon covers almost the entire sternum, the basis and ischium of WL 4, and the basis of WL 2–3; three first segments are visible from dorsal view; locking mechanism absent.

Pleopods (Fig. 8A). Setation arrangement (but not morphology) of pleopods may vary among individuals. The follow description is based on a representative female IV (CW = 3.0 mm). First pair: exopod short, with ten (nine long, one short) simple setae, and endopod five-segmented, first segment with two (one long, one short) simple setae, second segment with seven long distal simple setae, third segment with eight long distal simple setae, fourth segment with four short distal simple setae, fifth segment

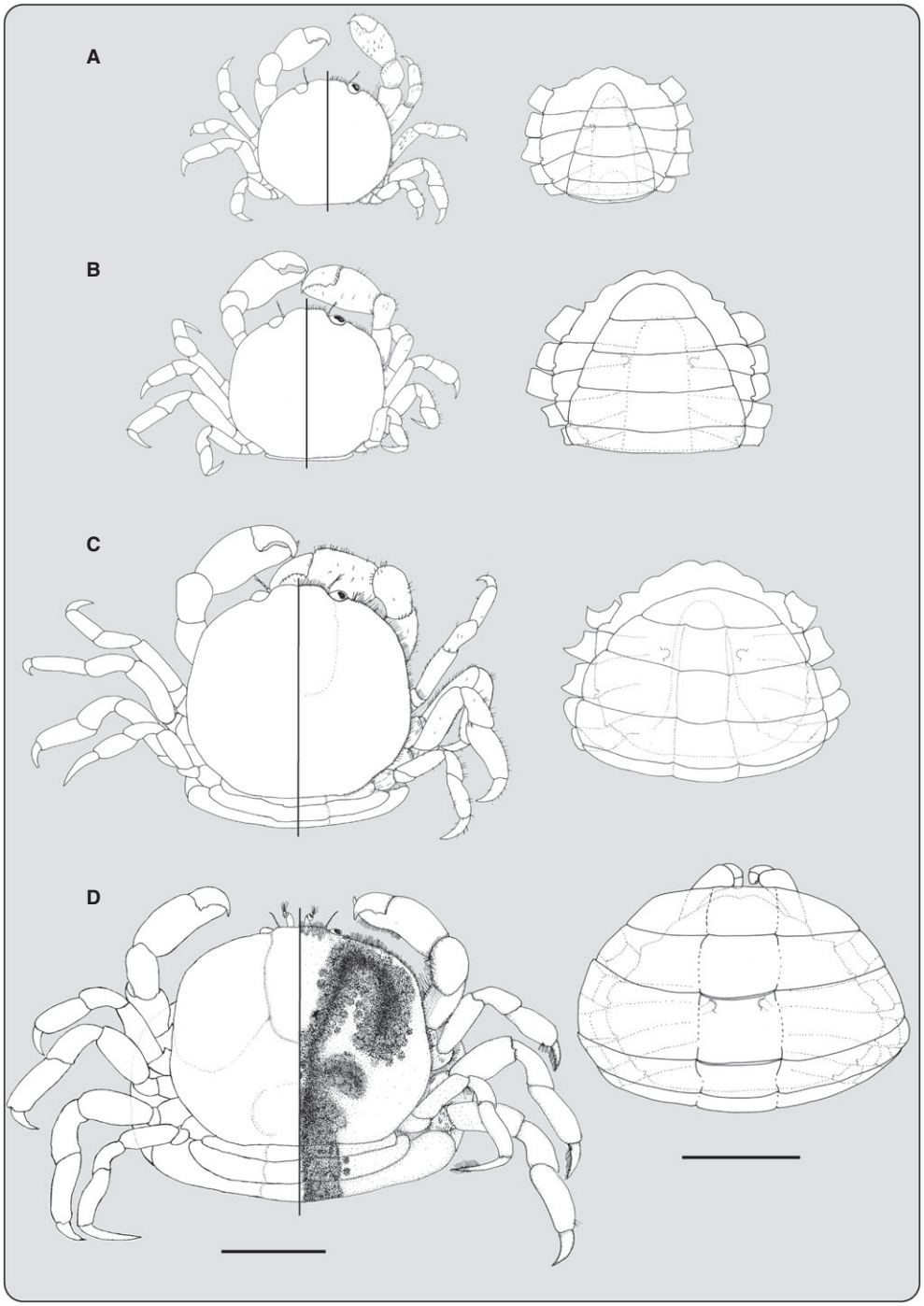

Figure 5. *Calyptraeotheres garthi*, dorsal view of whole animals (right side show the natural aspect) and ventral view of cephalothorax and abdomen in: (A) stage II female, (B) stage III female, (C) stage IV female, (D) stage V female. Scale bars: 2 mm.

with four long proximal, five (two long, three short) sub-distal, six long apical simple setae. Second pair: exopod stout, two-thirds of the endopod, covered with short simple and slightly longer plumose setae, and distally with 11 plumose and one simple long setae; endopod five-segmented, first segment with two short medial and three long distal simple setae, second segment with seven (five long, two short) distal

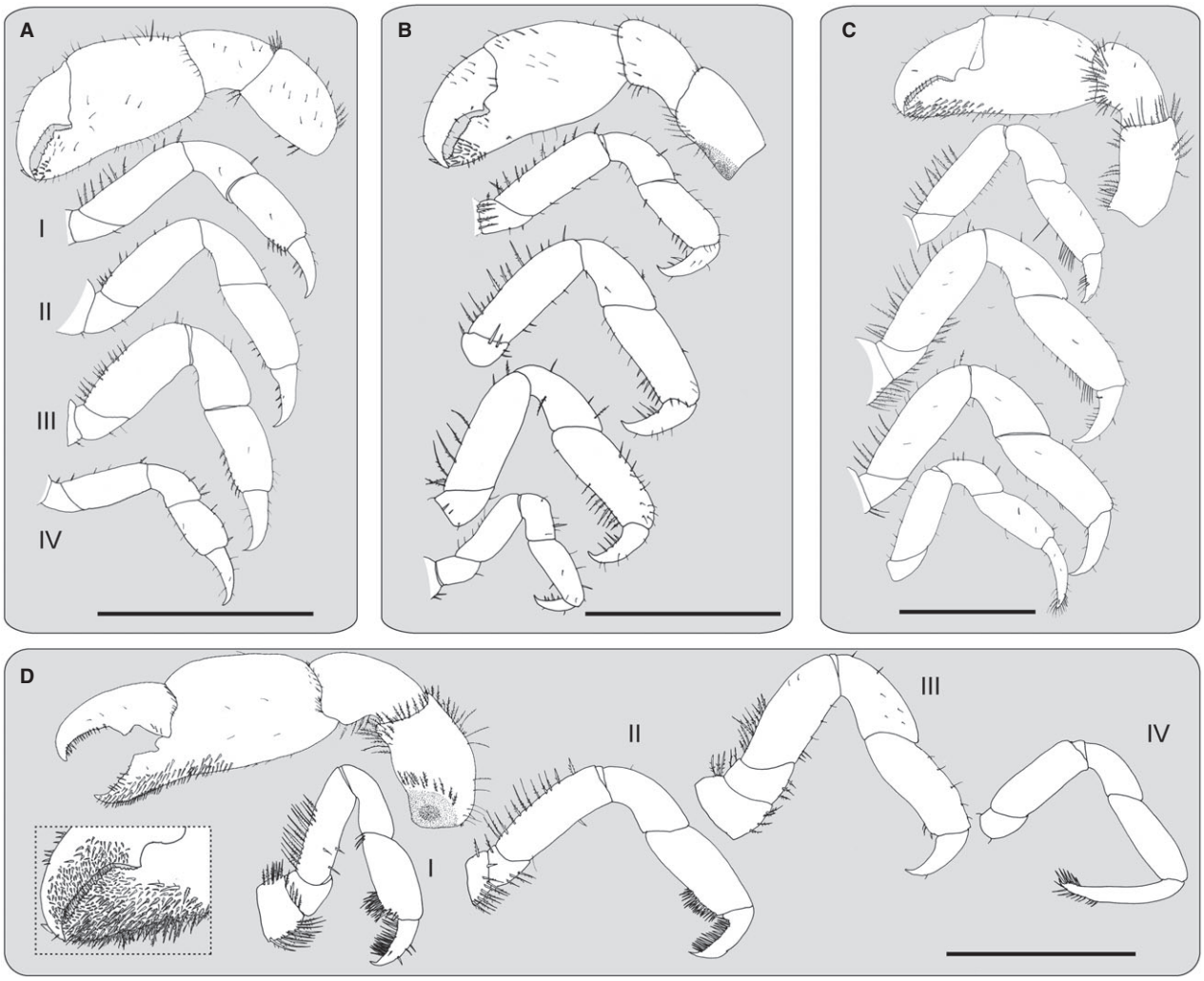

Figure 6. *Calyptraeotheres garthi*, cheliped and first (I), second (II), third (III), and fourth (IV) walking legs in (A) stage II female, (B) stage III female; (C) stage IV female, (D) stage V female, the dotted line box details the spines and setae on the inner surface of the cutting edges of fingers and propodus of the cheliped. Scale bars: (A–C) 1 mm, (D) 2 mm.

setae, third segment with two (one long, one short) proximal and three (two long, one short) distal simple setae, fourth segment with three (two long, 1 short) proximal and five (three long, two short) distal simple setae, fifth segment with three long proximal, six (four long, one short, 1 min) medial, and eight (six long, two short) distal simple setae. Third pair uniramous, now four-segmented, first segment naked, second segment with four (two long sparely plumose, one simple long, one simple short) proximal and four (two long simple, two short simple) distal setae, third segment with two proximal long, one medial short, and four (three short, one long), fourth segment bear four long proximal and 11 (eight long, three short) distal simple setae. Fourth pair uniramous, two-segmented, first segment with two long distal sparely plumose setae, second segment with six (two long, four short) medial, seven (four long, three short) sub-distal, and eight (six long, two short) distal simple setae.

Stage V: In this stage the carapace attains the greatest size and more rounded shape. The exoskeleton is whitish or colourless, although due to its translucence, the carapace reflects the colour of the eggs contained in the ovary.

Carapace (Fig. 5D). (CW = 5.81 ± 1.21 mm, $N = 55$) sub-quadrangular in shape, broad than longer; internal structures seen through carapace; ovary visible under most of carapace and it extends to the tip of the pleon, its colour varies from whitish (empty) and yellow (early in development) to deep red (prior deposition);

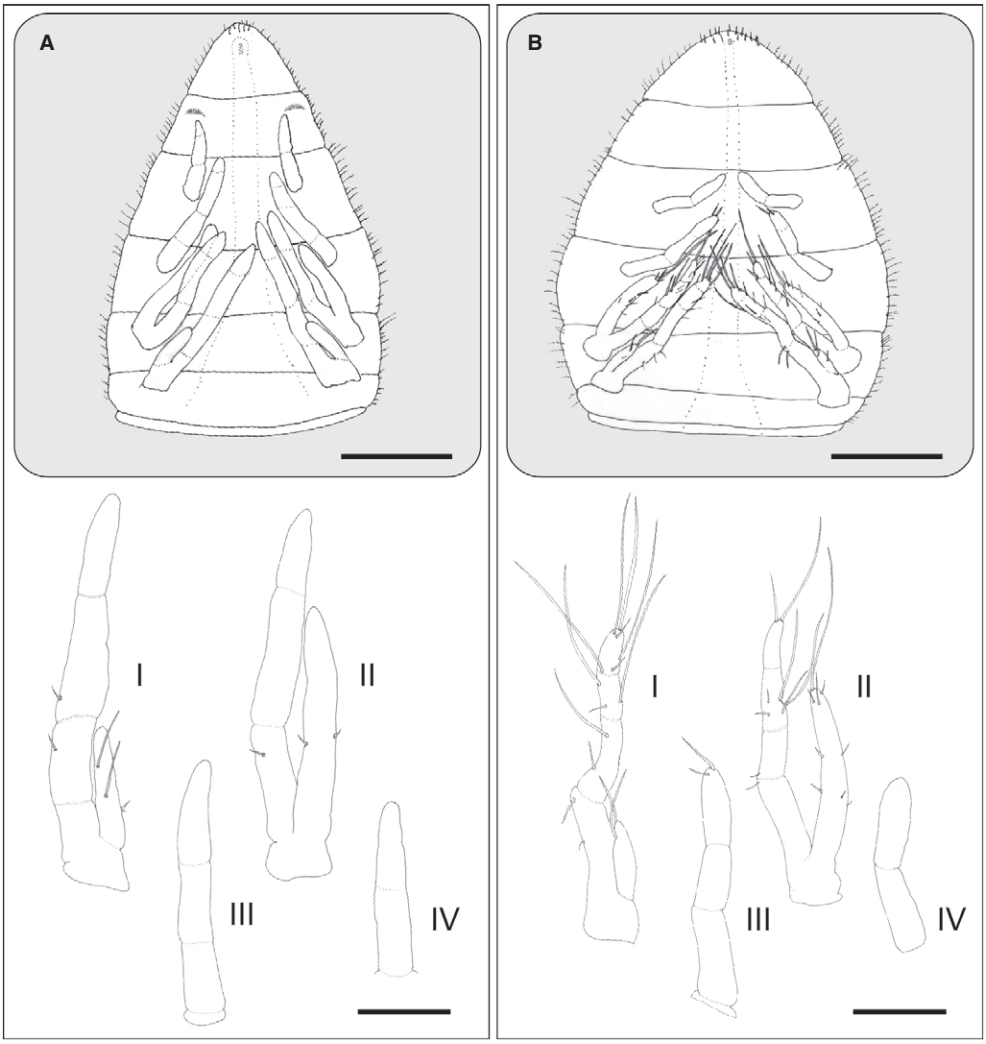

Figure 7. *Calyptraeotheres garthi*, ventral view of the abdomen (above), first (I), second (II), third (III), and fourth (IV) pleopods (below) in (A) stage II female, (B) stage III female. Scale bars: (above) 0.5 mm, (below) 0.2 mm.

seminal receptacles also dorsally visible as two small circular whitish structures in the meso-branchial region (not shown); stomach visible soon after the crab feed posterior to the front (not shown, but see Ocampo *et al.*, 2014); longitudinal cervical depressions more conspicuous; front less projected than in previous stages, eyes small but visible from dorsal view.

Chelipeds (Fig. 6D). Stouter than those of previous post-hard forms; propodeum longer than before. Simple setae and spines on cutting edges of fingers are notably denser than in previous stages. Similarly, simple setae on the inner surface of the fixed finger are more abundant and extend proximally.

Walking legs (Fig. 6D). WL 4 almost backward directed; dactylus of WL 4 as long as the propodus, and considerably longer than those of WL 1–2–3, with distally a tuft of simple setae; propodi of WL 1–2 with tuft of stiff simple setae on disto-ventral margin; dactyli of those WL with tuft of stiff simple setae ventrally.

Pleon (Figs 5D, 8B). (AW = 7.43 ± 1.75 mm, N = 55) sub-circular; oval in shape when separated from cephalothorax; concave, dorsally hollowed; as wide as or wider than carapace, it protrudes laterally beyond the ischium of WL and chelipeds and anteriorly partially covers the third maxilliped, telson five times as wide as long.

Pleopods (Fig. 8B). As before, setation varies among individuals being denser in those larger crabs. The follow description is based on a representative stage V individual (CW = 5.6 mm). First pair: exopod short, covered by 33 long and four short proximal simple setae, endopod five-segmented, first

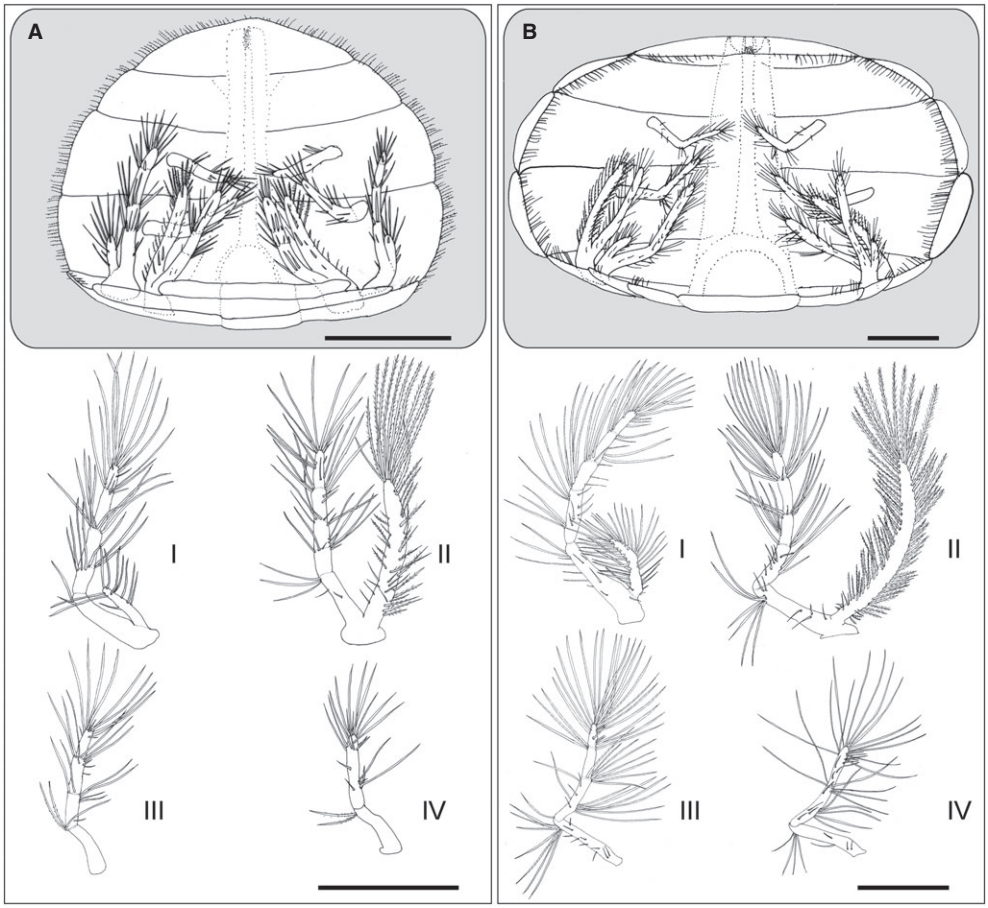

Figure 8. *Calyptraeotheres garthi*, ventral view of the abdomen (above), first (I), second (II), third (III), and fourth (IV) pleopods (below) in (A) stage IV female, (B) stage V female. Scale bars: (above) 1 mm, (below) 0.5 mm.

segment with three long and eight short simple setae, second segment with six long and five short distal simple setae, third segment with five long and three short simple setae, fourth segment with eight long and three short simple setae, fifth segment with 13 long and three short simple setae. Second pair: exopod stout, slightly shorter than the endopod, densely covered by short and long plumose setae, endopod five-segmented, first segment with six long distal and six short (four proximal, two distal) simple setae, second segment with nine (three long, six short) medial setae, third segment with nine long (four proximal, four medial, 1 distal) simple setae, fourth segment with six (five long, one short) simple setae, fifth segment with 11 (ten long, one short) proximal and 12 distal simple setae. Third pair uniramous, four-segmented, first segment now with seven long distal and ten short simple setae, second segment with one long proximal, nine (seven long, two short) medial, and two (one long, one short) simple setae, third segment with eight proximal and four distal long simple setae, fourth segment with five (two long, three short) proximal and 17 long distal simple setae. Fourth pair uniramous, two-segmented, first segment with two short proximal, two short medial, and four long distal simple setae, second segment with eight (five long, three short) proximal, seven (six long, one short) medial, four short sub-distal, and 18 (13 long, five short) distal simple setae.

Sexual dimorphism and allometric growth in *Calyptraeotheres garthi*

In the studied species, CW varied from 0.67 mm (the smallest invasive crab individual) to 7.90 mm (the largest stage V female crab individual) (Fig. 9A). Visual inspection of the population distribution (Fig. 9A) reveals considerable overlap in body size (CW) between pre-hard and hard-stage males. Consistently, no significant differences were found between the CW of hard and pre-hard males (*t*-test, $t_{74} = 1.56$,

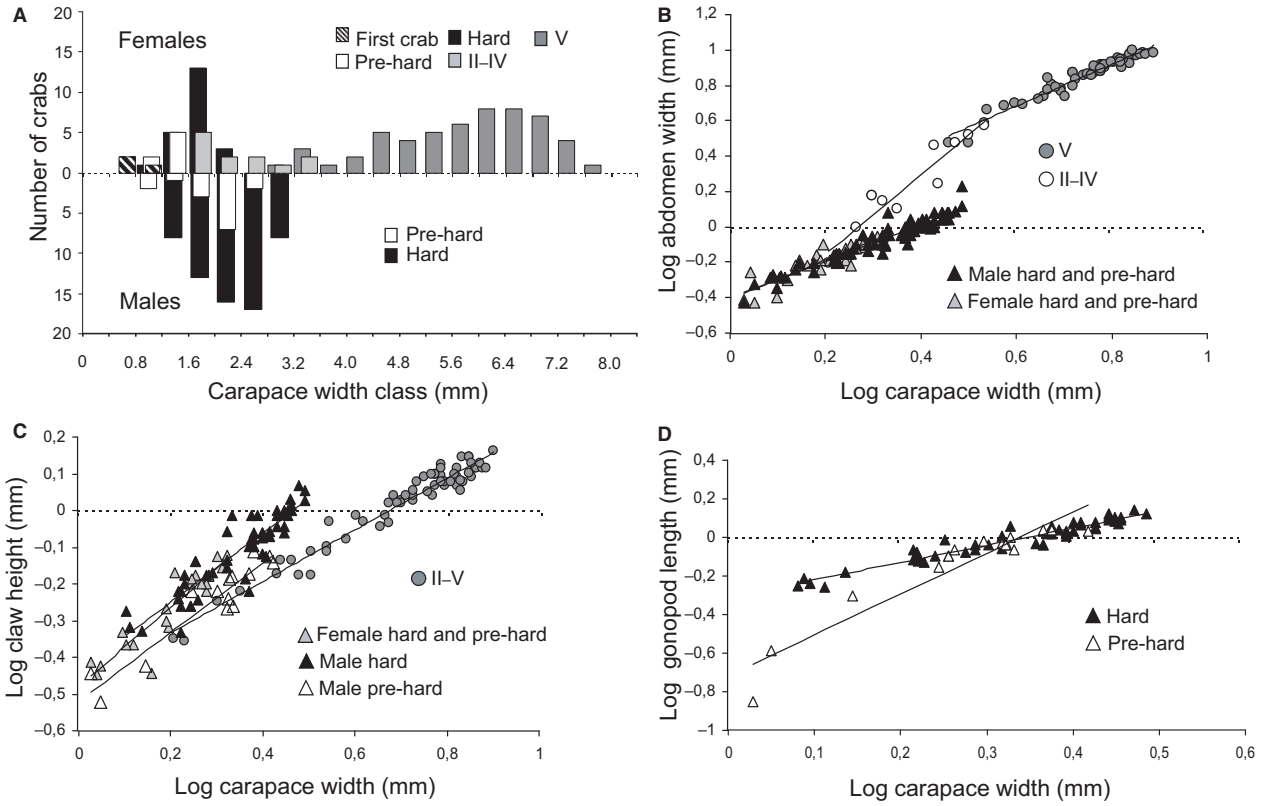

Figure 9. (A), population structure of *Calyptraeotheres garthi* at San Matías Gulf, North Patagonia, Argentina. Logarithm of (B) abdomen width, (C) right claw (= cheliped) height, and (D) right gonopod (= first male pleopod) length plotted vs. the logarithm of carapace width of *C. garthi* (see Table 1, for further details).

$P = 0.12$). In contrast, the CW between hard and pre-hard females was significantly different (pre-hard < hard females; t-test, $t_{27} = 3.40$, $P < 0.002$).

Significant differences were observed between the CW of stage V females and hard males (t-test, $t_{115} = 21.05$, $P < 0.0001$), hard females and hard males (t-test, $t_{82} = 3.92$, $P < 0.001$), and pre-hard females and pre-hard males (t-test, $t_{20} = 3.29$, $P < 0.005$). These differences indicate sexual dimorphism with respect to body size. Nevertheless, the pattern of size dimorphism varies: stage V female are larger than hard males, hard males are larger than hard females, and pre-hard males are larger than pre-hard females (Fig. 9A).

The ANCOVA analyses revealed no significant effect of ontogenetic stage in AW when we compared pre-hard and hard females (ontogenic stage term: $F = 0.143$, d.f. = 1, $P < 0.75$), and pre-hard and hard males (ontogenic stage term: $F = 0.109$, d.f. = 1, $P < 0.75$). Also, no significant effect of ontogenic stage were found in ClwH between pre-hard and hard females (ontogenic stage term: $F = 0.004$, d.f. = 1, $P < 0.95$), and stages II–IV and stage V females (ontogenic stage term: $F = 2.918$, d.f. = 1, $P < 0.1$). The interaction term of the ANCOVAs above was not significant ($P > 0.05$). In contrast, ANCOVAs showed either significant effects of the ontogenic stage ($P < 0.05$) and/or significant differences in the interaction term ($P < 0.01$) in all others studied comparisons i.e. AW between hard females and females in stages II–IV, and female in stages II–IV and V; ClwH between pre-hard and hard males, and hard females and females in stages II–IV; CH and ClwL between pre-hard and hard males, pre-hard and hard females, hard females and females in stages II–IV, and female in stages II–IV and V; GL between pre-hard and hard males.

The relative growth of selected dimensions varied according to sex and stage (Table 1, Fig. 9B, D) but isometry is the predominant pattern. Positive allometry was only recorded in the growth of AW of stages II–V females, CH of stages II–IV females and hard males, and GL of pre-hard males. In turn, negative allometry was observed in ClwL of stage V females and hard males, in ClwH of stages II–V females and GL of hard males.

DISCUSSION

The present is the first study that provides a detailed description of the entire set of post-larval stages in any member of the family Pinnotheridae. *Calyptraeotheres garthi* is also the first pea-crab species having all larvae (Ocampo *et al.*, 2011) and post-larval stages (present study) properly described and illustrated. A graphical summary of our results and conclusions (see below) is provided in Figure 10. There, size ranges of all stages and most relevant morphological characters in the post-larval life of *C. garthi* are highlighted. In the following, we discuss those morphological changes related to life habit (facultative or obligate endosymbiosis), molting cycle, and reproduction.

Morphology of the post-larval stages in *Calyptraeotheres garthi*

In *C. garthi*, not a single megalopa has ever been retrieved from the brooding chamber or any other internal cavity of limpets examined during this and previous studies (Ocampo *et al.*, 2012, 2013, 2014). The above support the notion that the smallest crab (and not the megalopa) is the invasive stage in this species, similar to that reported in other pea-crab species (Møller Christensen & McDermott, 1958; Pearce, 1966) but different from that found in *Pinnotheres halingi*, in which the megalopa appears to be the invasive stage (Hamel, Ng & Mercier, 1999). In *C. garthi*, the invasive crab exhibits morphological features that we believe represent adaptations to both host localization and colonization, similar to that reported before for *Zaops ostreum* (Møller Christensen & McDermott, 1958) and *Fabia subquadrata* (Pearce, 1966). These features include the presence of long and well developed swimming setae on the second and third pair of ambulatory legs and a body shape that is rather dorso-ventrally compressed. In addition, the invasive crab in *C. garthi* is soft as in *F. subquadrata* (Pearce, 1966), although, one out of three specimens herein analyzed did exhibit moderate carapace hardness in between pre-hard and hard forms, as observed in *Z. ostreum* (Møller Christensen & McDermott, 1958). Importantly, the average body size of the smallest crabs of *C. garthi* observed in this study was only slightly larger than that reported before for megalopae obtained from reared material (Ocampo *et al.*, 2011). The above further suggests that the invasive crab is also the first crab instar immediately following the megalopa stage in *C. garthi*.

The analysis of the size frequency distribution of the different post-larval stages in *C. garthi* supports the notion that the invasive crab stage later moults into the male and female pre-hard forms, as reported before for other pea-crabs (Møller Christensen & McDermott, 1958; Pearce, 1966). Crabs in this pre-hard stage loss all swimming traits, in agreement to that found in other pea-crab species (Pearce, 1964, 1966; Jones, 1977a). Furthermore, the external appearance of pre-hard crabs (i.e. a soft exoskeleton, rounded carapace, and slender claws and ambulatory legs) supports the notion that is at this stage when *C. garthi* adopt for the first time in their life an endosymbiotic habit. The amount of time that pre-hard crabs remain in the brooding chamber of limpets is presently unknown. Nonetheless, after this pre-hard stage, crabs moult into a hard-stage form that appears to exhibit adaptations to live, at least temporarily, outside of host individuals. In this hard-stage, various features similar to those found in invasive crabs 'reappear': e.g. the presence of laterally compressed ambulatory legs and long natatory setae on the second and third pair of pereopods. Additionally, during this hard stage, crabs exhibit a well calcified exoskeleton, stout claws, and a pigmented tegument. These features are particularly evident among the largest hard-stage males we examined. The general shape and features observed during the hard-stage and first crab of *C. garthi* are likely beneficial if crabs roam off host individuals in their natural environment. For instance, swimming setae might help both male and female crabs to move efficiently along the marine bottom (mainly conformed by rocks, mussels, algae and other sessile invertebrates – E. H. Ocampo, pers. observ.) in order to find host individuals and/or sexual partners. Additionally, the swimming ability of hard stages might help to prevent predatory attacks in the external environment, as reported in the free-swimming males of the pea-crab *Pinnotheres bidentatus* (Hsueh, 2001). Lastly, hard-stage individuals of *Z. ostreum* and *Nepinnotheres novaezelandiae* have been observed trapped between the valves of their bivalve host (Stauber, 1945; Jones, 1977b). Therefore, the dorso-ventrally flattened and hard carapace herein observed for *C. garthi* might also allow crabs to resist potential crushing by the shell of limpets while entering/leaving host individuals.

In males of *C. garthi*, no forms other than the pre-hard and hard stages have been found among examined specimens in this as well as in previous ecological studies (Ocampo *et al.*, 2012, 2014), in agreement with that reported for other pea-crab species (e.g. *N. novaezelandiae* – Jones, 1977a). This result indicates that the hard form is the terminal stage in males of this species. By contrast, our results suggest that females undergo a much more complex 'metamorphosis'-like process, as previously reported in other pea-crabs (e.g.

Table 1. Relative growth of selected features in males (hard and pre-hard) and females (stage V, stage II to IV, hard and pre-hard) of *Calyptraeotheres garthi*

Variable	Regression	N	r^2	SE_s	t_s	P (slope = 1)	Allometry
Abdomen width (AW)							
Female stage V	AW = 1.167CW – 0.024	55	0.945	0.039	4.333	< 0.0001	(+)
Female stages II–IV	AW = 2.221CW – 0.599	12	0.906	0.226	5.410	< 0.0005	(+)
Female hard and pre-hard	AW = 1.094CW – 0.407	29	0.796	0.106	0.886	> 0.05	(0)
Male hard and pre-hard	AW = 1.023CW – 0.399	77	0.912	0.037	0.624	> 0.05	(0)
Claw length (ClwL)/height (ClwH)							
Female stage V ClwL	ClwL = 0.807CW – 0.193	54	0.919	0.032	5.792	< 0.0001	(–)
Female stages II–IV ClwL	ClwL = 0.928CW – 0.261	9	0.942	0.086	0.824	> 0.05	(0)
Female stages V and II–IV ClwH	ClwH = 0.707CW – 0.476	63	0.952	0.020	14.47	< 0.0000001	(–)
Female hard ClwL	ClwL = 1.047CW – 0.268	18	0.876	0.097	0.484	> 0.05	(0)
Female pre-hard ClwL	ClwL = 0.778CW – 0.227	6	0.647	0.286	0.766	> 0.05	(0)
Female hard and pre-hard ClwH	ClwH = 1.086CW – 0.482	24	0.832	0.104	0.831	> 0.05	(0)
Male hard	ClwL = 0.880CW – 0.221	51	0.870	0.048	2.473	< 0.05	(–)
	ClwH = 0.945CW – 0.439	51	0.819	0.062	0.865	> 0.05	(0)
Male pre-hard	ClwL = 1.028CW – 0.287	14	0.936	0.078	0.364	> 0.05	(0)
	ClwH = 0.949CW – 0.524	14	0.892	0.095	0.536	> 0.05	(0)
Gonopod length (GL)							
Male hard	GL = 0.878CW – 0.308	51	0.924	0.035	3.367	< 0.005	(–)
Male pre-hard	GL = 2.092CW – 0.718	15	0.893	0.200	5.453	< 0.0005	(+)
Carapace height (CH)							
Female stage V	CH = 0.925CW – 0.126	42	0.924	0.065	1.127	> 0.05	(0)
Female stages II–IV	CH = 1.407CW – 0.445	10	0.932	0.133	3.067	< 0.05	(+)
Female hard	CH = 1.115CW – 0.324	17	0.487	0.344	0.877	> 0.05	(0)
Female pre-hard	CH = 0.551CW – 0.259	6	0.313	0.409	1.097	> 0.05	(0)
Male hard	CH = 1.156CW – 0.339	56	0.808	0.075	2.042	< 0.05	(+)
Male pre-hard	CH = 0.967CW – 0.288	15	0.801	0.132	0.242	> 0.05	(0)

The regression equations, sample sizes (N), correlation coefficients (r^2), standard errors of the slopes (SE_s), t-test statistics (t_s), significance (P) and the allometric status of each variable are shown. CW, carapace width.

N. novaezealandiae – Jones, 1977a; *Z. ostreum* – Møller Christensen & McDermott, 1958). Females pass through a series of post-hard soft-shelled forms (II–V) after the hard form and before attaining sexual maturity. In these post-hard stages female loss all 'free-life' body features observed in hard-stage forms. For instance, the exoskeleton progressively turns soft and membranous, the body shape becomes increasingly rounded, and the ambulatory legs and claws became smaller and slender, similar to that observed in the same stage but in other pea-crabs (e.g. Møller Christensen & McDermott, 1958). As in the pre-hard crabs, the external features that develop during the different and consecutive post-hard crabs support the notion that these crabs are well adapted to an endosymbiotic lifestyle (Stauber, 1945; Pearce, 1966).

In addition to the changes depicted above, there are other more subtle changes in the female morphology while transitioning from the stages II–IV. We believe that the development of a series of characters (see below) might be beneficial for crabs that have fully engaged in an endosymbiotic lifestyle. For instance, claw setation becomes progressively denser from stages II–V. Post-hard females of *C. garthi* feed by gently introducing their claws underneath the neck of their limpet hosts so to grasp small pieces of phytoplankton-rich mucous that they consume (Ocampo *et al.*, 2014). A dense setation on the inner surface of the claws and cutting edges of the movable and fixed claw fingers might allow crabs to capture food more efficiently, as previously suggested to occur in other pea-crabs (*Pinnotheres pisum* and *P. pectunculi* – Becker, 2010). Moreover, stage V females exhibit a large dactylus in the backward-pointed fourth ambulatory leg that additional observations have shown is used to anchor them to the host propodium (E. H. Ocampo, pers. observ.). Similar structures that develop from pereopods have been shown to assist females in feeding in others pea-crab species (e.g. *Tumidotheres maculatus* – Caine, 1975). We have not observed *C. garthi* females using their ambulatory legs to feed (Ocampo *et al.*, 2014). We believe that this remarkably elongated dactylus might help crabs to maintain their position within the host brooding chamber while the same host

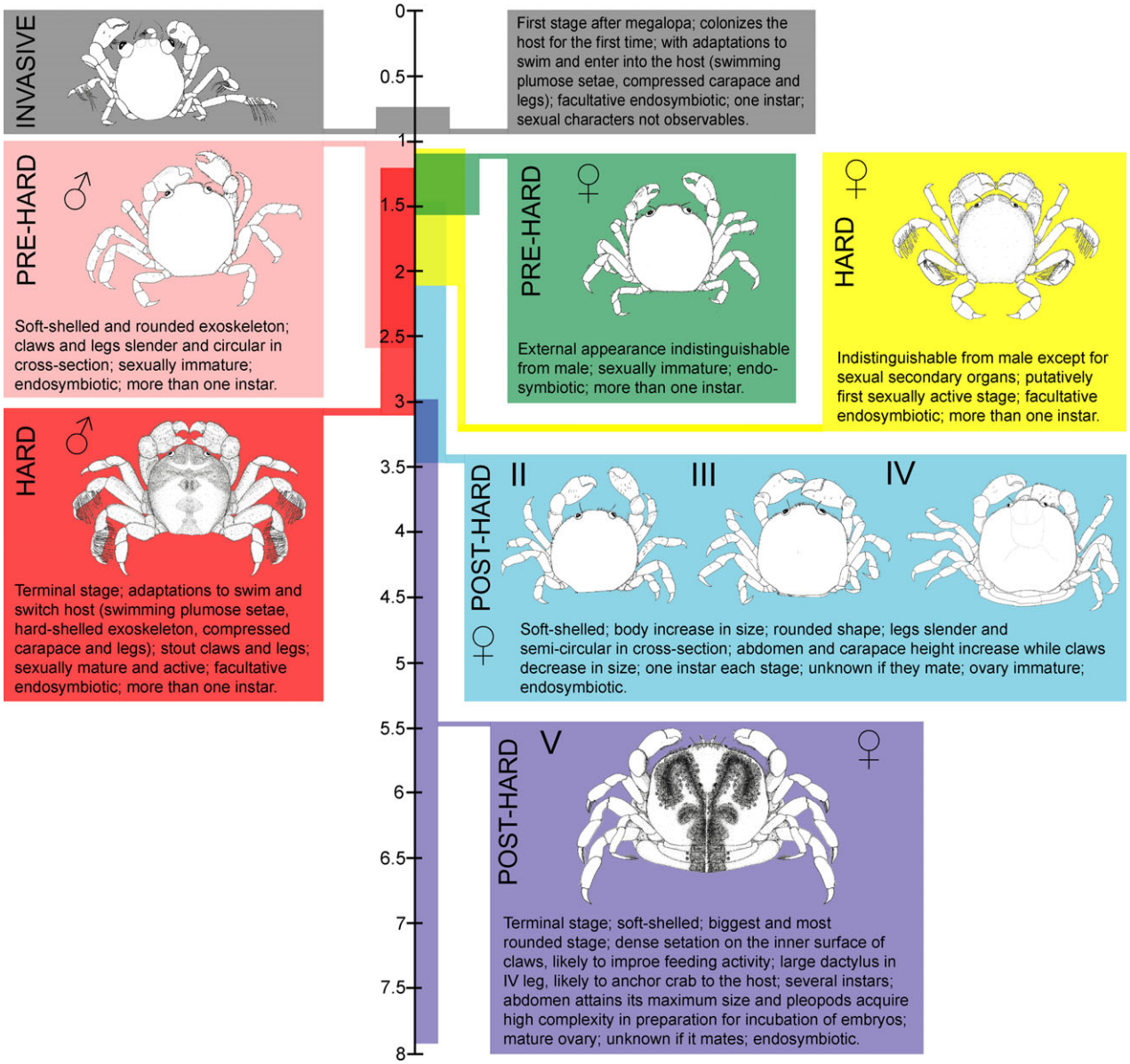

Figure 10. Graphic summary of most relevant morphological characteristics in the post-larval stages of *Calyptraeotheres garthi*. The extent of each colour bar on the vertical scale shows the body size range (mm) of each stage.

individuals move or accommodate some of their internal organs. In other pea-crabs species, modified dactyli have been reported to improve attachment to the host surface (e.g. *Dissodactylus mellitae* – Bell, 1984).

At present, is not well understood whether or not each stage comprises one or more instars in pea-crabs, including *C. garthi*. In a few pea-crab species, however, the wide range of body size and the direct observation of multiple moult events in the laboratory have demonstrated that the stage V comprise several instars in females (Møller Christensen & McDermott, 1958; Watanabe & Henmi, 2009). Similarly, pre-hard and hard-stage crabs also appear to comprise more than one instar in other pea-crab species. For instance, pre-hard males in the mussel crab *F. subquadrata* has been observed to undergo eight consecutive moults before attaining the hard form (Pearce, 1966). By contrast, the post-hard stages II to IV, as well as the first crab, seems to represent 'single-instar' stages in *Z. ostreum* and *F. subquadrata* (Møller Christensen & McDermott, 1958; Pearce, 1966). In agreement with the pattern inferred above for other pea-crab species, female stage V and male and female in pre-hard and hard stages in *C. garthi* exhibited a wide range of body

sizes. The above suggest that these stages pass through several instars attaining large size without experience considerable morphological changes. By contrast, both first crabs and stages II–IV females in *C. garthi* appear to change stages after one single moult event, as indicated by the short range of body size of these crabs.

Throughout their shared evolutionary history, the host likely exerts selective pressures that might favour or constrain the evolution of morphological traits in the symbiont (Renaud *et al.*, 1996). Taking into account the above and considering that the host used by *C. garthi* differs considerably in size and body plan from bivalves, differences on the post-larval cycle of *C. garthi* compared with bivalve-inhabiting related species should be expected. In contrast to the expectation above, however, our results reveal that the juvenile and adult life of males and females in *C. garthi* follow a sequence of stages similar to that reported for bivalve-dwelling species of pea-crabs (e.g. *Zaops ostreum* – Møller Christensen & McDermott, 1958; *Fabia subquadrata* – Pearce, 1966; *N. novaezelandiae* – Jones, 1977a). This information suggests that the post-larval cycle is evolutionary conserved among bivalve and gastropod-dwelling pea-crabs. Nevertheless, more studies are needed to conclusively determine if the post-larval cycle observed in the above species is also shared by pea-crab that inhabits in or on other macro-invertebrate hosts. For instances, the pea-crab *Dissodactylus primitivus*, a phylogenetically closely related species of *C. garthi* (Ocampo *et al.*, 2013; Palacios Theil *et al.*, 2016) that lives on the tegument of irregular echinoids, does not exhibit soft-shelled stages throughout its post-larval life (Pohle & Telford, 1982). In this species, individuals from all sizes and sexes have a well calcified exoskeleton (Pohle & Telford, 1982) suggesting that the post-larval life cycle discussed above does not fit all members of the family Pinnotheridae. Additional comparative studies focusing in pea-crabs using hosts species differing in body size, body plan, and ecology will help us to understand the role that hosts play in determining the evolution of morphological features in pea-crabs.

Sexual dimorphism and mating behaviour in *Calyptraeotheres garthi*

The pea-crab *C. garthi* is sexually dimorphic with respect to body size, but the extent and direction of dimorphism varies according to crab stage. For instance, in the studied population, hard-stage males were, on average, larger than hard-stage females. Classical sexual dimorphism in terms of body size, as that observed for hard stages in *C. garthi*, is expected in species in which male-male competition for receptive females via overt aggression is intense (Hartnoll, 1974; Shuster & Wade, 2003). In other crab families, sexual dimorphism with respect to body size is typically accompanied by the exaggeration of body parts, such as the claws (Hartnoll, 1974). In males, larger claws might significantly increase the likelihood of winning during agonistic competitive interactions for access to receptive females (Hartnoll, 1974, 1982). In *C. garthi*, however, no exaggeration of body parts (i.e. claws) was observed in the putatively sexually active hard crab stage. Indeed, no differences between the hard male and female stages were observed with respect to any external body part. Furthermore, preliminary observations of behavioral interactions among hard males maintained in groups together with females in the laboratory did not result in any type of male-male agonistic interactions (E. H. Ocampo, pers. observ.). Therefore, other than body size, there are no indications of intense male competition and/or monopolization of receptive females via overt aggression in this species. Instead, body size dimorphism during the hard-stage form in *C. garthi* might be the consequence of sex-specific growth rates during their post-larval development. Specifically, hard females might moult quickly into the first post-hard stage while males remain in this terminal hard form and might continue molting and increasing in body size without suffering substantial changes in their morphology. Controlled laboratory experiments are necessary to reveal more details about sex-specific growth schedules in hard stages of *C. garthi*.

By contrast to that observed between hard crabs, females of *C. garthi* were larger than males when we compared the body size of females in the terminal stage V and males in the terminal hard stage. Females attaining larger body size than males have been reported before in many symbiotic species (e.g. Poulin, 2007) including various pea-crabs (Hines, 1992; Campos, 1996; Ambrosio & Baeza, 2016). A larger size in females might be beneficial if it translates into larger egg clutches (Poulin, 2007). Indeed, in *C. garthi*, fecundity (number of embryos) is positively correlated with body size (Ocampo *et al.*, 2012). The reproductive investment is extraordinary high in this species (up to 89% of the body weight – Ocampo *et al.*, 2012), as observed before in other pea-crabs (Hines, 1992). Therefore, the relatively large body sizes that stage V females of *C. garthi* attain might significantly increase their reproductive investment. Moreover, a large egg clutch should be particularly important to achieve in symbiotic species, such as *C. garthi* and other pea-crabs. Symbiotic species are expected to suffer high larval and post-larval mortality rates compared to free-living species as the environmental offer for substrates in which to settle is

greater in the latter species (Hines, 1992). Therefore, a larger egg clutch (average number of eggs: 1794 in *C. garthi* – Ocampo *et al.*, 2012; 5680 in *Z. ostreum* and 7560 in *F. subquadrata* – Hines, 1992) likely helps to compensate for the high mortality that offspring of *C. garthi* (and other pea-crabs) might suffer while search for a proper limpet host individual to settle.

In addition to females obtaining benefits attaining a larger body size, the relatively small size might also provide a net of benefits to males of *C. garthi*. The small body size of hard males might be beneficial if male individuals leave often the host and actively move along the external environment searching for another hosts or sexual partners. For instance, the small size might help crabs of *C. garthi* to successfully pass through (leaving or entering) the tight gap between the shell and substratum that limpet hosts temporarily open to breath and/or feed (see Trottier & Jeffs, 2015). A previous study conducted in *C. garthi* revealed that hard males do not stay, at least for long periods of time, inside of the same host individual (Ocampo *et al.*, 2012). Also, our present results show that hard-stage males develop free-life features (swimming setae, flattened carapace, hard exoskeleton, pigmentation) as observed in other pea-crabs (Orton, 1920; Pearce, 1966). The above strongly suggest that in this hard stage, male crabs achieve an efficient capability to abandon and then roam outside of the host. Therefore, the small size might facilitate movements exploring the external surroundings and/or other hosts located in the vicinities and this activity ultimately might propitiate mating opportunities.

In general, the information above support the notion that hard males have a rather vagile life habit, likely roaming among host individuals in search of receptive females. Indeed, in agreement to that reported in other pea-crabs (Becker, Klaus & Tudge, 2013), males of *C. garthi* likely attain sexual maturity in the hard stage. Our results showed that the gonopods follow a classical two-phase allometric growth pattern observed in many other brachyuran crabs (Hartnoll, 1974) including pea-crabs (Peiró *et al.*, 2011) in which the gonopod of juvenile individuals scales fast to later grow much slower during the adulthood (Hartnoll, 1974). The body size at which the two phases shift represent an estimation of the size at behavioural and/or physiological sexual maturity in crabs (Hartnoll, 1982). In *C. garthi*, a break in the plot depicting the growth of the gonopod (Fig. 9B) was clearly observed between the pre-hard and hard stages, suggesting that males became mature (and possibly ready to mate) after molting from the pre-hard to the hard form.

Hard females in *C. garthi* exhibit the same free-life features observed in hard males, similar to that found in other pea-crab species (Stauber, 1945; Pearce, 1966). The above results suggest that females might also abandon host individuals in search of other host and/or sexual partners. Importantly, in few pea-crab species hard females have been observed to form 'copulatory swarmings' with hard males in open waters (Pearce, 1964, 1966). Whether in *C. garthi* hard females do abandon the host to copulate in open waters or, by contrast, male–female hard pairs first found a proper host and then copulate inside the host is presently unknown. Unfortunately, our results and observations do not allow us to reveal this and other details regarding the reproductive behaviour of *C. garthi*. For instance, we also do not know if hard females are sexually receptive and actively search for sexual partners or if they mature later in life after attaining the post-hard stages. Studies conducted in other pea-crabs are not conclusive with respect to the timing of female copulation (see Møller Christensen & McDermott, 1958; Pearce, 1966; Trottier & Jeffs, 2015). While females from some species appear to mate just during the hard stage (Orton, 1920; Stauber, 1945; Pearce, 1966) other, by contrast, might mate in subsequent post-hard stages (Møller Christensen & McDermott, 1958; Trottier & Jeffs, 2015). Controlled laboratory or field experiments together with an exhaustive ontogenic analysis of the female genital organs might help us to unmask these poorly known details of the sexual biology in this remarkable family.

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