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BEHAVIORAL INTERACTIONS BETWEEN *TRITAETA GIBBOSA* (CRUSTACEA, AMPHIPODA) AND *OCNUS PLANCI* (ECHINODERMATA, HOLOTHUROIDEA)

E. LAETZ[1]*, C. O. COLEMAN*[2]*, G. CHRISTA*[1]*, H. WÄGELE*[*1]

[1] *Zoologisches Forschungsmuseum Alexander Koenig, Adenauer Allee 160, 53113 Bonn, Germany*
[2] *Museum für Naturkunde Berlin, Invalidenstraße 43, 10115 Berlin, Germany*
* *Corresponding author: h.waegele@zfmk.de*

PARASITISM
SYMBIOTIC RELATIONSHIP
TRITAETA, OCNUS
ANATOMY
HISTOLOGY
BEHAVIOR

ABSTRACT. – Although dexaminid amphipods have been observed interacting with numerous other taxa, the particular relationships they share with echinoderms remain unexplored. This study examines interactions between *Tritaeta gibbosa* and the holothurian *Ocnus planci,* which the amphipod inhabits. Each amphipod creates its pit and propels itself through the mantle by actively pulling the mantle tissue using its pereopods. Many individuals were observed living in high densities in all areas of the *O. planci* mantle, with higher preference of the oral and "dorsal" sides, a trend that was corroborated by behavioral experiments. Experiments on behavior and anatomical studies of the amphipod and the holothurian host were performed in order to clarify the mechanisms behind settlement and pit formation, placement and location, as well as the amphipod's morphological adaptations to this peculiar life style. To investigate parasitism and allow for future identification, *T. gibbosa, O. planci* and *Cucumaria montagui* were also barcoded (CO1 and 16S), unfortunately with lower success.

INTRODUCTION

While interactions between amphipods and echinoderms have been investigated in the past, little is known about the particular relationships of the dexaminid amphipod, *Tritaeta gibbosa* Bate, 1862, with its documented hosts Porifera, Ascidiacea or Echinodermata (Della Valle 1893, Walker 1895, Fage 1928, Chevreux & Fage 1925, summarized by Vader 1978). Ruffo (1958) first collected solitary *Tritaeta* individuals from the sea cucumber *Holothuria tubulosa* Gmelin, 1791 and the brittle star *Ophiothrix fragilis* Abildgaard, *in* O.F. Müller, 1789. Changeux (1960) observed *T. gibbosa* living within skin folds of the holothurian *Ocnus planci* Brandt, 1835, (syn. *Cucumaria planci* Von Marenzeller, 1893) and *Holothuria stellati* Delle Chiaje, 1823. Most recently, McClintock *et al*. (2009) described a similar relationship from the Antarctic amphipod *Polycheria antarctica* Stebbing, 1875 f. *acanthopoda* Thurston, 1974, living in pits they dig in the tunica of the ascidian *Distalpia cylindrica* (Lesson, 1830).

Although *T. gibbosa* is documented from numerous hosts, none of these reports elucidate the mechanisms behind this interaction from ecological, anatomical, or physiological perspectives. This study aimed to clarify some of these mechanisms, reveal how and why *T. gibbosa* interacts with holothurian hosts, and to fill in unknown morphological characters that might show adaptations to this enigmatic life style. In order to verify possible parasitism of the amphipod by feeding on the holothurian, gut content barcoding was utilized.

MATERIAL AND METHODS

Ocnus planci, *Tritaeta gibbosa* and *Cucumaria montagui* Fleming, 1828 specimens were collected using a benthic trawl net at a depth of 40-60 m off the coast of Banyuls-sur-Mer, France in September 2011 and April 2012. *Holothuria tubulosa* was collected from *Posidonia oceanica* Linné, 1767 beds at a depth of about 20 m. All specimens were kept in plastic tanks equipped with fresh seawater and a thin layer of sediment from the collection site, for a maximum of 12 days. Experiments on live animals were performed in these plastic tanks at the Laboratoire Arago of Banyuls-sur-Mer (France).

Holothurian species were identified by ossicle analysis. Mantle samples were dissolved in a KOH and fresh water solution for 48 hours. The ossicles were then examined under a light microscope. For identification, we followed the identification keys in *Faune de France* (Koehler 1921).

Following the behavioral experiments, samples of each inhabited *Ocnus* as well as *T. gibbosa* were taken for scanning-electron microscopic (SEM) and histological examination. The samples for SEM analysis were preserved in 96 % ethanol while the histological samples were preserved in formaldehyde/seawater (10 %). Morphological details of five *T. gibbosa* specimens were analyzed with a scanning electron microscope (Hitachi S – 2460 N, Bonn, and a Zeiss EVO LS 10, Berlin). Samples were washed in acetone and freeze-dried. The histological samples were embedded in hydroxymethylmetacrylate (Kulzer Technovit 7100), sectioned (4 μm), stained with Toluidine blue and analyzed using a Zeiss Axio Imager.Z2M compound microscope, a Zeiss AxioCam HRC camera and AxioVision version 4.8 software.

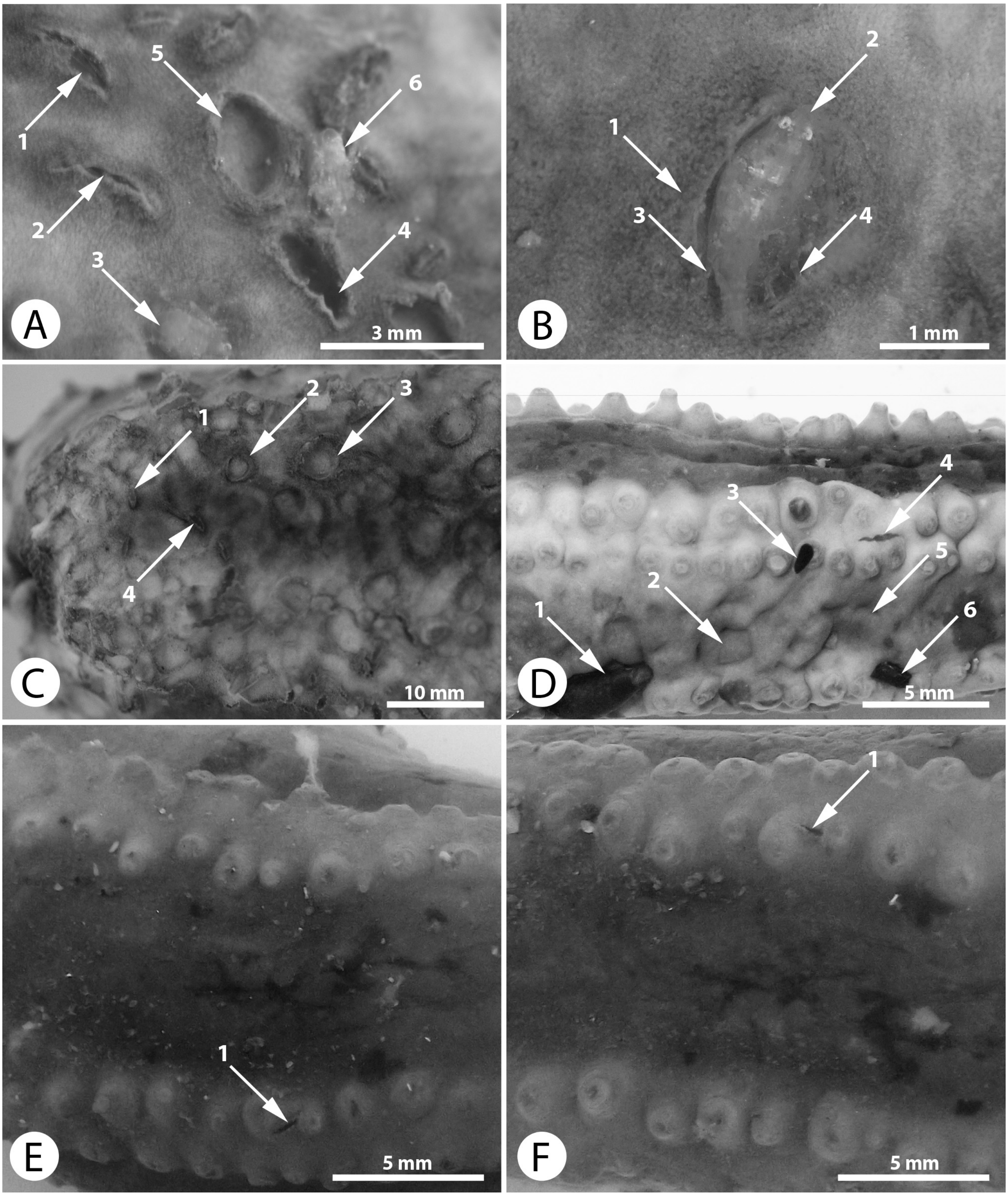

Fig. 1. – *Tritaeta gibbosa* pits within the *O. planci* mantle. **A**: Various pit forms. Arrow 1: undisturbed amphipod, with its pit open. Arrow 2: disturbed amphipod. Arrow 3: amphipod beginning to form a new pit after it has been removed from another one. Arrow 4: pit of an amphipod removed immediately before the photo was taken. Arrow 5 pit of an amphipod removed 30 min prior to the photo being taken. Arrow 6: amphipod removed and about to begin embedding, with its dorsal side touching the *O. planci* mantle. **B**: An embedding *T. gibbosa*. Arrow 1: darker tissue surrounding the amphipod, showing that this amphipod is embedding in a former pit location. Arrow 2: amphipod head, with the two bright eyes. Arrows 3 and 4: amphipod pereopods gripping the holothurian mantle. **C**: Abundant scars after amphipod removal. Arrows 1 and 4: amphipods, embedded in the mantle. Arrows 2 and 3: scars from former pits, where the amphipods had been removed ten days before this photo was taken. **D**: Debris collected by *O. planci*. Arrows 1, 3 and 6: differently sized debris attached and collected by podia (arrow 3), or lightly held by the mantle (arrows 1, 6). When debris is removed, no scars remain (arrows 2, 5). **E**: Position of *T. gibbosa* specimen 2 embedded in *O. planci* specimen 1 after 96 hours (see Table I). **F**: Same amphipod after 120 hours. The total distance traveled was 15 mm.

Ocnus planci, *C. montagui* and *T. gibbosa* DNA was extracted by using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at –20 °C. Amplification reactions were performed using 0.5 µl of genomic DNA, 2.5 µl sterilized water, 1 µl Qiagen® Q-Solution, 5 µl of double concentrated Qiagen® Multiplex PCR Master Mix and 0.5 µl of 5 pmol/µl of each of the concentrated primers. The amplification of partial COI sequence for *Tritaeta gibbosa* was carried out using primers LCO (5' GGTCAACAAATCATAAAGATATTGG 3', Folmer *et al*. 1994) and HCO (5' TAAACTTCAGGGTGACCAAAA AAT A 3', Folmer *et al*. 1994), and were denatured for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 45 s, 51 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. Amplification of partial COI sequence for the two holothurians was performed using the specific primers COI-F (5' CCTGCAGGAGGAGGAGGAGAYCC -3, Palumbi *et al*. 1991) and COI-R (5' CCAGAGATTAGAGGGAATCAGTG 3', Palumbi *et al*. 1991), and were denatured for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. Amplification of partial 16S sequence for the two holothurians was carried out using specific primers for these taxa: 16s-L (5' GACGAGAAGACCCTGTGGAGC3', El Naggar *et al*. 2008) and 16s-R (5' ACTTAGATAGAAACTGACCTG 3', El Naggar *et al*. 2008), and were denatured for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. All PCR fragments were purified by adding 10 µl of chilled 96 % Ethanol and 1 µl of 3 mol Na-acetate. This was then centrifuged for 15 min at 13,000 rpm. After removing the supernatant, 10 µl of 70 % Ethanol were added and the samples were centrifuged for 5 min at 13,000 pm. The pellets were then dried, resolved in 20 µl sterile water and sequenced with primers used for amplification by Macrogen Inc. (Amsterdam, Netherlands).

RESULTS

Live Experiments

The investigation of living specimens of *Ocnus planci* (Cucumariidae) revealed that *T. gibbosa* individuals were embedded in pits within the holothurian mantle (Fig. 1A) with legs pointing into the water column. The amphipods usually gripped the pit rim with their pereopods (Fig. 1B) and created water currents with their pleopods allowing respiration. Upon inspection with a probe, each amphipod was able to close the pit by moving the holothurian mantle over itself to form a covering, with only a small slit remaining to indicate their presence in the mantle (Fig 1A). Once removed from the pit, the amphipod died, the pit disappeared and the mantle flattened out within 24 hours. Scars from previous pit locations were visible as discolored, flattened structures with a distinct ring where the pit rim had been for more than 10 days (Fig. 1A).

Of the 16 *Ocnus planci* specimens observed, only 3 were uninhabited by *T. gibbosa,* the others housing 1-140 individual amphipods (Fig. 1C). Each amphipod was between 0.5 and 2.5 mm long. They were found on every radial (the rows of tube feet or podia) and every plate (the area between each radial) at both the oral and aboral ends. However, amphipod populations usually were concentrated at the oral end between radials C and D (the "dorsal" plate, Plate C). They were also found at the juncture between the mantle and the feeding tentacles, although no *T. gibbosa* were found in non-mantle tissue (Fig. 1C).

Stones or debris of varying shapes and sizes were usually associated with each of the *Ocnus* specimens investigated (Fig. 1D). This debris was only slightly embedded in the mantle tissue and easily removed with minimal force. These impressions then quickly disappeared with no sign of scarring. Stones, plastic beads and pieces of wood of the same size as an average *T. gibbosa* were placed on an infected *O. planci* specimen to determine

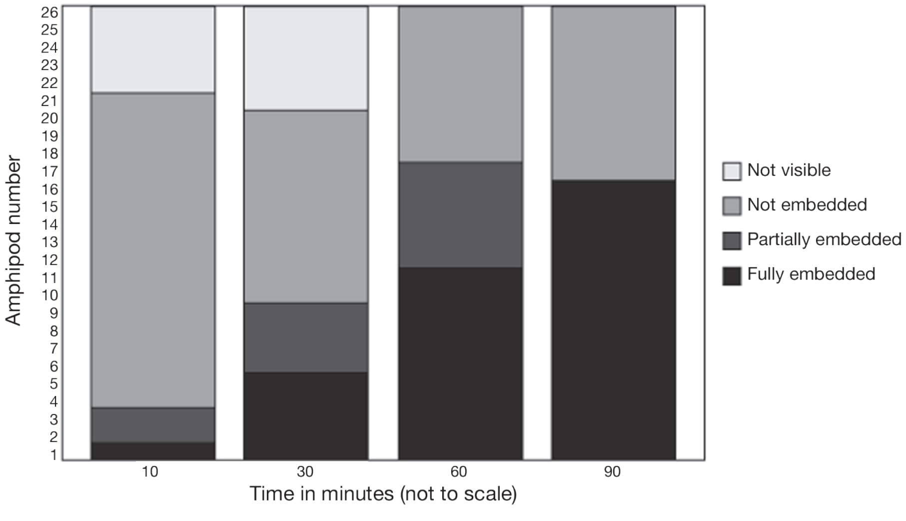

Fig. 2. – *Tritaeta gibbosa* re-embedding progress. The starting location and pit formation progress versus time in minutes for each amphipod. As the experiment was left undisturbed while in progress, not all amphipods were visible at all times.

Table I. – *Tritaeta gibbosa* embedding progress and success. ? formation success unknown. * This animal started to make a pit but was unsuccessful, dying during the process and not completing pit formation.

Amphipod numbers	Starting location	Formation success	Final location
1-8	*O. planci*	yes	pit
9-10	*O. planci*	?	pit
11-16	Water	yes	pit
17	Water	yes	*
18-19	Water	?	water
20-26	Water	no	water

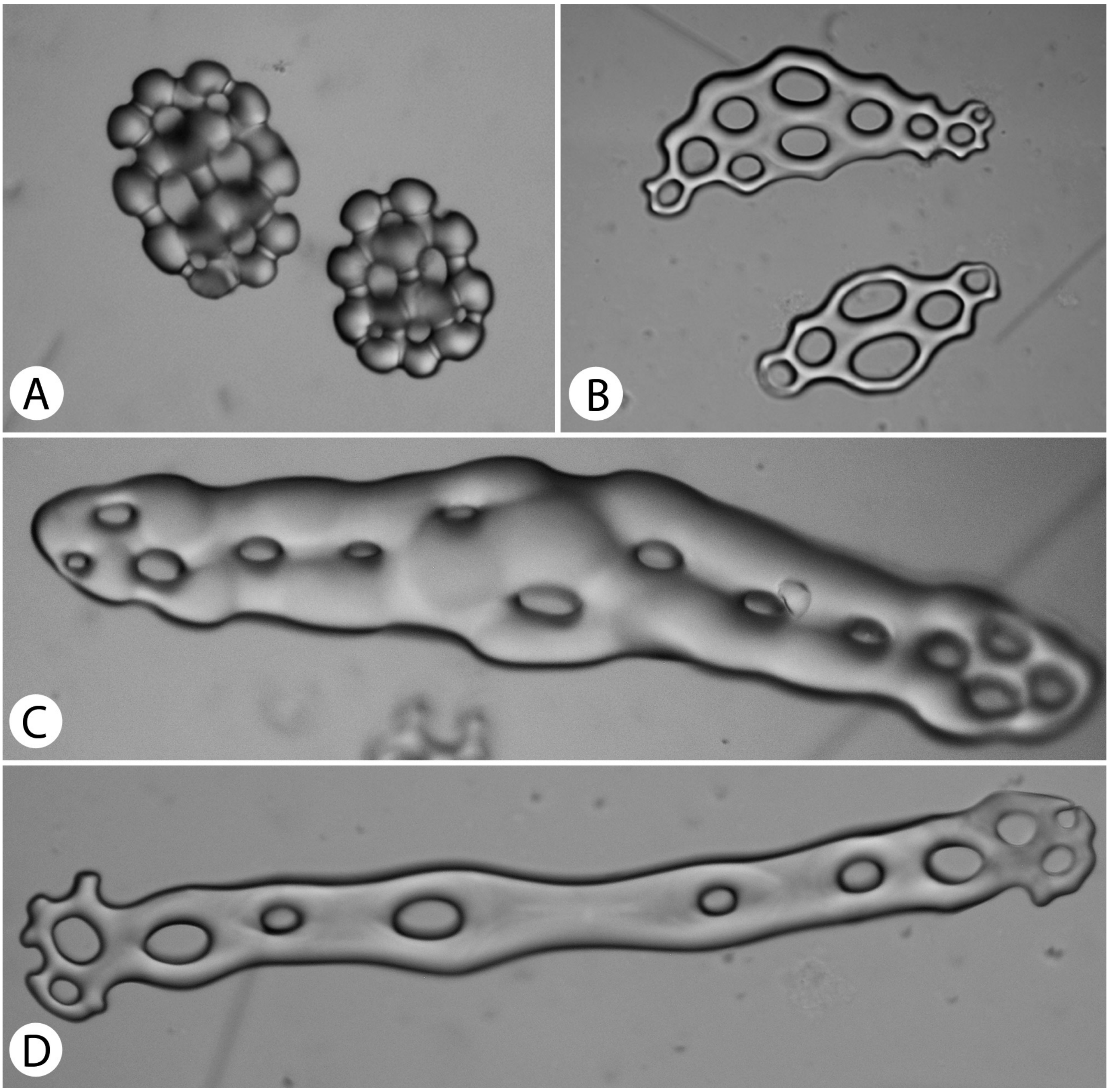

Fig. 3. – Ossicle identification. **A**: *Ocnus planci*, typical honey-comb shaped ossicles. **B**: *Cucumaria montagui* ossicles with characteristic large, infrequent holes. **C**: *O. planci*, elongate ossicle with numerous, small holes. **D**: *C. montagui* elongate ossicle with larger and less frequent holes than in *C. planci*.

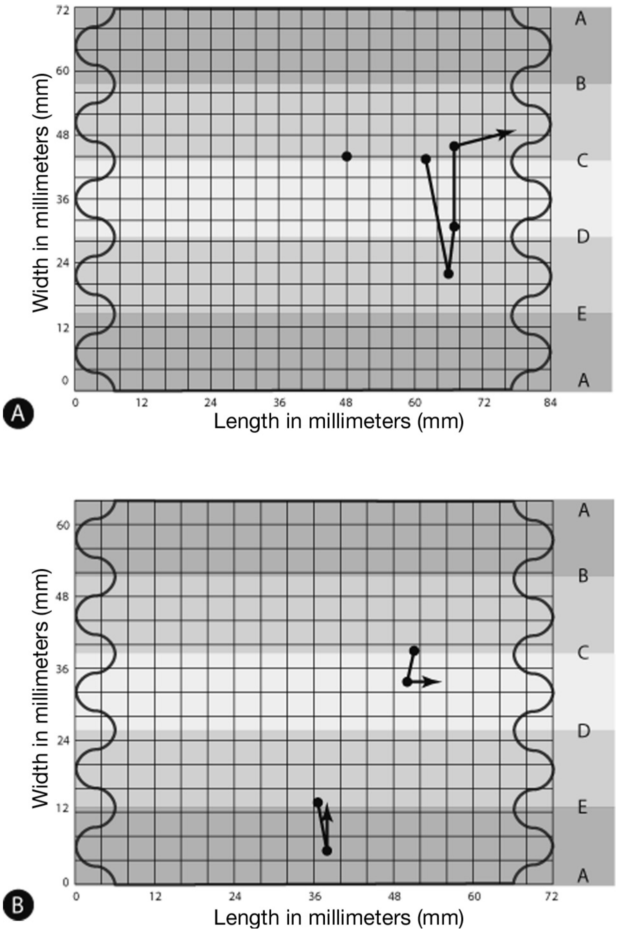

Fig. 4. – *Tritaeta gibbosa* movements within the *Ocnus planci* mantle (see also Table II). **A**: *O. planci* specimen #1 unrolled to form a flat surface. The single point indicates amphipod 1, the amphipod that did not move during the course of this experiment. The connected line segments show the trajectory of amphipod 2. Measurements were taken every 12 hours. **B**: *O. planci* specimen #2, unrolled. Both amphipods moved and their trajectories are displayed by connected line segments.

if the holothurian mantle tissue responded to objects of amphipod size by making pits. These objects were not engulfed and incorporated into pits however, suggesting the amphipod is actively involved in the pit formation process.

To establish if *T. gibbosa* could re-enter the *O. planci* mantle, 26 *T. gibbosa* were removed from an inhabited *O. planci*. They were immediately placed on a previously uninhabited specimen, from the same trawl. 10 were placed directly on the mantle, in a line on Plate C. The other 16 were placed in the water column surrounding the *O. planci* specimen at a distance of about 1 cm. The time it took to re-embed within the mantle, the re-embedding locations and the number of successful amphipods were recorded every 10 minutes. 16 out of the 26 amphipods were able to successfully and completely re-embed (61.5 %) with the fastest amphipod completely embedding in 9 minutes (Fig. 2). The mean re-embedding time was about 60 minutes. After 120 minutes, amphipods that had not successfully embedded were dead and therefore the experiment was stopped. One amphipod that began to form a pit after 60 minutes was not embedded the next day. Each amphipod's starting and final locations as well as the final embedding success rates were recorded (Table I). Of the 16 successful amphipods, 8 belonged to those placed directly in the row on the *O. planci,* 6 were from the surrounding water and 2 could have been either from the row or from the water column. The amphipods placed in the water surrounding *O. planci* were rather inactive and rarely swam.

To establish if *T. gibbosa* could embed within the mantle of other holothurian species, the same procedure outlined in the earlier experiment was used on *Holothuria tubulosa* (Holothuriidae) and *Cucumaria montagui* (Cucumariidae). Embedding within *Cucumaria montagui* mantles had an average time of 11 minutes, however *T. gibbosa* was not able to embed in *Holothuria tubulosa*, even when placed directly on the intended host. The ossicles from each species were examined to confirm our identifications of holothurian species and are depicted in Figure 3.

To determine if *T. gibbosa* can move within an *O. planci* mantle, 4 amphipods were transferred from their original host to two previously uninhabited *O. planci* specimens. Once embedded, they were allowed 24 hours to acclimatize. The original placement locations as well as the actual embedding locations were recorded. They were monitored and measured every 12 hours for 5 days to determine their current locations and the distances they traveled. Amphipod movements were mapped by depicting the holothurian as a two dimensional surface (unrolling it along Radial A to form a flat surface), placing a grid over the plane and then graphing each movement. After 48 hours, the first movement was observed. This individual continued moving until the experiment concluded for a total distance of 55.83 mm (Fig. 4A). One *T. gibbosa* did

Table II. – *Tritaeta gibbosa* movement through the *Ocnus planci* mantle observed in the two different holothurian specimens.

O. planci #	*T. gibbosa* number	Movement	Movement start time	Total distance traveled	Average speed (mm/hour)
1	1	no	–	–	–
1	2	yes	48	55.83	0.78
2	3	yes	96	14.07	0.42
2	4	yes	96	10.10	0.59

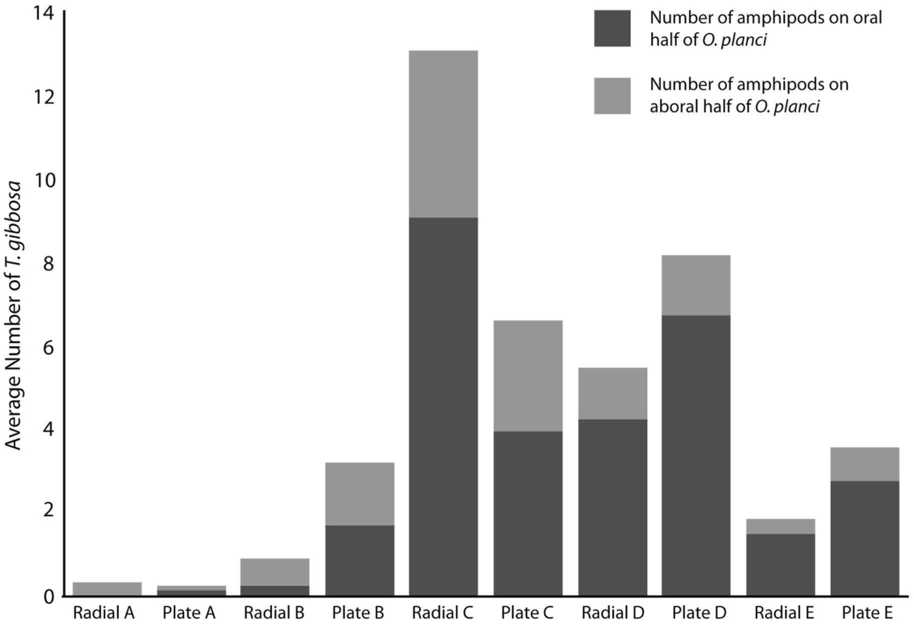

Fig. 5. – Observed initial locations and optimal area. The average number of *Tritaeta gibbosa* on each colonized *Ocnus planci* specimen (10 specimens), were divided by a modifier allowing the direct comparison between radials and plates. The darker grey bar indicates the number of amphipods found on the oral half of the holothurian and the lighter grey bar indicates the amphipods found on the aboral end.

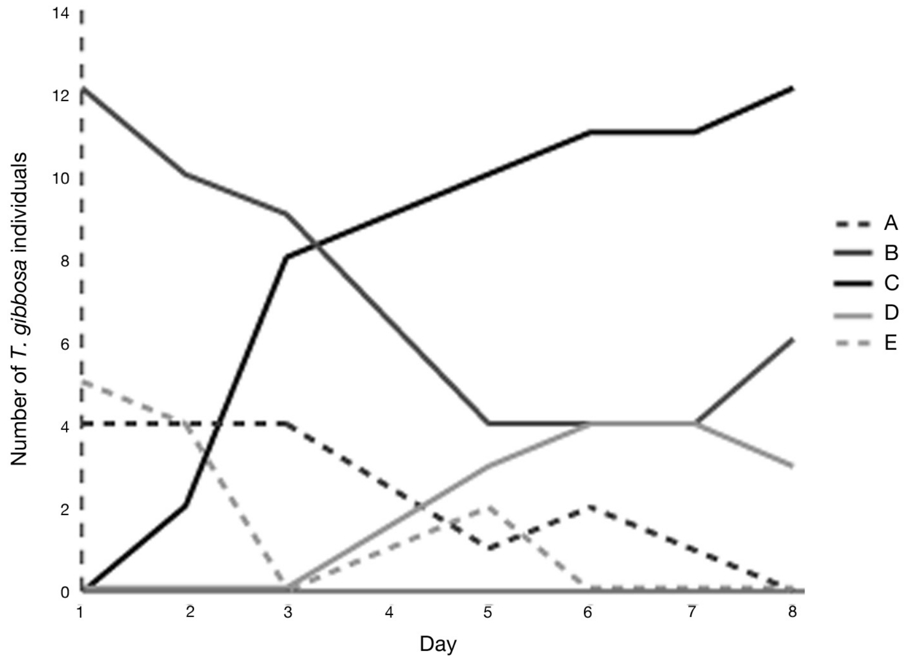

Fig. 6. – Average re-colonization of the optimal area. Following the removal of all amphipods found in the predicted optimal area, the oral end (Plate C, see Fig. 5), the population in this area was recorded as the other amphipods were allowed to re-colonize. Each line represents the population in that plate (y-axis) for each day (x-axis). At the beginning of the experiment Plate C has a population of zero, which increases to an average of 12 amphipods by day eight. The sub-optimal area populations (Plates A and E) drop from 4 and 6 to 0 specimens.

not move at all (Fig. 4A) and the final two started moving after 96 hours (Fig. 4B). The speed and distance each of the three amphipods that traveled were calculated for each 24-hour period, with an average of 16 mm per 24-hour period (measured after first movement) or 0.67 mm/hour (Table II). The farthest distance traveled in 24 hours was 21.38 mm.

Observations of the wild caught specimens revealed a distinct distribution pattern. While *T. gibbosa* individuals were found throughout the holothurian mantle on all radials and plates, they were densely aggregated toward the oral end on the "dorsal" plate C (Fig. 5). To determine if this area was actually preferred, all *T. gibbosa* individuals on the preferred oral half between radials C and D were removed from two heavily colonized *O. planci* specimens (Specimen #1 and #2, having 36 and 39 amphipods respectively) after their original distributions were mapped. The remaining amphipods (48 % of the original 36 amphipods in *O. planci* Specimen #1 and 47 % in Specimen #2) were monitored to determine whether there was directional movement toward this zone. The location of each amphipod and population in each area was charted for 8 days (Fig. 6). As each radial is much narrower than each plate (the area between each radial) the average population in each zone was divided by a modifier that accounts for the total surface area that could be inhabited (3.3 for each radial and 7.6 for each plate). This standardized each population value to account for the available surface area that could be colonized and allow comparison between each zone. A distinct pattern of relocation to the optimal area was observed, as 50 % (Specimen #1) and 42 % (Specimen #2) of the amphipods moved to plate C after 8 days. This suggests *T. gibbosa* does prefer the oral end between radials C and D.

Histological investigation of **Ocnus** *and embedded* **Tritaeta**

In its natural, undisturbed state, *Ocnus planci* mantle structure consists of five layers: the outer epithelium, a subepithelial layer of connective tissue directly beneath the epidermis with densely aggregated amoeboid cells (thickness about 70 μm), the outer dermis containing ossicles, connective tissue and amoeboid cells (thickness about 1 mm), the inner dermis consisting of cartilaginous fibers and microfibers, and the body wall (Fig. 7A). This coincides with anatomical descriptions by Thurmond *et al.* (1996).

When colonized by an amphipod, the mantle profile changes in a distinctive manner. The outermost three layers are clearly altered, however the innermost two (inner dermis and body wall) were not affected. The outer epithelium was not interrupted or disrupted, and, while the subepithelial layer of connective tissue was intact, it was nearly 90 % thinner underneath the amphipod than in undisturbed samples (reducing the thickness of the layer to about 10 μm). The amoeboid cell aggregations seen in the undisturbed profile were dispersed here. The third layer, the outer dermis, was also far thinner (65 %, or about 430 μm thick), and contained densely packed ossicles and less connective tissue (Fig. 7B, E-H).

T. gibbosa positioning within the *O. planci* mantle, as observed in live animals, was confirmed by the histological sections. The dorsal surface faces the cucumber's body cavity, and the ventral surface faces the water column (Fig. 7D-F). The amphipod epidermis is covered by a cuticle with no indication of special glandular structures excreting solvents or other chemicals that may help the amphipod embed.

Morphological observations of **Tritaeta gibbosa**

In order to identify morphological structures that might help the amphipod to embed, a thorough description of the external anatomy is provided.

Both pairs of antennae have long and slender flagellae, those belonging to antenna 1 even reaching the second pleon segment (Fig. 10H). On the basis of each of the flagellar articles are about 4 slender setae. They are longer than two articles combined and different from most amphipod species. Detrital particles are attached to these setae in the fixed specimens.

The upper lip (labrum) is fleshy and with a dense cover of microtrichs on the apicolateral margins.

The mandibles (Fig. 8A) lack a palp, but otherwise follow the basic gammaridean morphology (Watling 1993). The incisors are multidentate and their posterior teeth are especially stout. The lacinia mobilis of the left side is similar to the incisor dentate and it is situated parallel to the cutting edge. The lacinia mobilis of the right mandible (Fig. 8C) is different in shape, with the teeth appearing sharper and containing an additional tooth located proximally. The lower lip (hypopharynx) lies between the mandibular body and maxilla 1. The setae of the inner plates of maxilla 1 and maxilla 2 are directed into the hypopharyngeal gap (Fig. 8A, B). On the outer plates of maxilla 1 are robust serrate setae, which meet ventrally of the hypopharyngeal gap. The palp of maxilla 1 surpasses the outer plate with a serrated distal margin and posteroventral surface covered by long setae. The outer plate of maxilla 2 (Fig. 8B) is ovoid and bordered by long slender and smooth, minutely setulated setae located only apically; the setation of the inner plate is less than half the length of those from the outer plate. The maxillipeds (Fig. 8A) posteriorly close the cavity formed by the mandibles and the maxillae.

Gnathopod 1 has a stout ovoid subchelate propodus, with a sharp, straight and only inconspicuously serrate palm. The tip of the propodus is locked between 2 robust setae at the end of the palm. The medial faces of the carpus and propodus bear several rows of ventrally directed setae, a few setae are inserted at the lateral faces. The dorsal margin of the propodus in the male sex seems to have a unique, deep, narrow notch of unknown function not seen in other amphipods. In gnathopod 2 (Fig. 9A) the propodus is subtriangular in shape with a long oblique, weakly convex palm. Similar to gnathopod 1 there are rows of long setae on the medial face of the propodus. The margins of the basis to merus of pereopods 3 to 7 (Fig. 9B) are parallel; these articles are not much expanded distally as in many other amphipod taxa. The carpus of these appendages, however, is rather short (about 1/3 of the merus) and is expanded posterodistally. It bears 3 long robust setae in the posterodistal angle. Each of the propodi is narrower than the carpus and the curved dactylus reaches between the robust spines. On the posterodistal margin of the propodus close to the dactylus are 2 small robust setae (Fig. 9B). Different from the amphipod ground pattern, where the pereopods 5-7 are rotated so that the dactyli are showing anteriorly, in *T. gibbosa* (as in some other dexaminids) they are directed posteriorly.

Three different microstructures were observed along the external cuticle of the body segments. Short sensilla (Fig. 10A, B) lay symmetrically on each side in relative abundance along all tergite edges, gradually decreasing in number approaching the transversal midline. They

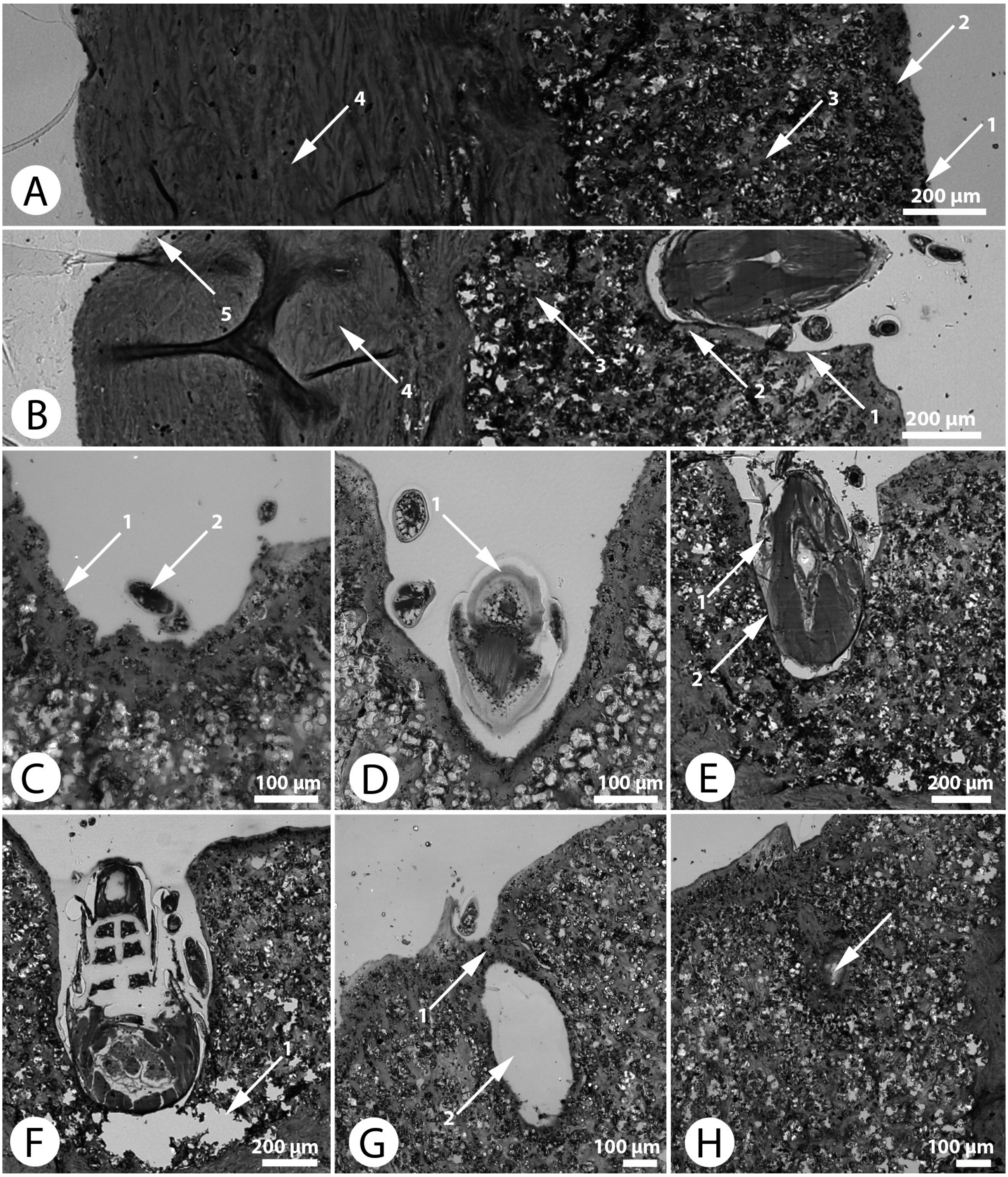

Fig. 7. – Histological analysis of the pit and mantle in *Ocnus planci*. **A**: Undisturbed *O. planci* mantle profile showing the dermal layers in their normal state. Arrow 1: outer epithelium. Arrow 2: sub-epithelial connective tissue layer. Arrow 3: ossicle layer. Arrow 4: inner dermis containing mostly connective tissue. The lining of the body cavity is not visible in this section. **B**: *O. planci* mantle profile when disturbed by a *T. gibbosa* pit. Arrow 1: presence of the unbroken outer epithelium, lining the pit wall. Arrow 2: diminished subepithelial layer, arrow 3: diminished ossicle layer, arrow 4: inner dermis. Arrow 5: lining of the body cavity. **C**: Distal part of pit and posterior end of amphipod. Arrow 1: note intact epithelium. Arrow 2: cross-section of one of the amphipod's posterior appendages. **D**: Distal part of pit and posterior part of amphipod (Arrow 1). Note that subepithelial layer already appears thinner and the ossicle layer looks condensed. **E**: Cross-section through the amphipod's thorax. Arrow 1: longitudinally sectioned thoracopod, reaching out of the pit. Note the thinnest part of the subepithelial layer (Arrow 2) throughout the pit (about 5 μm) and the thin ossicle layer. **F**: Proximal part of thorax, beginning of the head. Note the thinnest part of the ossicle layer throughout the pit, with only about 250 μm thick. Part of the layer missing due to artifact of sectioning (Arrow 1). **G**: Proximal end of the amphipod and pit with outer epithelium and subepithelial layer reconnected after the amphipod has ended (arrow 1). Arrow 2: continuation of the pit under this reconnection. **H**: The end of the pit (arrow).

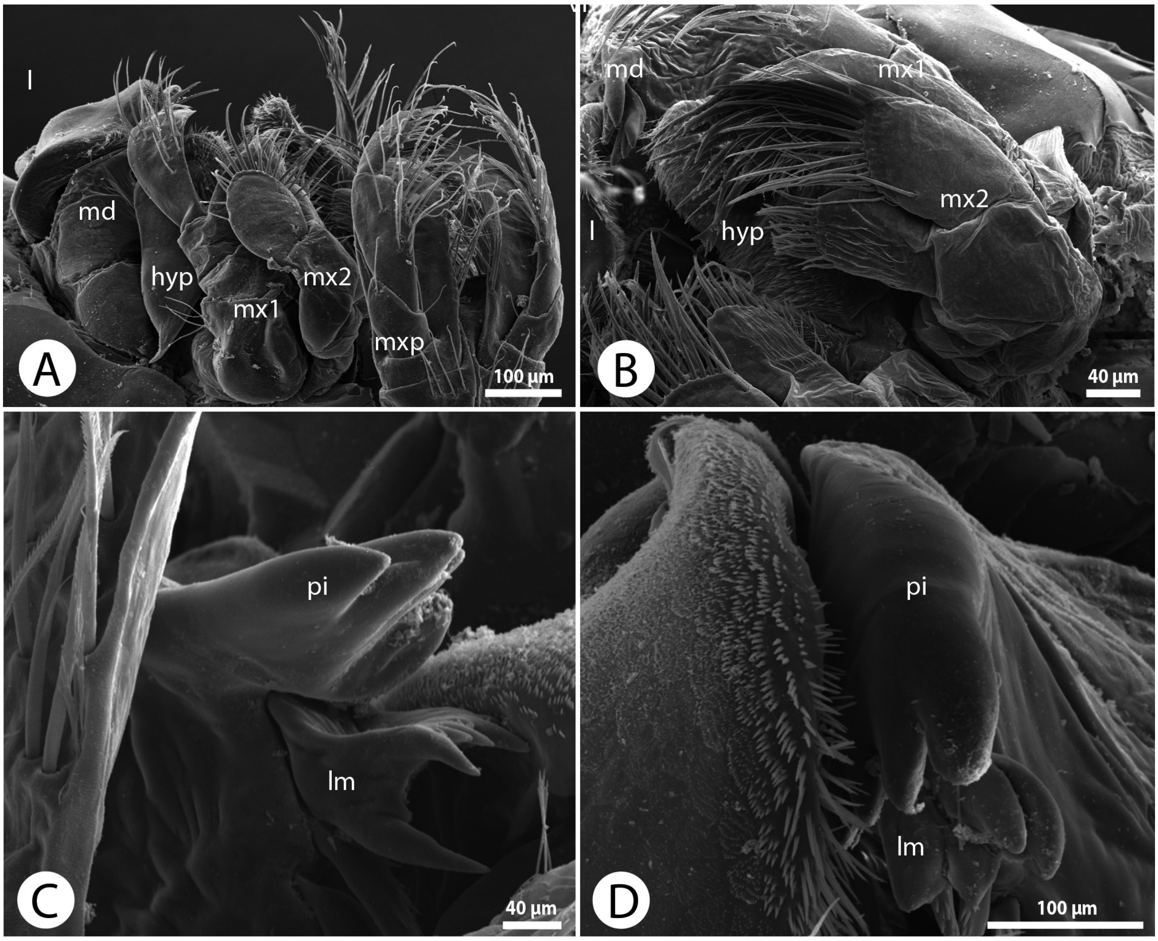

Fig. 8. – SEM micrographs of *Tritaeta gibbosa* mouthparts. **A**: Lateroposterior view showing the maxillipeds (mxp), maxilla 1 and 2 (mx1, mx2), lower lip (hyp) and the labrum (l). **B**: Detail of maxilla 1 and 2 (mx1, mx2), the lower lip (hyp) and left mandible (md). **C**: Right mandible with pars incisiva (pi) and lacinia mobilis (lm). **D**: Left mandible with pars incisiva (pi) and lacinia mobilis (lm).

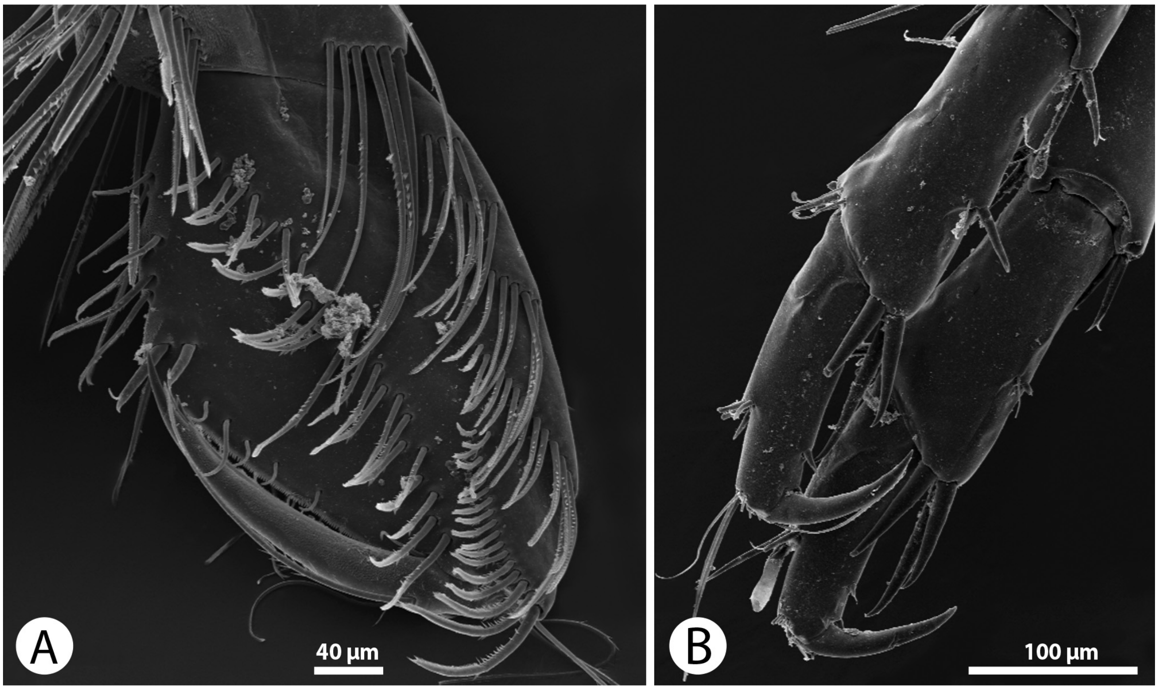

Fig. 9. – SEM micrographs of *Tritaeta gibbosa* appendages. **A**: Propodus and dactylus of gnathopod 2. **B**: Carpus to dactylus of pereopods 5 and 6.

are more frequent at the anterior end of the animal and decrease in number toward the posterior end. Almost every sensillum observed had numerous ridges at the base, a slightly branched appearance, and originated from a porus in the cuticle.

Another type of microstructures, only occurring on the dorsal sides of the pleonites, consisted of type II microtrichs. These cleft, blade-like sensilla are located in two rows, on either side of the transversal midline (Fig. 10D-G). On the first pleonite, the rows were short, containing 3-4 blades (Fig. 10D). The absence of a fourth pore on the left side suggests that the asymmetry is not an artifact. On the second pleonite the rows were far longer having 8 blades each (Fig. 10E). The third type of microstructures were plumose setae, which also originated from cuticular pores (Fig. 10C, G). These differ from the type II microtrichs, as they are highly branched, much larger and found in rows on the pleon.

Barcoding results on **Tritaeta** *and the holothurians*

The CO1 analyses of three *Tritaeta* specimens revealed ambiguous results. Two specimens with identical sequences differed from the third one by a genetic difference of 10.9 %. BLAST search revealed a closer relationship to *Parhyale hawaiiensis*, the other to *Orchomenella franklini,* both members of the Gammaridea.

The primers used in this study for barcoding the holothurians did not work properly. No results for CO1 were obtained. A BLAST search of the single 16S sequence of *Ocnus planci* revealed a close affinity to the few *Cucumaria* sequences in GenBank. The same primers applied to *Tritaeta gibbosa* showed no positive results, thus indicating that the amphipod does not feed on holothurians. But these results are certainly preliminary due to the barcoding and primer affinity problems.

DISCUSSION

Tritaeta gibbosa has been documented as a commensal species associated with Porifera (Fage 1928, Della Valle 1893), Ascidiacea (Walker 1895, Chevreux & Fage 1925, Sezgin 1999), and Echinodermata (Ruffo 1958, Changeux 1961), but almost nothing is known about these interactions. This study attempts to address the ecological and behavioral aspects of this relationship.

Contrary to previous observations (Changeux 1961), *T. gibbosa* does not live within *O. planci* skin folds, artifacts that occur under stressful laboratory conditions, but in distinct pits that can actively be formed and closed by the amphipod.

The ability to re-inhabit a holothurian host has been shown, with a high success rate in our experiments under laboratory conditions. The speed and positioning of the amphipod presents many physiological challenges and suggests a mechanism never observed in other amphipods. The ability to form a complete pit in nine minutes demonstrates an amazing and unique behavior.

Pit formation is not a passive process the holothurian begins when prompted by an external trigger. This option can be excluded by the experiments performed with dead material placed on the outer surface of *Ocnus planci*. Pleon contractions were observed during pit formation, however it remains unclear if this actually enables or aids the formation of a pit. According to our observations, the pereopods seem to contribute most to the embedding process. Pereopods 3-7 were observed gripping holothurian mantle tissue in numerous cases. During the embedding process, they grasped the tissue surrounding the forming pit, potentially pulling the tissue around them to form the pit. Once embedded, the amphipods also gripped the edges of the pit, potentially keeping themselves positioned in the pit and allowing them to move within the mantle. When disturbed, this grip permitted the amphipod to close the mantle around itself. Similar observations were made by Changeux (1960).

The shortened propodus and curved dactylus of pereopods 3-7 are clearly prehensile and well equipped for holding the animal in position inside the pit and clinging to the pit margins: the curved dactyli could grip the edges of the pit and pinch them with the help of the 3 robust setae on the distal carpus angle, assisted by the 2 additional small spiniform setae on the posterodistal margin of the propodus. As in some dexaminid amphipods the pereopods 5-7 are turned so that the dactyli are directed posteriorly. This aberrant rotation of these appendages might result in a better grip on the surface of the host. McClintock *et al*. (2009) described a very similar behavior in another dexaminid species, *Polycheria antarctica* f. *acanthopoda,* which live in pits they dig in the tunica of the ascidian *Distaplia cylindrica*. The authors were not able to observe exactly how the amphipods create their pits, but they described that the animals rotated to their dorsal side and pulled themselves through the host tissue by using their appendages to cling to the margins of the pit.

The lack of gland cells along the *T. gibbosa* body surface also support a physical mechanism of pit formation, however the possibility of a chemical process should not yet be eliminated.

Tritaeta gibbosa mandible morphology was investigated thoroughly to ascertain its role in pit formation, especially due to the fact that some amphipods feed on holothurians (Coleman 1990). *T. gibbosa* mandible morphology suggests effective cutting, due to the presence of multidentate lacinia mobilis on both mandibles, which can act as additional cutting edges. The incisors and the lacinae mobilis form a functional unit of four interlocking cutting edges that could deal with the rigid integument of the holothurian, similar to the interlocking morphology of the mandibles described by Mayer *et al*. (2009) for Gam-

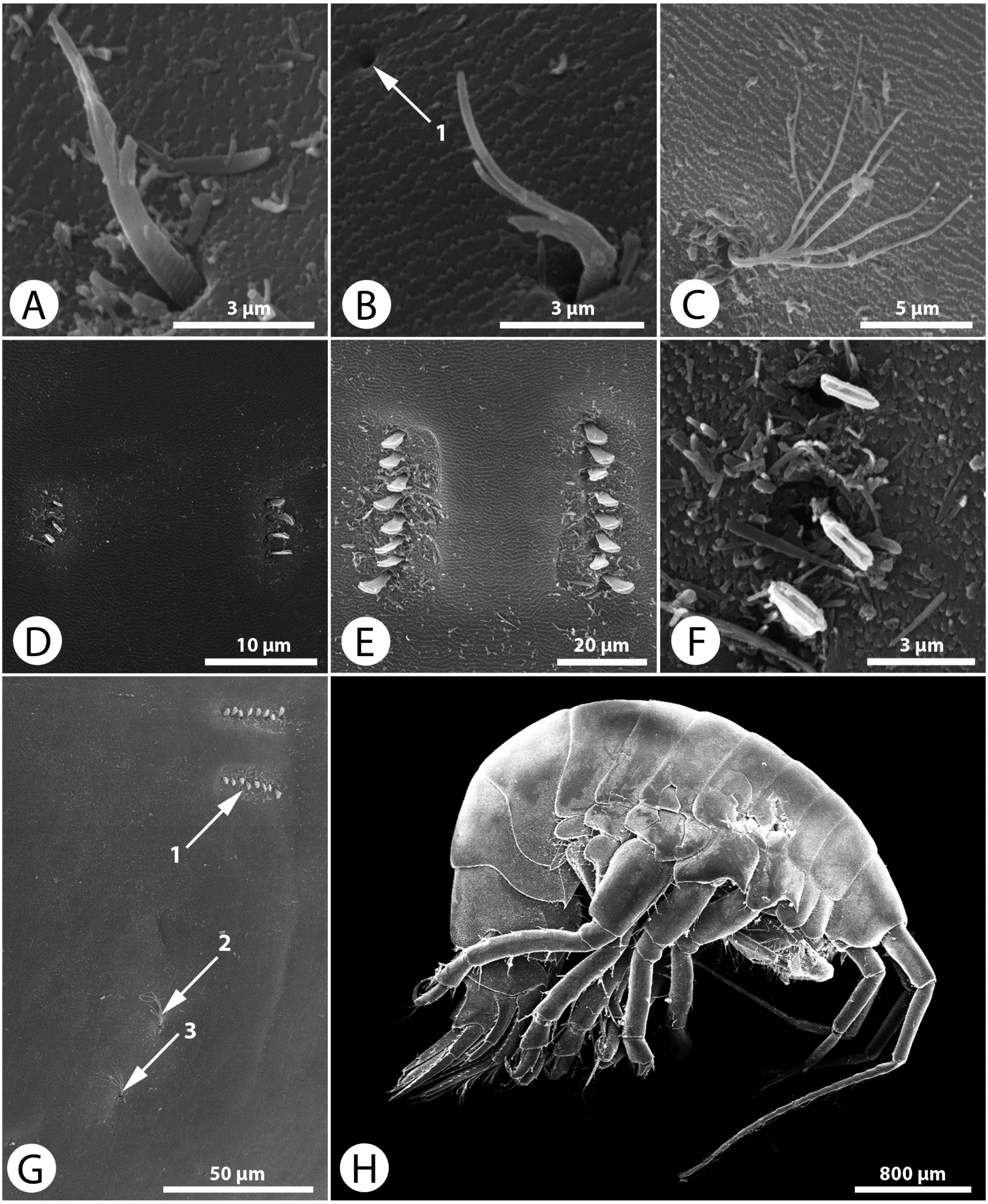

Fig. 10. – SEM analysis of surface microstructures on *Tritaeta gibbosa*. **A**: Short branched sensilla along the tergite edges. **B**: Short branched sensillum. Arrow 1: pore, unrelated to this sensillum. **C**: Plumose setae on the abdominal tergites. **D**: Microtrichs along the dorsal midline of the first pleonite with three on one side and four on the other. **E**: Microtrichs on the second pleonite with an even number on each side of the dorsal midline. **F**: Microtrichs. Note the cleft on the upper surface. **G**: The organization of surface microstructures on the dorsal pleonites. The paired microtrich rows (arrow 1) straddled the dorsal midline. Plumose setae, two on each side, were located between each microtrich row and the tergite edge (arrows 2 and 3). **H**: Lateral view of *T. gibbosa*.

maridea. The amphipod *Bathypanoploea schellenbergi* Holman & Watling, 1983, feeds on holothurians and has a similar effective mandibular cutting mechanism (Coleman 1990), however the author has not observed if *Bathypanoploea* really uses the mandibles to cut a hole in the holothurian integument. As *T. gibbosa*'s mouth was facing the water during the pit formation process and was not observed in contact with the holothurian, it is unlikely that the mouth plays any role in pit formation. Dissection and barcoding analyses verified a lack of holothurian material in the amphipod gut.

Tritaeta gibbosa was not observed exiting the mantle tissue during the migration experiments, meaning all movement must have occurred by moving through the holothurian mantle, a partly calcified and therefore rather tough medium. Photographic evidence suggests the pereopods might pull the mantle, thus inducing movement. SEM micrographs show many sensilla, both type II microtrichs similar to those observed by Wong & Williams (2009) in *Gammarus* sp., and plumose setae resembling those seen in *Hyalella* sp., by Zimmer *et al*. (2009). These sensilla may be used to guide the amphipod to a proper settlement location, sense the environment within the pit and aid in locomotion through the pit. Wong *et al*. (2009) suggested that microtrichs may be associated with burrowing behaviors in gammarids, acting in unison to sense water currents and their microenvironment. In contrast to the gammarids studied by Wong *et al*. (2009), *T. gibbosa* had very few microtrichs, distributed in fewer locations (only somites 9 and 10) and they occurred symmetrically along the dorsal midline although they were observed in different abundances. These differences may contribute to different functions or a differing ability to perform the same tasks. *T. gibbosa* is also much smaller than the gammarids investigated by Wong *et al* (2009). Zimmer *et al*. (2009) gave no indication as to function of the plumose setae in the studied *Hyalella*.

The high abundance of amphipods at the oral end between radials C and D in the preliminary survey of wild caught *O. planci*, suggests a preference for this area. This was corroborated in subsequent experiments with re-colonizing *T. gibbosa* specimens which showed distinct directional movements towards the optimal area. The reasons behind this preference have yet to be clarified and there is no indication of which is more preferred, the oral end or the "dorsal" plates.

Observations of wild caught specimens and our experimental studies show *Ocnus planci* as a definitive host. Our laboratory experiments also confirm *Cucumaria montagui* as a possible host, although the only collected specimen was not infected when collected. *O. planci* and *C. montagui* live sympatrically, and it is possible that in natural settings *O. planci* is the preferred host. Under laboratory conditions, *T. gibbosa* was not able to inhabit *Holothuria tubulosa*, and thus contradicts former observations (Ruffo 1958, Changeux 1961). While *Ocnus planci* and *C. montagui* both have a relatively smooth outer surface, *Holothuria tubulosa* has dermal spikes and many skin projections giving it a velvety appearance. This different mantle texture might be one reason for colonization success or failure.

The smooth setae on the mouthparts of *Bathypanoploea schellenbergi* are considered adaptations to better deal with the mucus of the holothurian integument (Coleman 1990). *Tritaeta gibbosa* has similar smooth setae on the maxilla 2, however, it is not clear what *T. gibbosa* feeds on. We did not find any holothurian ossicles in the alimentary canal of the few dissected animals and barcoding preliminarily confirm this. The foregut and midgut of the animals were empty. It seems very likely that *T. gibbosa* feeds on detritus. The inner faces of the propodi of gnathopods 1 and 2 have a dense setation that are used in many species, and possibly also *T. gibbosa*, to brush detrital particles off of the body, the mouthparts and the long setose antennae (e.g. *Paraceradocus* sp., see Coleman 1989a) for consumption. The functional complex of the mandibles, maxilla 1 and 2 and the maxilliped does not differ much from the general amphipod body plan (Coleman 1989b, 1990), with the exception of the loss of the mandibular palp, which would allow these animals to feed on big as well as small food particles.

It seems that the association of *T. gibbosa* and holothurians is not obligate, as the species has also been found on other taxa, such as sponges (Pirlot 1932, Bacescu & Mayer 1960) and ascidians (Lincoln 1979). Sezgin (1999) reported free-living *T. gibbosa* within and above *Posidonia oceanica* and *Codium fragilis* in the Black Sea, indicating that *T. gibbosa* can survive in the water column without a host organism. This was not observed in the laboratory however, as every free amphipod quickly died. This could be due to a shock-like response to removal from their host, or unsuitable laboratory conditions.

Sitting in a pit that can be closed by the pereopods is certainly an adaptation to avoid predation by fish and crabs (McClintock *et al*. 2009). Unknown disadvantages that may occur due to this lifestyle are probably offset by the advantages of this lifestyle. The number of different environments where *T. gibbosa* is found may explain their distant and not obligate relationship with holothurians as well as their ability to live freely in the water column. This may also be reflected in the genetic variance, which certainly has to be investigated further.

With the thorough analyses presented here, we contribute to the behavioral, ecological and morphological understandings of this peculiar relationship between an amphipod species and its holothurian hosts. Nevertheless, there are still questions concerning further (chemical?) cues that help in host recognition, embedding, as well as the evolutionary traits and benefits that led to these symbiotic or commensal interactions.

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