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External morphology of the cycliophoran dwarf male: a comparative study of
Symbion pandora* and *S. americanus

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Abstract Cycliophora is a recently described phylum to which only two species have been assigned so far, *Symbion pandora* and *S. americanus*. The cycliophoran life cycle is complex and alternates between asexual and sexual stages. Although not recognized as an entirely independent free-swimming stage when the phylum was first described, the dwarf male has a remarkably complex bodyplan albeit its very small size (approx. 30-40 μ m in length). Aiming to increase the knowledge on the gross morphology of the cycliophoran dwarf male, specimens from *S. pandora* and *S. americanus* were analyzed by scanning electron microscopy. In both species, anterior and ventral ciliated fields, as well as paired lateral sensorial organs were identified, thus confirming previous observations. However, new details are described herein such as the penial pouch that encloses the penis. We compare our findings on both *Symbion* species with the data currently available and other metazoan dwarf males.

Introduction

Cycliophora is a metazoan phylum recently erected to accommodate minute marine invertebrates that live commensally on clawed lobsters. So far, only two species have been described, *Symbion pandora* Funch and Kristensen, 1995 and *Symbion americanus* Obst, Funch & Kristensen, 2006, which live on the mouthparts of the Norwegian lobster *Nephrops norvegicus* Linnaeus, 1758, and the American lobster, *Homarus americanus* H. Milne-Edwards, 1837, respectively. However, cryptic speciation has been suggested for *S. americanus* by recent molecular studies (Obst et al., 2005; Baker & Giribet, 2007; Baker et al., 2007).

Cycliophorans are characterized by a very complex life cycle that alternates between a sexual and an asexual phase, involving various stages. The most prominent stage is the sessile feeding stage, which lives attached to the setae of the host lobster and filters small food particles from the water (Funch et al., 2008). This stage generates several other life cycle stages by internal budding (Funch & Kristensen, 1995, 1997; Kristensen, 2002). Feeding individuals are capable of producing Pandora larvae, Prometheus larvae, or females, one at a time, inside a brood chamber. These stages are later released to commence a free-swimming life style. In the asexual phase of the life cycle the Pandora larva settles close to the maternal feeding stage and develops asexually into a new feeding stage. In the sexual phase of the life cycle a free male stage, the Prometheus larva, settles on the trunk of a feeding stage, probably the maternal individual. The Prometheus larva then produces 1-3 dwarf males inside its body. The dwarf male is regarded as a free stage and is morphologically characterized by a heavy ciliated body and a ventral-posterior penis, among other features (Obst and Funch, 2003). The fertilized female is thought to settle on the mouthparts of the same

host where it encysts (Funch, 1996). A dispersal stage, the chordoid larva, hatches from this cyst, settles on a new host individual, and develops into a new feeding stage.

The phylogenetic position of Cycliophora has been unclear ever since its original description, in which a relationship to Entoprocta and Ectoprocta had been hypothesized (cf. Funch & Kristensen, 1995). More recently, several studies have suggested a close relationship to either Entoprocta (e.g., Zrzavý et al., 1998; Sørensen et al., 2000; Obst, 2003; Funch et al., 2005; Passamanek & Halanych, 2006; Paps et al., 2009; Hejnol et al., 2009) or gnathiiferan taxa such as Rotifera (e.g., Winnepenninckx et al., 1998; Giribet et al., 2000; Peterson & Eernisse, 2001; Giribet et al., 2004; Glenner, 2004). Because of the incongruent data from gene sequence analyses, further details on the morphology of all life cycle stages are needed for a better understanding of the cycliophoran phylogenetic interrelationships.

The cycliophoran dwarf male has already been described using light and electron microscopy, and at the cytochemical level using confocal laserscanning microscopy (Funch and Kristensen, 1997; Obst and Funch, 2003; Neves et al., 2009a, 2009b, 2010). However, only one male of *Symbion pandora* has so far been described by scanning electron microscopy, and its external morphology was apparently altered during the freeze-drying technique used to prepare specimens (cf. Obst and Funch, 2003). Also the high loss of specimens during preparation, due to the minute size of the males, limited the success of this approach. In the study presented herein, we compare the external morphology of the dwarf males of *S. pandora* and *S. americanus*.

Materials and Methods

Feeding stages with attached Prometheus larvae of the cyclophoran species *Symbion pandora* were obtained from mouth parts of Norway lobsters, *Nephrops norvegicus*, collected off the coast of Sagres, Portugal (January 23rd, 2008) by local fishermen. Cyclophorans of the species *S. americanus* were obtained from mouth parts of the American lobster, *Hommarus americanus*, collected from Maine (USA; between October 1st and 15th, 2006). After fixation for 1h in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PBS), specimens were washed 3×15 min in 0.1M PBS containing 0.1% sodium azide and stored at 4°C.

PFA-fixed males of *Symbion americanus* were washed in distilled water and concentrated on a Millipore filter (12 mm pore size) using a Swinnex filter (Millipore, Massachusetts, USA) holder to minimize the loss of specimens. The specimens were then post-fixed in 1% OsO₄ for 1h at room temperature, and subsequently washed with distilled water. Afterwards, specimens were dehydrated in an ethanol and an acetone series and critical point dried (CPD) using carbon dioxide. Finally, the membrane with the attached animals was mounted on an aluminium stub with sticky carbon pads, and sputter-coated with platinum/palladium alloy. Alternatively, dwarf males of *S. americanus* and *S. pandora* were attached to circular coverslips coated with poly-L-lysine (solution 0.01%, molecular weight 150,000-300,000; Sigma-Aldrich, Brøndby, Denmark). After post-fixation with OsO₄ as described above, the specimens were dehydrated in an ethanol series and immersed in hexamethyldisilazane (HMDS) (Sigma-Aldrich, Brøndby, Denmark) for about 12 hours in a desiccator. Finally, the coverslips with the dwarf males were allowed to dry inside the desiccator for approx. 15 minutes, mounted on an aluminium stub, and sputter-coated as described above. All specimens were analysed and digitally photographed using a JEOL JSM-6335F field emission scanning electron microscope (Tokyo, Japan).

Results and discussion

The external morphology of 10 specimens of *Symbion pandora* and 16 specimens of *S. americanus* investigated herein showed the typical features that characterize the cycliophoran dwarf male (Figs. 1, 2), including the anterior and ventral ciliated fields, the paired lateral sensory organ, and the ventral-posterior penis (see Obst and Funch 2003; Obst et al., 2006). The penis is located inside a pouch-like structure, which is laterally constricted and forms an elongated slit at the cuticle level (Figs. 1a,b, 2a,b). Only the tip of the penis is protruding from the penial pouch. The body region at the base of the penis, in the posterior-most end of the slit, is the only difference between males from the two cycliophoran species. In *S. pandora* the cuticle at the base of the penis shows some ridges (Fig. 1b), whereas the same structure in *S. americanus* is smooth (Fig. 2b). This fact was observed for all specimens investigated and is consistent even for the different drying processes used in this study. No differences were recognized in any other structure due to the different drying processes.

In both cycliophoran species, a ventral ciliated field starts in the very anterior end of the body of the dwarf male and extends posteriorly until the beginning of the penial pouch (Figs. 1a, 2a). No clearly distinguishable longitudinal rows of cilia are found in the somewhat horseshoe-shaped ventral ciliated field. An additional distinct ciliated field likewise emerges in anterior position. This is a frontal ciliated field that spans dorsally through the most anterior third of the male body length (Figs. 1c, d, 2c, d) and is clearly separated from the ventral ciliated field by a depression. Sensory cilia located laterally as an extension from the frontal ciliated field are found to be very long (10-15 μ m) in *S. pandora* and *S. americanus* (Figs. 1a, c, d, 2a, c, d) (Obst and Funch,

2003). The non-ciliated parts of the male integument do not exhibit the typical polygonal sculpture observed in other cyclophoran life cycle stages (e.g., the feeding stage). Different shapes of the body of the dwarf male were found for both species. Some specimens have a roundish, oval body (Figs. 1a, 2a), whereas other specimens present a more elongated, rectangular shape (Fig. 1c, 2c).

Our results corroborate the view of the dwarf male as a totally independent stage in the life cycle of both hitherto described cyclophoran species. The external phenotype of dwarf males of the two species, *Symbion pandora* and *S. americanus*, is very similar. The main difference noticed is related to the cuticle at the base of the penis, which shows some ridges in *S. pandora* but not in *S. americanus*. Interestingly, the presence of those ridges in *S. pandora* is confirmed by transmission electron microscopy (cf. Obst and Funch 2003). However, there are no data available on the ultrastructure of males of the species *S. americanus*, which makes further comparisons between both species impossible. Since the ridges were neither observed in HMDS- nor in CPD-dried specimens of *S. americanus*, this finding is apparently not caused by the different drying processes used for each species.

Earlier descriptions of the dwarf male of *Symbion pandora* and *S. americanus* revealed details of sensory elements other than the lateral sensory cilia (Obst and Funch 2003, Obst et al. 2006). In *S. pandora* a frontal sensorial organ was identified by TEM but not by SEM, and several palps and a tactile papilla (Fig. 3) were described only for one specimen using SEM. Frontal palps were also described for *S. americanus* (Obst et al. 2006). These sensorial structures could not be identified in any of the males of *S. pandora* or *S. americanus* in our study. However, our results do not argue against the existence of such structures since the frontal ciliated field is very dense and the structures mentioned are possibly covered by cilia.

The various shapes found for the male body were already observed in previous studies under light microscopy (RCN pers. obs.). Since the external morphology is not distinct among males with different shapes, all specimens should be fully formed, i.e., at the same developmental phase. Thus, the differences found in the shape of the male body are not indicative of any physiological constraints. It is possible that those differences are related to the number of males developed at the same time and the exact position they adopt inside the attached Prometheus larva.

External morphology and functional implications

In the original description of Cyclophora, the extremely reduced dwarf male had not been recognized as a free swimming, independent stage. Instead, an intermediate stage, now termed the Prometheus larva, was originally proposed as the free mature male (cf. Funch and Kristensen 1995, 1997). The cyclophoran dwarf male is now known to have a sophisticated bodyplan that includes a highly complex musculature, a relatively large brain, a pair of ventral longitudinal nerve cords, fully developed gonads and mating structures, sensory organs, and various types of glands (Fig. 3). Accordingly, the details on the external morphology as presented herein strengthen the view of the cyclophoran dwarf male as a free swimming stage with an important role during sexual reproduction.

So far, fertilization in cyclophorans has never been observed and questions such as when, where, and how this process occurs remain unanswered. The presence in the male of extensive locomotory ciliated fields together with the elongated sensory cilia and an elaborated muscle architecture are indicative of its swimming and crawling capabilities (Fig. 3). Therefore, these locomotory and sensory structures might be necessary for the male to carry out its role in the reproductive process, i.e., find the

female and copulate (Funch and Kristensen 1999, Obst and Funch 2003). It is possible that the transfer of sperm cells takes place during the encounter of the male with the female while free swimming stages. The mechanisms involved in the transfer of the sperm cells to the oocytes, however, remains unknown.

The penis of the dwarf male of both species is a cuticular hollow tube without any aperture at the very distal end (Fig. 3; see also Obst and Funch 2003). The intriguing question is how the sperm cells exit the male body through the penial structure. The fact that the tip of the penis is less than one third of the diameter of the sperm cells raises the possibility that this organ serves as an anchoring device that facilitates copulation rather than being a true copulatory organ (Obst and Funch 2003). The anchoring cirrus organ could then be used by the male to momentarily pierce the female and somehow liberate the sperm cells from the inside of its body. Both hypotheses are supported by the fact that a complex set of muscles are present in the posterior body region in connection with the base of the penial (or anchoring?) structure (Funch and Kristensen 1997, Obst and Funch 2003, Neves et al. 2009b).

The penial pouch in which the penis lies is a newly described feature for the cycliophoran dwarf male. This structure may be very important during the release of sperm cells by the cycliophoran dwarf male. If, however, the penis would be shown to be an anchoring organ, the penial pouch may act as a receptaculum where the sperm cells accumulate before being transferred to the female's body. This assumption is obviously speculative but based on the aforementioned morphological details of the penial structure.

The cycliophoran male compared to other metazoan dwarf males

Dwarf males are known from several metazoan lineages such as echiurans, polychaetes, siboglinids, monogonont rotifers, barnacles, and spiders (Windoffer and Westheide 1988, Ricci and Melone 1998, Rouse et al. 2004, for reviews see Gilbert and Williamson 1983, Volrath 1998). Frequently in textbooks (e.g., Ruppert et al. 2004), dwarf males are described as rudimentary sexual intervening forms that are only equipped with the strictly necessary structures to seek a female and copulate. The cycliophoran dwarf male, however, has a very complex body architecture (cf. Funch and Kristensen 1997, Obst and Funch 2003, Neves et al. 2009b). The location of the penis inside a pouch is to our knowledge a new feature among metazoan dwarf males. In some cases, the penial structure is found inside the body (e.g., in polychaetes or monogonont rotifers; see Windoffer and Westheide 1988, Clément and Wurdak, 1991) or may be entirely absent (e.g., in echiurans or the siboglinid *Osedax*; Schuchert and Rieger 1990, Rouse et al. 2004, Worsaae and Rouse 2010). Therefore, more data are necessary to clarify the male-female interaction and the true function of the penial structure and the penial pouch.

In the future, males of both *Symbion* species should be compared at the ultrastructural level. A better understanding of, e.g., spermatogenesis and sperm morphology will permit further comparisons with other lophotrochozoan taxa, which could result in further insights into the phylogenetic placement and evolution of Cycliophora.

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Figure Legends

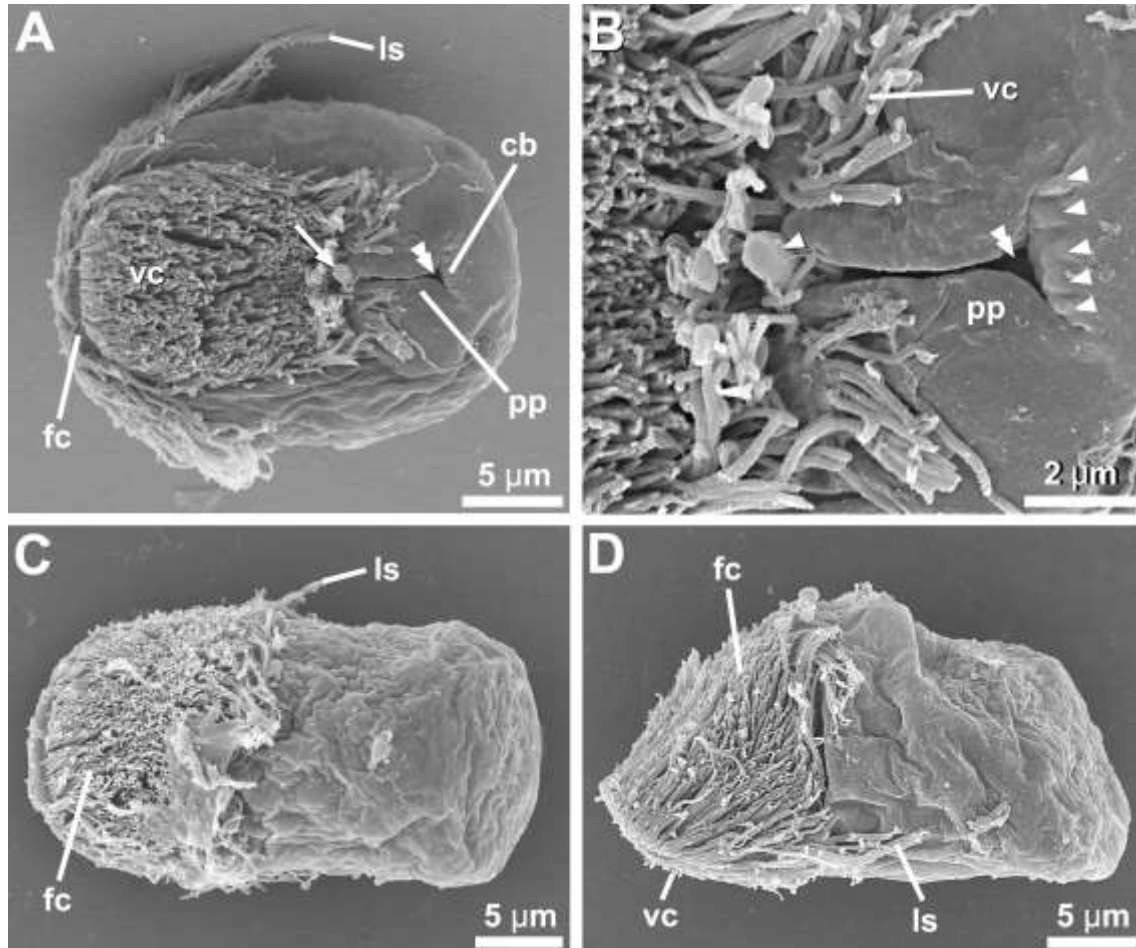
Fig. 1. *Symbion pandora*, dwarf male. Anterior faces to the left in all aspects. **a** *Ventral view*. The tip of the penis (arrow) is extruded while the base is hidden inside the lumen (double arrowhead) of the penial pouch (*pp*). The lateral sensoria (*ls*) are composed of long cilia. **b** Close-up of the penial pouch of the specimen shown in a. Note the ridges (arrowheads) of the cuticle in the region at the base of the penis (*cb*). **c** *Dorsal view*. **d** *Lateral view*. The frontal ciliated (*fc*) field extends dorsally, covering the anterior-most third of the male body, and a ventral ciliated field (*vc*) spans from the most anterior body region until the penial pouch.

Fig. 2 *Symbion americanus*, free male. Anterior faces to the left in all aspects. **a** *Ventral view*. The penis (arrow) lies inside the lumen (double arrowhead) of the penial pouch (*pp*). Long cilia compose the lateral sensoria (*ls*). **b** Close-up of the penial pouch of a specimen in *ventral view*. Note that the cuticle in the region at the base of the penis (*cb*) is devoid of ridges. **c** *Dorsal view*. **d** *Ventro-lateral view*. The frontal ciliated (*fc*) field covers the dorsal side in the anterior-most third of the male body. On the ventral side, a ciliated field (*vc*) spans from the anterior-most part of the body until the penial pouch.

Fig. 3 Line drawing of the cyclophoran dwarf male (reprinted with permission from Neves et al., 2009b). *Lateral view*, anterior faces to the left. Scale bar represents 10 µm. *cg*, cerebral glands; *dm*, dorsal muscle; *dp*, dorsal papilla; *dvm*, dorsoventral muscles; *fc*, frontal ciliated field; *fp*, frontal palp; *fs*, frontal sensoria; *ga*, cerebral ganglion; *hm*, horseshoe-shaped muscles; *ls*, lateral sensoria; *mg*, medial glands; *pe*, penial structure;

397 *pg*, prostate glands; *psm*, posterior short muscles; *pp*, penial pouch; *sp*, sperm cells; *te*,
 398 testis; *vc*, ventral ciliated field; *vlm*, ventrolateral muscles.

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