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Serotonin immunoreactivity in the nervous system of the Pandora larva, the Prometheus larva, and the dwarf male of *Symbion americanus* (Cycliophora)

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Abstract

Cycliophora is a recently described phylum of enigmatic metazoans with a very complex life cycle that includes several sexual and asexual stages. *Symbion pandora* and *Symbion americanus* are the only two cycliophoran species hitherto described, of which morphological and genetic knowledge is still deficient to clarify the phylogenetic position of the phylum. Aiming to increase the database on the cycliophoran neural architecture, we investigated serotonin immunoreactivity in the free swimming Pandora larva, the Prometheus larva, and the adult dwarf male of *S. americanus*. In the larval forms, serotonin is mainly expressed in a ring-shaped pattern at the periphery of the antero-dorsal cerebral ganglion. Additionally, several serotonergic perikarya emerge from both sides of the cerebral ganglion. Thin neurites project anteriorly from the cerebral ganglion, while a pair of ventral longitudinal neurites emerges laterally and runs along the anterior-posterior body axis. Posteriorly, the ventral neurites fuse and extend as a posterior projection. In the dwarf male, serotonin is found mainly in the commissural neuropil of the large anterior cerebral ganglion. In addition, serotonin immunoreactivity is present in the most anterior region of the ventral neurites. Comparative analysis of spiralian nervous systems demonstrates that the neuroanatomy of the cycliophoran larval stages resembles much more the situation of adult rather than larval spiralians, which may be explained by secondary loss of larval structures and heterochronic shift of adult components into the nervous system of the Pandora and the Prometheus larva, respectively.

Keywords: Morphology; Life cycle; Evolution; Phylogeny; Spiralia; Lophotrochozoa

1. Introduction

Cycliophora is one of the most recently described metazoan phyla and to date it only accommodates two

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species, *Symbion pandora* Funch and Kristensen, 1995, and *Symbion americanus* Obst, Funch and Kristensen, 2006. Cycliophorans are highly host specific; *S. pandora* lives commensally on the mouthparts of the Norway lobster, *Nephrops norvegicus* Linnaeus, 1758, whereas *S. americanus* is found only on the American lobster, *Homarus americanus* H. Milne-Edwards, 1837 (Funch and Kristensen 1995; Obst et al. 2006). However, recent molecular studies have suggested cryptic speciation in *S. americanus* (Baker and Giribet 2007; Baker et al. 2007).

The cycliophoran life cycle is one of the most complicated amongst metazoans and consists of an asexual and a sexual generation (Funch and Kristensen 1995, 1997; Kristensen 2002). The most prominent stage is a feeding individual that lives permanently attached to the host mouthparts, filtering food particles from the surrounding water (Riisgård et al. 2000; Funch et al. 2008). The feeding stage is able to generate, one at a time, a Pandora larva, a Prometheus larva or a female. Once freed, the Pandora larva settles very close to the maternal individual and develops asexually into a new feeding stage, thus completing the asexual part of the life cycle. The sexual cycle is more complicated and involves the Prometheus larva, which settles on a feeding stage and develops 2–3 dwarf males by inner budding (Neves et al. 2009a, 2010a). The dwarf males are responsible for fertilization of the large oocyte present in the body of the female. The female settles in sheltered areas on the mouthparts, probably of the same host individual (Funch and Kristensen 1997; Obst and Funch 2003, 2006; Obst et al. 2006). Only the adhesive disc and the cuticle of the inseminated female persist and form a cyst-like structure inside of which the embryo develops into the chordoid larva (Funch 1996). The chordoid larva hatches from the cyst, colonizes a new host, settles, and develops into a new feeding stage.

The Prometheus larva of *S. americanus* exhibits a pair of posterior retractable appendages, the so-called toes, that, together with deep, ring-like scars in the cuticle of the trunk of the feeding stages, are absent in *S. pandora* (Obst et al. 2006). These morphological differences are used to distinguish *S. americanus* from *S. pandora*, which otherwise show high similarity in the anatomy of their life cycle stages. A large, bilobed brain is located dorso-anteriorly in all free-swimming stages, i.e., the Prometheus larva, the female, the male, the Pandora larva, and the chordoid larva (Funch and Kristensen 1997; Obst et al. 2006). The latter possesses, however, a more complex brain than the other stages (Funch 1996). Paired neurite bundles project dorsally and anteriorly from the brain of the chordoid larva of *S. pandora* and innervate dorsal sensory structures and the anterior ciliated band, respectively. In addition, two pairs of ventral neurites run longitudinally in the body of the chordoid larva and lateral projections emerge from the posterior region of each outermost ventral neurite (Wanninger 2005; Neves et al. 2010b). These projections probably innervate small, postero-lateral sensory organs (Funch 1996).

Contrary to the chordoid larva, the main sensory structures of the Pandora larva and the Prometheus larva consist of anterior clusters of long compound cilia, the so-called frontalia and ventralia. Frontal sensory palps and a small ventro-posterior pore have been described for the Pandora larva and the Prometheus larva of *S. americanus* but not for *S. pandora* (Funch and Kristensen 1997; Obst et al. 2006). In addition, a posterior tuft of cilia surrounding a large pore is present in the Pandora larva of both cycliophoran species and in the Prometheus larva of *S. americanus*. This structure might constitute the exit for the buccal funnel from the Pandora larva, which persists in the adult feeding stage, and for the dwarf males from the Prometheus larva, respectively (Obst et al. 2006).

The cycliophoran dwarf male is characterized by a large cerebral ganglion that occupies about one third of the body volume and is composed of a dorsolateral pair of ganglia (Obst and Funch 2003; Neves et al. 2009b). Both ganglia contain a cluster of perikarya and are interconnected by a commissural neuropil. Medially, the brain possesses a small anterior extension and glial cell bodies. The male nervous system also includes a pair of longitudinal nerves that projects latero-ventrally from the brain to the base of the postero-ventral penis. This penial structure may constitute a mere cirrus organ used by the male to pierce the female during a putative encounter. Prominent ventral and frontal ciliated fields cover the male body, and sensorial tufts of cilia are situated laterally and frontally. Additional sensorial elements are the frontal palps and the dorsal papilla (Obst and Funch 2003; Obst et al. 2006; Neves et al. 2009b).

To date, the neuroanatomy of the feeding stage is the least resolved of all life cycle stages. For *S. pandora*, the existence of a ganglion at the base of the buccal funnel and another ganglion partially surrounding the oesophagus, as suggested by earlier studies based on light microscopy, have never been confirmed by transmission electron microscopy analyses (Funch and Kristensen 1997). By contrast, only thin nerve fibers were found at the base and laterally to the buccal funnel, as well as in the vicinity of the anus. Concerning *S. americanus*, a mass of nervous tissue situated at the base of the buccal funnel was interpreted as the cerebral ganglion (Obst et al. 2006). Additional detailed research is thus necessary to assess these observations.

In the original description, cycliophorans were considered to be phylogenetically related to Entoprocta and Ectoprocta (Funch and Kristensen 1995). Since then, several studies have suggested a relationship to gnathiferan taxa (Winnepenninckx et al. 1998; Giribet et al. 2000, 2004; Peterson and Eernisse 2001; Zrzavý et al. 2001; Zrzavý 2002), or Entoprocta alone (Zrzavý et al. 1998;

Sørensen et al. 2000; Obst 2003; Passamanek and Halanych 2006). Interestingly, recent molecular studies suggest Ectoprocta as the sister group of a cycliophoran-entoproct assemblage (Paps et al. 2009; Hejnol et al. 2009), a hypothesis that is not supported by morphological data. Additional information on the morphology of crucial organ systems of the various life cycle stages is therefore essential to assess the molecular-based hypotheses on the phyletic relationships of Cycliophora. As for the nervous system, some light (LM), transmission electron microscopy (TEM), and immunocytochemical data are currently available, but are restricted to selected life cycle stages (Funch 1996; Funch and Kristensen 1997; Obst and Funch 2003; Obst et al. 2006; Wanninger 2005; Neves et al. 2010b). In order to increase the database on the distribution of neurotransmitters in the nervous system of cycliophorans we describe herein serotonin immunoreactivity in the Pandora larva, the Prometheus larva and the dwarf male of *S. americanus* and compare our data to those on the chordoid larva of *S. pandora* and to phyla currently considered potential cycliophoran sister groups. Potential homology of character states between Cycliophora and other spiralian taxa is critically assessed with the aim to shed more light on the evolution of the phylum.

2. Materials and methods

2.1. Collection of specimens and fixation

Specimens of the American lobster, *Homarus americanus*, were collected off the coast of Maine (USA) between 1 and 15 October 2006, and on 14 May 2009 by local fishermen. Mouthparts were dissected from the hosts and placed in Petri dishes with seawater and feeding stages with attached Prometheus larvae were isolated. Alternatively, setae with the epizoic cycliophorans were gently shaved and transferred to 6-well culture plates with seawater. Both sets were kept inside a refrigerator at 5 °C, with water changed every other day. Within several days, crawling Pandora and Prometheus larvae were found and collected with a Pasteur pipette. All specimens were narcotized by adding drops of a 7% $MgCl_2$ solution and fixed for 30 min at room temperature in freshly prepared 4% paraformaldehyde (PFA) in 0.1 M PBS. After that, specimens were washed 3×15 min in phosphate buffered saline (PBS) containing 0.1% sodium azide (NaN_3) and subsequently stored at 4 °C.

2.2. Immunolabelling

For serotonin staining, antibodies were applied following protocols described earlier (Wanninger 2005; Neves et al. 2010b). The Pandora and Prometheus larvae were permeabilized for 1 h at 4 °C in 0.1 M PBS containing 5% Triton X-100 and 0.1% NaN_3 (=PBT). Then, unspecific binding sites were blocked for 24 h at 4 °C in 6% normal goat serum (Sigma, Brøndby, Denmark) in PBT (=PTA). Sexually mature dwarf males were dissected out of the Prometheus larva prior to permeabilization. The polyclonal anti-serotonin antibody from rabbit (Immunostar, Hudson, USA) was diluted in PTA and applied at a 1:500 working concentration for 24 h at 4 °C. The specimens were subsequently rinsed four times over 6 h at 4 °C in PTA and were then incubated in a goat anti-rabbit Alexa Fluor 594 antibody (Invitrogen, Molecular Probes, Eugene, USA) diluted 1:400 in PTA for 24 h at 4 °C. This was followed by four washes overnight in 0.1 M PBS. For serotonin and acetylated α-tubulin double labelling, incubations with the primary antibodies were performed using the anti-serotonin antibody from rabbit (dilution 1:500) mixed with an anti-acetylated α-tubulin antibody from mouse (Sigma, Brøndby, Denmark, dilution 1:500) in PTA for 24 h at 4 °C. Secondary antibodies were applied in a solution that included the goat anti-rabbit Alexa Fluor 594 antibody (dilution 1:400) and a goat anti-mouse Alexa Fluor 488 antibody (Invitrogen, Molecular Probes, Eugene, USA; dilution 1:300). To visualize cell nuclei, specimens were incubated in PBS with DAPI (Invitrogen, Taastrup, Denmark) at a final concentration of 5 µg/ml for 30 min at room temperature. Finally, specimens were washed 3×15 min in PBS and embedded on glass slides in either Fluoromount G (SouthernBiotech, Birmingham, USA) or Vectashield (Vector Laboratories, Burlingame, USA). Nine Pandora larvae and six Prometheus larvae were analyzed after a single serotonin labelling experiment, and six Pandora larvae and five Prometheus larvae were double labelled with fluorescence-coupled antibodies directed against serotonin and acetylated α-tubulin. Twelve dwarf males were analyzed after a serotonin and acetylated α-tubulin double-labelling experiment. All experiments showed consistent results. Negative controls were performed by omitting either the primary or the secondary antibody against serotonin and yielded no specific signal.

2.3. Confocal laserscanning microscopy and 3D imaging

Analysis was performed with a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal laserscanning unit (Leica, Wetzlar, Germany). Optical sections were generated with a Z-step size ranging between 0.28 and 0.32 µm and the resulting stacks were merged into maximum projection images. The image stacks were processed with the image editing software Imaris v. 5.7.2 (Bitplane AG, Zürich, Switzerland) to create the 3D surface-rendered models.

2.4. Scanning electron microscopy

PFA-fixed Pandora larvae and Prometheus larvae of *S. americanus* were post-fixed in 1% OsO_4 for 1 h at room temperature and subsequently washed with distilled water. Afterwards, specimens were dehydrated by gradually stepping them into 100% acetone and critical point dried using a BAL-TEC 030 dryer (Bal-Tec AG, Balzers, Liechtenstein) with carbon dioxide as intermediate. Finally, the specimens were mounted on aluminium stubs with sticky carbon pads and sputter-coated with platinum/palladium alloy. Because of their minute size, PFA-fixed males were concentrated on a Millipore filter (12 μm pore size) using a Swinnex filter holder (Millipore, Massachusetts, USA). The membrane with the attached males was mounted on the sticky carbon pads and sputter coated after post-fixation, dehydration, and critical point drying as described above. Alternatively, dwarf males were attached to poly-L-lysine-coated coverslips (solution 0.01%, molecular weight 150,000–300,000; Sigma-Aldrich, Brøndby, Denmark). Specimens were post-fixed with OsO_4, dehydrated in an ethanol series, and immersed in hexamethyldisilazane (Sigma-Aldrich, Brøndby, Denmark) for about 12 h in a desiccator. After that, the coverslip with the dwarf males was allowed to dry inside the desiccator for approx. 15 min. Then, the coverslips were mounted on aluminium stubs and sputter coated. All specimens were analysed and digitally photographed using a JEOL JSM-6335F or a JSM-840 field emission scanning electron microscope (Jeol, Tokyo, Japan).

3. Results

3.1. Gross morphology of the Pandora larva and the Prometheus larva

The external morphology of the Pandora larva and the Prometheus larva shows a ventral ciliated field and a posterior ciliated tuft (Fig. 1A and C). In addition, a pair of posterior toes is present in the Prometheus larva (Fig. 1C and D). All Pandora larvae have a newly formed buccal funnel inside (Fig. 1B), thus corroborating the findings of the original description of *S. americanus* (cf. Obst et al. 2006). Orientation of the Prometheus larva is given following earlier descriptions with the ciliated side marking ventral and the swimming direction indicating anterior (Obst and Funch, 2003).

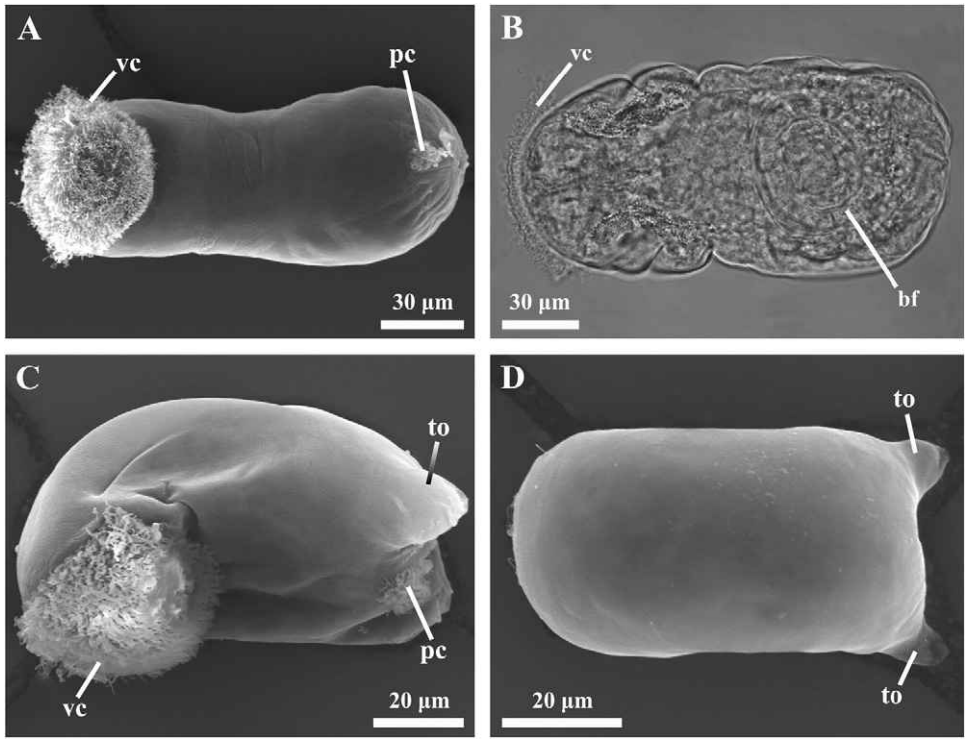

Fig. 1. The free-swimming Pandora larva (A and B) and Prometheus larva (C and D) of *Symbion americanus*. Scanning electron (A, C and D), and light (B) micrographs. Anterior faces left in all aspects. (A) Pandora larva, ventral view. This larval form possesses a large, antero-ventral ciliated field (vc) and a posterior ciliated tuft (pc) as locomotive organs. (B) Dorsal view. Note the buccal funnel (bf) in the posterior region of the larval body. (C) Lateral view. A pair of retractable appendages, the so-called toes (to), is located posteriorly. A large antero-ventral ciliated field (vc) and a posterior tuft of cilia (pc) serve as locomotive organs. (D) Dorsal view.

3.2. The serotonergic nervous system of the Pandora larva

Serotonin immunoreactivity in the Pandora larva of *S. americanus* is found mainly in the antero-dorsal cerebral ganglion, which is revealed as a bilateral ring-shaped structure (Fig. 2A–C and E). Several perikarya (most likely three) emerge from both sides of the cerebral ganglion; two perikarya with long cell extensions project latero-posteriorly adjacent to each other, and a single perikaryon emerges posteriorly. Thin neurites project anteriorly and antero-ventrally from the cerebral ganglion (Fig. 2A–C, E). An additional unpaired structure with weak serotonergic signal

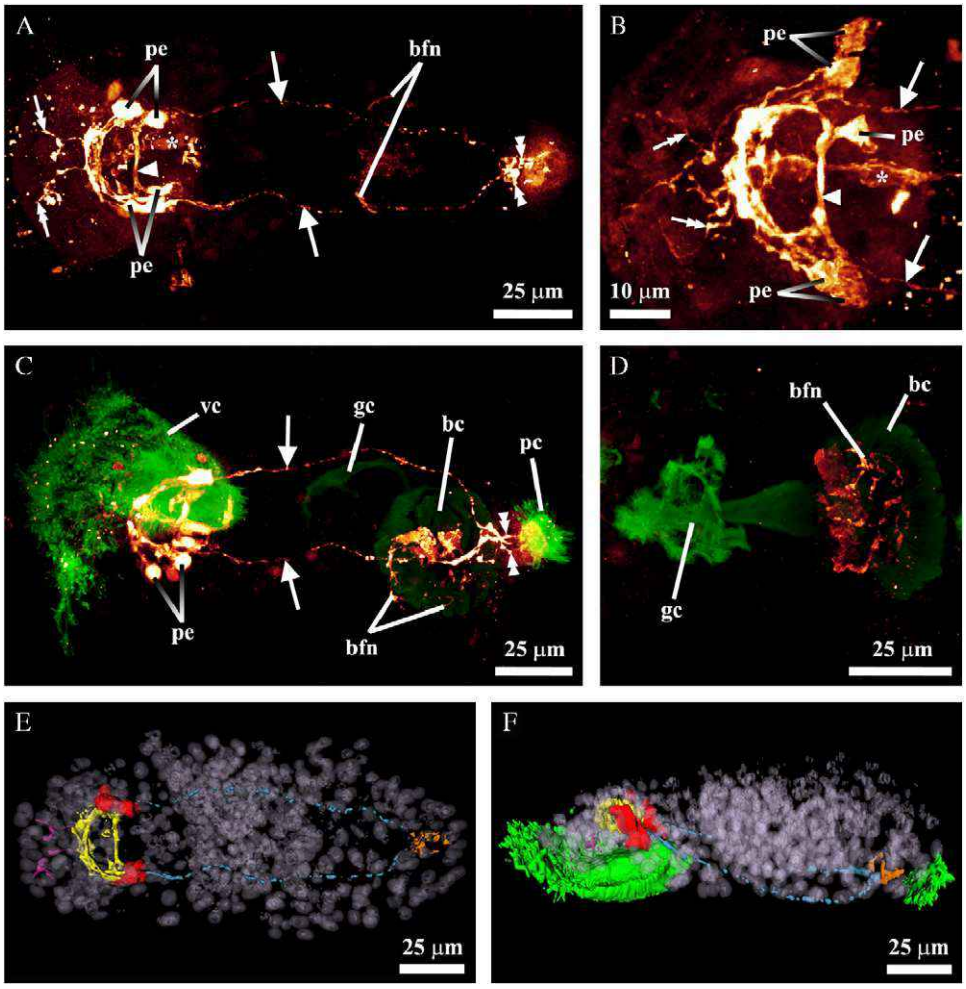

Fig. 2. The serotonergic nervous system of the Pandora larva of *Symbion americanus*. Maximum projection images of confocal microscopy stacks (A–D) and 3D reconstructions (E, F). Anterior faces left in all aspects. (A) Dorsal view showing the ring-shaped serotonergic cerebral ganglion from which several (probably three) serotonergic perikarya (pe) project latero-posteriorly and thin neurites project anteriorly (double arrows). A commissure (arrowhead) lines the posteriormost part of the ring-shaped pattern. Note the ventral longitudinal neurites (arrows) emerging laterally from the cerebral ganglion and converging at the posteriormost region of the larval body. Short neurites project posteriorly from the point of convergence (double arrowheads). Serotonergic neurites are present in the buccal funnel (bfn). An asterisk marks a putative glandular structure. (B) Dorsal view. Detail of the ring-shaped serotonergic cerebral ganglion with several serotonergic perikaria (pe) emerging laterally, and thin neurites projecting anteriorly (double arrows). (C) Ventro-lateral view. Double-stained specimen; cilia labelled green. Note the large antero-ventral ciliated field (vc) and a small posterior tuft of cilia (pc). Cilia inside the buccal funnel (bc) and gut (gc) are also stained. Serotonergic neurites are found in the buccal funnel (bfn). (D) Ventral view. Double-stained specimen; cilia labelled green. Detail of the developing digestive system with cilia inside the buccal funnel (bc) and the gut (gc). Note the serotonergic neurites in the buccal funnel (bfn). (E) Ventral view. Somatic nuclei indicate the outline of the larval body (grey). (F) Lateral view of a double-stained specimen. Colour code for E and F: green, cilia; grey, nuclei; light blue, ventral longitudinal neurites; orange, posterior projections; magenta, anterior projections; red, perikarya; yellow, cerebral ganglion.

emerges mid-posteriorly from the cerebral ganglion (Fig. 2A–C). A serotonergic neurite originates latero-posteriorly from both sides of the cerebral ganglion and runs ventrally along the anterior-posterior axis of the larva. The two ventral neurites interconnect at the posteriormost region of the larval body and are extended by short posterior projections (Fig. 2A–E). An additional serotonergic region is found in the posteriormost third of the larval body, where cilia from the buccal funnel are located (Fig. 2A, C and D). This signal probably stems from serotonergic neurites present in the buccal funnel. Double-stained specimens also show that the cerebral ganglion and the anterior neurites are located in the region of the ventral ciliated field, and that the posterior extensions of the ventral neurites terminate in the region of the posterior ciliated tuft (Fig. 2C and F).

3.3. The serotonergic nervous system of the Prometheus larva

The overall architecture of the serotonergic nervous system of the Prometheus larva is highly similar to that of the Pandora larva. Serotonin immunoreactivity is most prominent in a ring-shaped pattern in the commissures and perikarya of the antero-dorsally located cerebral ganglion (Fig. 3A–E). On both sides of the cerebral ganglion, one pair of perikarya protrudes in postero-lateral and an additional perikaryon in posterior direction. Thin serotonergic neurites project anteriorly from the cerebral ganglion towards the anteriormost pole and in the antero-ventral regions of the larval body (Fig. 3A, B, D, E). As in the Pandora larva, an unpaired structure with weak serotonergic immunoreactivity is found emerging mid-posteriorly from the cerebral ganglion (Fig. 3A–D). A single pair of ventral serotonergic neurites runs longitudinally along the body axis, connecting each other in the posteriormost region of the larval body (Fig. 3A–E). Posterior projections extend the ventral neurites beyond the joining point where they interconnect in the posteriormost region (Fig. 3A, B, D and E). Serotonergic neurites innervating the toes were not found in any of the 12 specimens analyzed. Anteriorly, both ventral neurites connect latero-posteriorly to the cerebral ganglion.

Double-stained specimens confirm the location of the cerebral ganglion and anterior neurites in the region of the ventral ciliated field and the posterior extensions of the ventral neurites in the region of the posterior ciliated tuft (Fig. 3D and F). A third set of cilia is found in the median region of the larval body. These cilia most probably belong to one or two developing dwarf males, in which serotonin expression is still lacking (Fig. 3D).

3.4. Gross morphology and serotonergic nervous system of the dwarf male

The gross morphology of the dwarf male investigated in this study is in accordance with previous descriptions (cf. Obst et al. 2006; Neves et al. 2009b). Frontal and ventral ciliated fields as well as a lateral sensory organ are very prominent and a postero-ventrally located penis is present in all specimens (Fig. 4A and B). Serotonin immunoreactivity in the nervous system of the dwarf male is detected mainly in the cerebral ganglion. The respective signal is revealed as a thin, sickle-shaped pattern that is confined to the commissural neuropil of the cerebral ganglion (Fig. 5A–E). Serotonin is also expressed in the latero-ventral neurites, which extend posteriorly from the cerebral ganglion until the region antero-lateral to the testis (Fig. 5D–F). The immunoreactive signal originating from these extensions is scattered and weaker than that observed in the cerebral ganglion.

4. Discussion

4.1. Comparison of the serotonergic nervous system of the three cycliophoran larval types: the Pandora larva, the Prometheus larva, and the chordoid larva

Earlier studies have pointed out the similarities between the nervous system of the Pandora larva and the Prometheus larva (Funch and Kristensen 1997; Obst et al. 2006). According to these descriptions, a bilobed cerebral ganglion with two large clusters of ganglion cells is located dorso-anteriorly in both larval types, although no further details on the peripheral nervous system were provided. Our data show a number of congruencies in the serotonin distribution of both larvae, thus corroborating the original descriptions. Accordingly, a serotonergic ring is found at the periphery of the cerebral ganglion of both larval types. Moreover, the peripheral serotonergic nervous system and the presence of several serotonergic perikarya protruding from the cerebral ganglion constitute additional similarities. The anterior projections from the cerebral ganglion described herein may be responsible for the innervation of the ventral ciliated field and/or the sensory cilia and palps of both larval types (Funch and Kristensen 1997; Obst et al. 2006). The posterior projections emerging from the joining point of the two ventral neurites may constitute the innervation of the posterior ciliated tuft that characterizes these larvae. The structures with weak serotonergic signal that emerge from the median region of the cerebral ganglion of the Prometheus larva and the Pandora larva are intriguing. Since no ultrastructural details are available

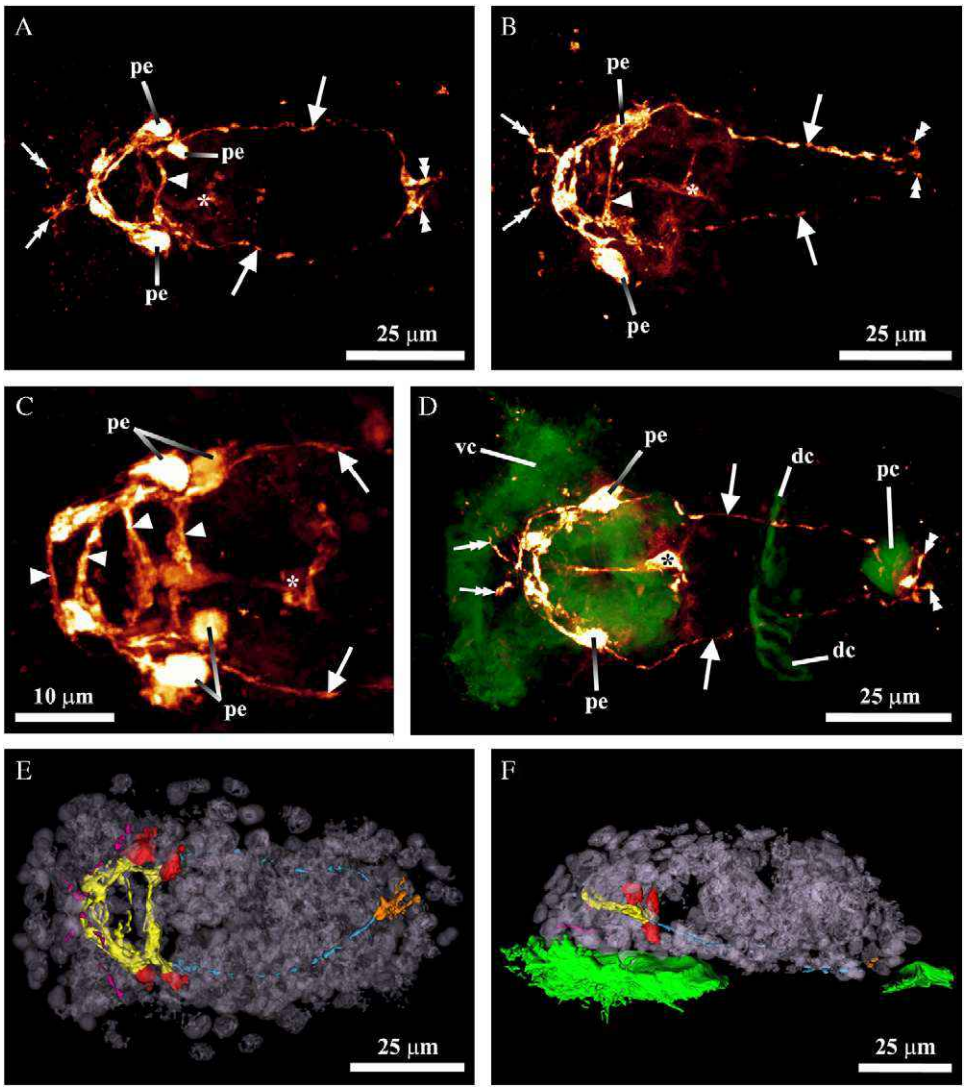

Fig. 3. The serotonergic nervous system of the Prometheus larva of *Symbion americanus*. Maximum projection images of confocal microscopy stacks (A–D) and 3D reconstructions (E and F). Anterior faces left in all aspects. (A) Dorsal view showing the ring-shaped serotonergic cerebral ganglion with several serotonergic perikaria (pe) emerging latero-anteriorly and thin neurites projecting anteriorly (double arrows). A commissure (arrowhead) lines the posteriormost part of the ring-shaped pattern. Ventral longitudinal neurites (arrows) emerge laterally from the cerebral ganglion and converge at the posteriormost region of the larval body. Note that short neurites project posteriorly from that point of convergence (double arrowheads). Asterisk marks a putative glandular structure. (B) Ventral view. Note that immunoreactivity appears weaker in the point of convergence of the ventral longitudinal neurites than in the specimens shown in A and D. (C) Dorsal view. Detail of the ring-shaped serotonergic cerebral ganglion with several serotonergic perikarya (pe) emerging laterally. Note the several serotonergic commissures (arrowheads). (D) Dorsal view. Double-stained specimen; cilia labelled green. Note the large, antero-ventral ciliated field and the small posterior tuft of cilia. The small ciliated areas (dc) located in the mid-part of the larval body belong to developing dwarf males. Note the strong serotonin signal from the putative glandular structure (asterisk). (E) Ventral view. Somatic nuclei (grey) indicate the outline of the larval body. (F) Lateral view of a double-stained specimen. Colour code for E and F: green, cilia; grey, nuclei; light blue, ventral longitudinal neurites; orange, posterior projections; magenta, anterior projections; red, perikarya; yellow, cerebral ganglion.

for any of the two larval types, further studies are necessary to clarify from which structure this signal stems.

The internal body organization of the Prometheus larva and the Pandora larva differ from each other, and this is also reflected in their different roles in the cycliophoran life cycle. The Pandora larva generates a buccal funnel inside its body, while the Prometheus larva produces one to three dwarf males. Nonetheless, the external morphology is very similar concerning

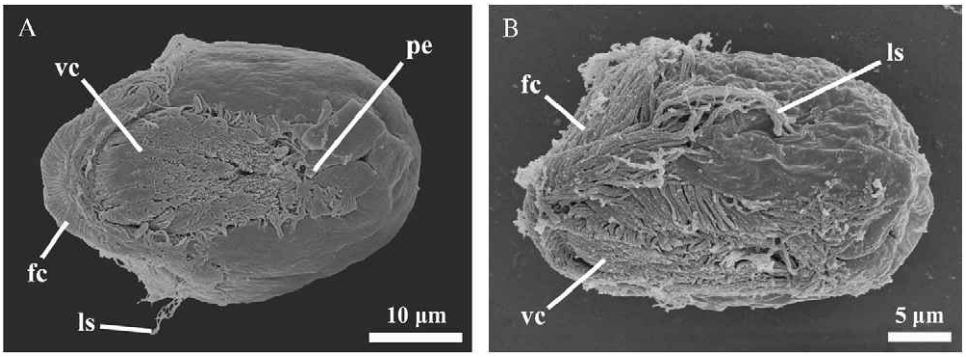

Fig. 4. The dwarf male of *Symbion americanus*, scanning electron micrographs. Anterior faces left in all aspects. (A) Ventral view showing the large ventral ciliated field (vc) and the ventro-posterior location of the penis (pe). (B) Lateral view with the long cilia that compose the lateral sensorial organ (ls). Note the frontal ciliated field (fc) which covers approximately one third of the dorsal region of the male body.

locomotive and sensory organs and this may explain the similarities in the organization of the nervous system of both larvae. Similarities between the nervous system of the Pandora larva, the Prometheus larva, and the female were also pointed out in earlier studies (Funch and Kristensen 1997; Obst et al. 2006). Although then no details were known about the peripheral nervous system, the brain was considered highly similar in all those free swimming stages, which contributed to the view of the female as a neotenic adult (Funch and Kristensen 1997).

The cycliophoran chordoid larva was originally described as a modified trochophore, although a typical spiralian apical organ is absent (Funch 1996). The nervous system of this larval stage in *S. pandora* is well studied at the immunocytochemical level; serotonin immunoreactivity is found in the bilobed cerebral ganglion, its anterior projections, and in two pairs of longitudinal ventral neurites (Wanninger 2005; Neves et al. 2010b). Different from the condition found in the Pandora larva and the Prometheus larva of *S. americanus*, no lateral perikarya protrude from the cerebral ganglion of the chordoid larva of *S. pandora*. Therefore, the serotonergic cerebral ganglion of the Pandora larva and the Prometheus larva differ significantly from that of the chordoid larva. Moreover, the latter larva has four distinct ventral neurites that fuse posteriorly, while the others have only two.

4.2. Serotonin immunoreactivity and neuroanatomy of the dwarf male

Since the dwarf male is characterized by a cerebral ganglion of relatively large size with respect to its entire body volume (Obst and Funch 2003), the serotonin distribution we found was somewhat unexpected. Contrary to the larval stages, serotonin in the cerebral ganglion of the male is confined to the median commissural neuropil. Moreover, serotonin immunoreactivity in the male did not reveal the presence of thin neurites projecting antero-ventrally from the cerebral ganglion, as found in the Pandora larva and the Prometheus larva.

The ventral longitudinal neurites of the dwarf male were described in earlier studies as extending from the cerebral ganglion towards the base of the penial structure (Obst and Funch 2003). These ventral neurites have been proposed to innervate the several muscles situated in the posterior region of the male body, particularly in the vicinity of the penis (see also Neves et al. 2009a, 2009b, 2010a). Our results, however, show that serotonin immunoreactivity in the ventral longitudinal neurites is only present until the posterior end of the ventral ciliated field, which coincides with the tip of the penis. Therefore, serotonin seems to be present only in the anterior half of the ventral neurites. Since a posterior ciliated tuft is absent in the dwarf male, this finding supports our assumption that this structure is innervated by the ventral neurites in the Pandora and the Prometheus larva.

4.3. Cycliophoran and spiralian neuroanatomy

The neuroanatomy of the various cycliophoran life cycle stages was initially studied only by light and transmission electron microscopy, but recent approaches using immunocytochemistry in combination with confocal laserscanning microscopy have increased the amount of data and revealed previously unknown neural features (see Table 1 and references therein). These data suggest that a bilobed cerebral ganglion with anterior projections and a pair of ventral longitudinal neurites are part of the neuroanatomical groundpattern of larval cycliophorans.

A number of spiralians have recently been studied at the immunocytochemical level, including entoprocts (Fuchs et al. 2006; Wanninger et al. 2007; Fuchs and Wanninger 2008), nemertines (Hay-Schmidt 1990), mollusks (Friedrich et al. 2002; Voronezhskaya et al.

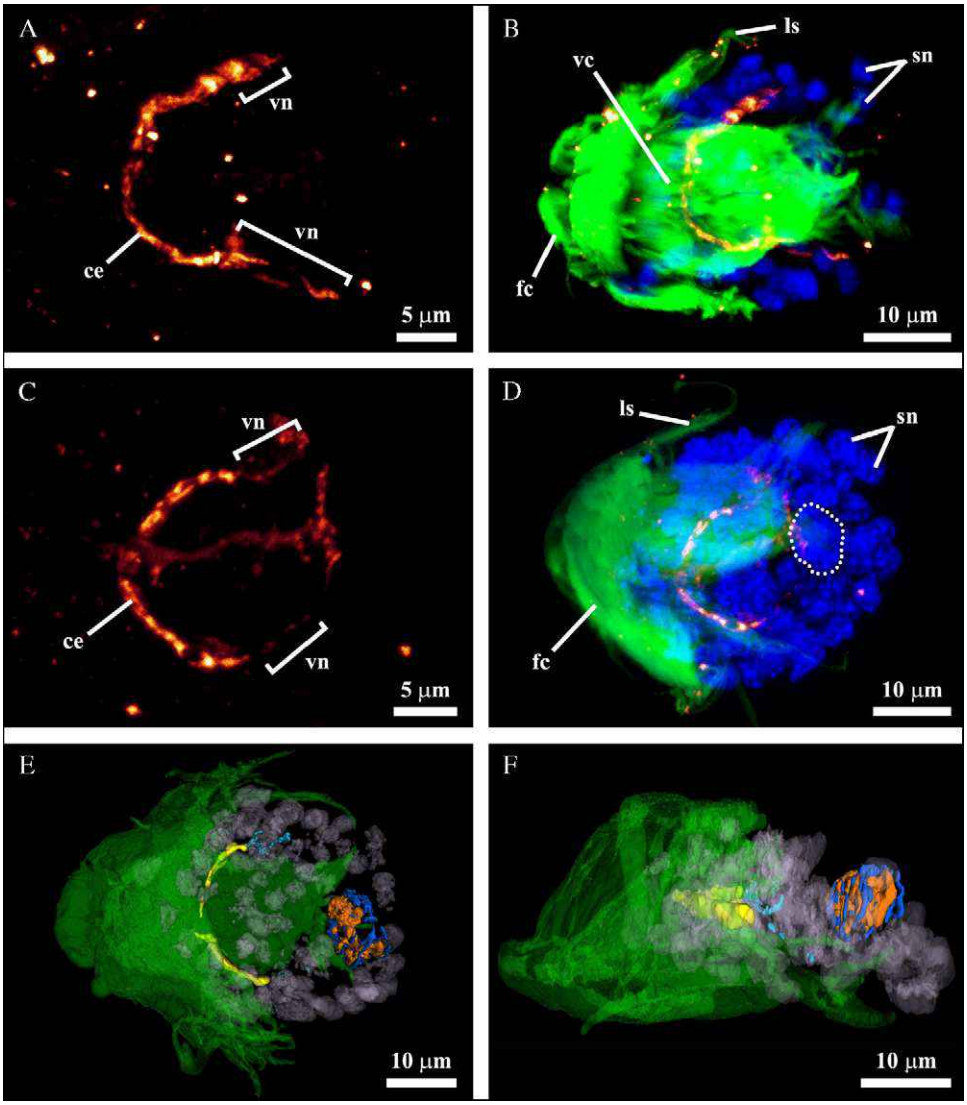

Fig. 5. The serotonergic nervous system of the dwarf male of *Symbion americanus*. CLSM projections of triple labelled specimens for serotonin (red), cilia (green), and nuclei (blue) (A–D), and 3D reconstructions (E and F). Anterior faces left in all aspects. (A) Ventral view showing the sickle shaped part of the neuropil of the serotonergic ganglion (ce). Note the ventral longitudinal neurites (vn) emerging from the serotonergic cerebral ganglion. (B) Same specimen as in A. Note the position of the serotonergic nervous system relative to the ventral ciliated field (vc) and the lateral sensory organ (ls). Somatic cell nuclei (sn) indicate the outline of the larval body. (C) Dorso-lateral view showing the serotonergic cerebral ganglion (ce) and the ventral longitudinal neurites (vn). (D) Same specimen as in C. Note the position of the serotonergic nervous system relative to the frontal ciliated fields (fc). White dots line the testes. (E) Dorsal view. (F) Lateral view. Colour code for E and F: dark blue, cilia of sperm cells; green, locomotive and sensorial cilia; grey, somatic cell nuclei; light blue, ventral longitudinal neurites; orange, sperm cell nuclei; yellow, cerebral ganglion.

2002), sipunculans (Wanninger et al. 2005, 2009; Kristof et al. 2008), the *incertae sedis* genus *Diurodrilus* (Worsaae and Rouse 2008), and polychaete annelids including echiurans (Hessling 2002, 2003; Hessling and Westheide 2002; Orrhage and Müller 2005; McDougall et al. 2006; Brinkmann and Wanninger 2008, 2009). Accordingly, most spiralian larvae are characterized by having an apical organ with four serotonergic flask-shaped cells and a nerve ring that innervates the prototroch (reviewed in Wanninger 2009). All these structures are absent in larval cycliophorans, which thus possess no obvious spiralian larval neural features. In polychaetes, one pair of ventral nerve cords with commissures form the early rudiments of the adult ventral nervous system, even if the adults have more than two (e.g., annelids; Brinkmann and Wanninger 2008) or only a single ventral nerve cord (as in echiurans and sipunculans; Hessling 2002, 2003; Kristof et al.

Table 1. Data currently available on the anatomy and the distribution of immunoreactive substances in the nervous system of the various life cycle stages of Cycliophora. Neural features identified for each stage result from light (LM) and/or transmission electron microscopy (TEM).

Life cycle stage	LM or TEM data source	Immunocytochemistry/confocal microscopy data source			Neural feature: present (+); not found (−)			
		FMRF amide immunoreactivity	Serotonin immuno-reactivity	Synapsin immuno-reactivity	Bilateral cerebral ganglion	Anterior projections from the cerebral ganglion	Dorsal projections from the cerebral ganglion	Ventral longitudinal neurites
Pandora larva	Funch and Kristensen (1995, 1997)[a] and Obst et al. (2006)[b]	Not available	This study[b]	a) [b]	+	+	−	+
Prometheus larva	Funch and Kristensen (1995, 1997)[a]; Obst et al. (2006)[b]	Not available	This study[b]	Not available	+	+	−	+
Chordoid larva	Funch and Kristensen (1995, 1997)[a], Funch (1996)[a], and Obst et al. (2006)[b]	Wanninger (2005)[a] and Neves et al. (2010b)[a]	Wanninger (2005)[b] and Neves et al. (2010b)[a]	Neves et al. (2010b)[a]	+	+	+	+
Female	Funch and Kristensen (1995, 1997)[a]	Not available	Not available	b) [b]	+	+[c]	+	+
Dwarf male	Funch and Kristensen (1997)[a], and Obst and Funch (2003)[a]	c) [b]	This study[b]	d) [b]	+	−	−	+
Feeding stage	Funch and Kristensen (1995, 1997)[a], and Obst et al. (2006)[b]	e) [a]	e) [a]	e) [a]	?	?	−	?

a) Only one specimen investigated. Synapsin-positive signal is found in the bilobed cerebral ganglion and the ventral longitudinal neurites (unpublished results).
b) Only one specimen investigated. Synapsin-positive signal is found in the bilobed cerebral ganglion and its dorsal and antero-ventral projections, and in the ventral longitudinal neurite (unpublished results).
c) Analysis performed in parallel with the study presented herein. FMRFamide-like immunoreactive signal is similar to that of serotonin.
d) Unpublished results. Synapsin-positive signal is restricted to the bilobed cerebral ganglion.
e) ? indicates inconsistent results. A scattered and weak signal is found only in the buccal funnel (unpublished results).
[a]Study performed on *Symbion pandora*.
[b]Study performed on *Symbion americanus*.
[c]Contrary to the other life cycle stages, these projections emerge from the ventral side of the cerebral ganglion and run latero-anteriorly.

2008). A pair of ventral nerve cords has thus been considered as part of the neuroanatomy of the last common spiralian ancestor (Wanninger 2009). However, modifications from this hypothetical ground-pattern have occurred in several spiralian lineages, e.g., in the tetraneural condition found in mollusks and the creeping-type larva of entoprocts (Friedrich et al. 2002; Voronezhskaya et al. 2002; Wanninger et al. 2007). The cycliophoran chordoid larva exhibits an intermediate condition, since it has four serotonergic ventral neurites that fuse in the posteriormost third of the larval body into one single pair, with both neurites being interconnected by a terminal commissure (Wanninger 2005). Contrary to this, the ventral neurites of the Pandora larva and the Prometheus larva have no commissures and fuse in the posteriormost region of the larval body. This condition, together with the presence of an anterior, bilateral cerebral ganglion and the lack of an apical organ, resembles much more the situation found in adult rather than larval spiralians (cf. Nielsen 2008). Accordingly, it appears that specific spiralian larval neural features, such as an apical organ

with few (probably four) serotonergic flask-shaped cells and a nerve ring that innervates the prototroch, were secondarily lost in cycliophoran larvae (see Wanninger 2009).

In the future, the anatomy and distribution of immunoreactive substances in the nervous system of the feeding stage and female should be studied. This will permit further comparisons with other lophotrochozoan taxa and could result in further insights into the evolution of Cycliophora.

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