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# The larval nervous system of the penis worm *Priapulid caudatus* (Ecdysozoa)

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The origin and extreme diversification of the animal nervous system is a central question in biology. While most of the attention has traditionally been paid to those lineages with highly elaborated nervous systems (e.g. arthropods, vertebrates, annelids), only the study of the vast animal diversity can deliver a comprehensive view of the evolutionary history of this organ system. In this regard, the phylogenetic position and apparently conservative molecular, morphological and embryological features of priapulid worms (Priapulida) place this animal lineage as a key to understanding the evolution of the Ecdysozoa (i.e. arthropods and nematodes). In this study, we characterize the nervous system of the hatching larva and first lorica larva of the priapulid worm *Priapulid caudatus* by immunolabelling against acetylated and tyrosinated tubulin, pCaMKII, serotonin and FMRFamide. Our results show that a circumoral brain and an unpaired ventral nerve with a caudal ganglion characterize the central nervous system of hatching embryos. After the first moult, the larva attains some adult features: a neck ganglion, an introvert plexus, and conspicuous secondary longitudinal neurites. Our study delivers a neuroanatomical framework for future embryological studies in priapulid worms, and helps illuminate the course of nervous system evolution in the Ecdysozoa.

## 1. Introduction

The animal nervous system is the specialized set of cells, tissues and organs responsible for integrating external and internal stimuli and coordinating adequate responses. During evolutionary time, the nervous system has acquired an astonishing level of complexity in bilaterally symmetrical animals (Bilateria), with the appearance of centralized and highly organized neural clusters, such as brains and nerve cords [1]. The presence of centralized nervous systems in distantly related bilaterian groups has raised a vivid debate on the homology (common ancestry) of these structures [2–11], and therefore about the morphological and functional diversification of the nervous system across bilaterian lineages. Insects, and to a minor extent other arthropods, have been key players in almost all these controversies, due to the tripartite organization of their brains and the presence of prominent anterior neuropils called mushroom bodies. These two sophisticated neural features have been homologized with similar anatomical structures in vertebrates and annelids [2,4–7,12], and thus used as argument for the presence of circuit ground patterns that also characterize brains in lineages that have diverged from the last common bilaterian ancestor. However, a proper understanding of the evolution of the arthropod nervous system also requires a detailed morphological, embryological and molecular investigation of often-neglected related bilaterian lineages, in

particular those that occupy informative nodes in the phylogeny. Such studies will reveal a better understanding of the evolutionary changes that led to nervous system diversity and how the nervous system architecture relates to the molecular and behavioural repertoire.

Arthropods, onychophorans and tardigrades (Panarthropoda), together with nematodes and nematomorphs (Nematozoa), are members of the Ecdysozoa [13]. Recently, molecular phylogenies have placed the priapulid worms (Priapulida), kinorhynchans and loriciferans in a group called Scalidophora, as the sister group taxa to the remaining ecdysozoans (i.e. nematoids and panarthropods) [14–17]. Priapulids, commonly referred to as penis worms, are exclusively marine, mud-dwelling or interstitial animals [18,19]. Despite being among the most abundant fossils in the Early Cambrian [20–22], Priapulida comprise only 19 known extant species [18,19,23]. Adults are sausage-shaped, annulated worms with bodies divided into an anterior retracting introvert with a terminal mouth, and a posterior trunk with a terminal anus and, in some species, a caudal appendage [19]. After external fertilization, priapulid eggs undergo holoblastic radial cleavage, deuterostomic development and formation of a ventral mouth, which are all inferred ancestral characters for the Ecdysozoa [24–27]. Embryonic development results in the formation of a larva, which matures into the adult worm through successive rounds of moulting [28–31]. Morphological and developmental evidence, together with their slow rate of molecular evolution [32] and phylogenetic position support the role of priapulid worms as a key group to understanding the earliest steps of ecdysozoan evolution, and thus deducing ancestral characters to morphologically more diverse ecdysozoan taxa, such as insects.

Studies on the nervous system of the Priapulida are, however, scarce, and mostly focused on adult stages or mature larval stages [30,33–37]. Only recently, immunohistological techniques have been applied to adult specimens of the meio-benthic species *Tubiluchus troglodytes* in order to study the nervous system [38]. In adults and mature priapulid larvae, the central nervous system (CNS) is intraepidermal and comprises a circumoral brain, an unpaired ventral nerve cord and two main ganglia, the neck ganglion at the joint between the introvert and the trunk and the caudal ganglion at the most posterior region of the body [38,39]. Notably, descriptions of a putative priapulid from the Mid-Cambrian, *Ottoia prolifica* [22], identify a paired reflective strand along the ventral midline and have been interpreted as a paired ventral cord [40]. Associated with the CNS, there are nerve plexuses in the pharynx, body wall and caudal appendage, as well as a stomatogastric nerve plexus in the digestive tract [33–35,38,41]. Immunodetection of serotonin and RFamide-like peptides demonstrated the presence of different neural subpopulations in almost all components of the priapulid nervous system [38]. In contrast with our current knowledge of the more mature stages, virtually nothing is known about the embryonic development and early post-embryonic morphology of the nervous system of priapulid worms, which are ultimately essential to understanding the evolution of the great diversity of nervous systems observed in other representatives of the Ecdysozoa.

To gain a better knowledge of the early stages of nervous system formation in priapulid worms, we analysed the immunostaining domains of five antibodies commonly used to characterize neural structures in ecdysozoan animals [2,38,42–51] in hatching larvae and first lorica larvae of the

species *Priapululus caudatus* Lamarck, 1816. Immunodetection of acetylated tubulin, tyrosinated tubulin, phosphorylated calcium/calmodulin-dependent protein kinase II (pCaMKII), serotonin and FMRF-like peptides (FLPs) demonstrates that the nervous system of hatching priapulid embryos consists of a circumoral brain, a main ventral nerve, a caudal ganglion and several less conspicuous neurite bundles associated with the buccal scalds, neck and sensory trunk tubuli. The first moulting event implies a significant maturation of the nervous system, with a general increase in the number of neuronal cells and nerve fibres, and the appearance of the neck ganglion. Our study is an important contribution to the study of the Priapulida and improves our understanding of the diversification of the nervous system in the Ecdysozoa, and thus of the evolution of some of the most elaborated neural structures found in animals.

## 2. Material and methods

### (a) Animal collection, fertilization and larva fixation

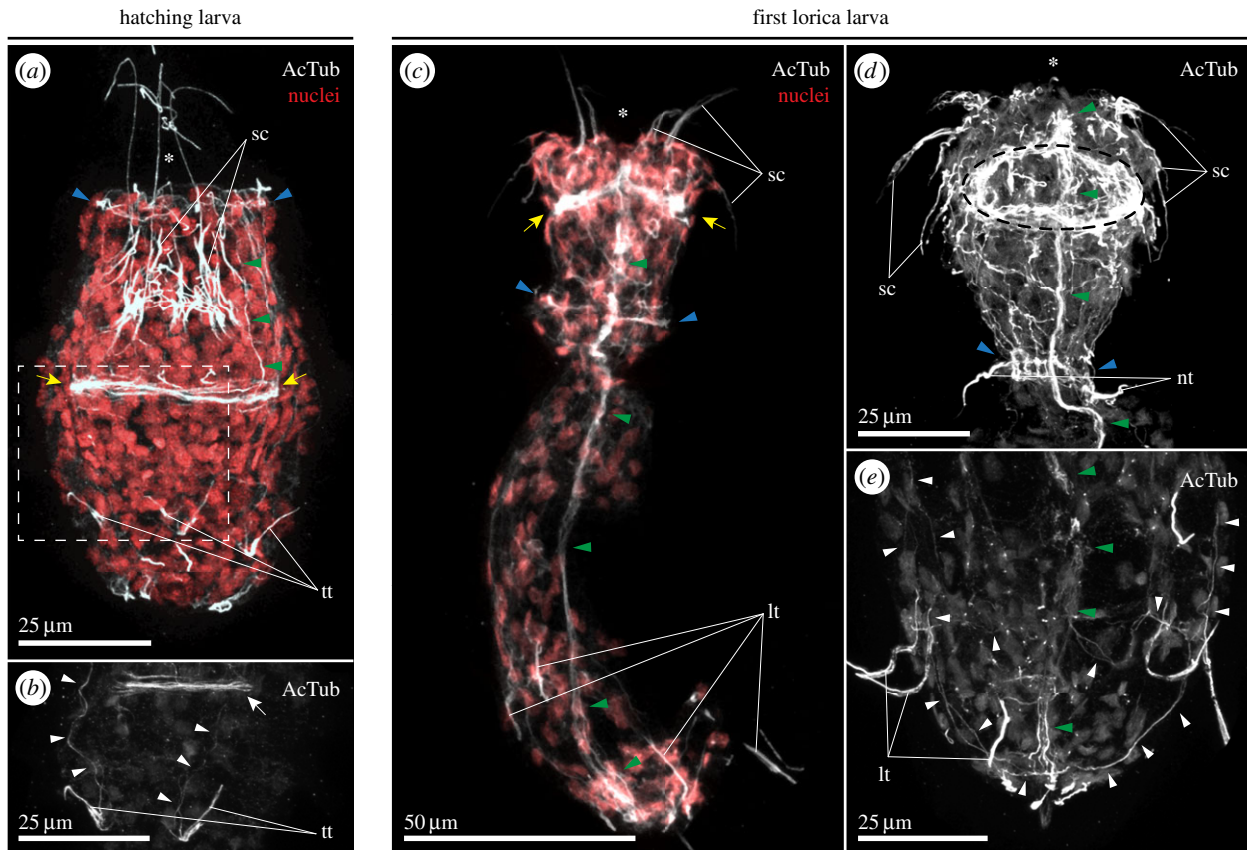
Adult gravid specimens of *P. caudatus* were collected from Gullmarsfjorden (Fiskebäckskil, Sweden) during the autumn. Dissection of the gonads, fertilization of the oocytes and culture of the embryos were performed as described elsewhere [24]. Embryos hatched 9 days after fertilization, and hatching larvae moulted to the first lorica larvae approximately two weeks thereafter, without any added food source. Before fixation, larvae were relaxed in 0.1% tricaine in filtered seawater (FSW) for 30 s, and immediately fixed in 4% paraformaldehyde (PFA) in FSW for 1 h at room temperature. Fixative was washed out in phosphate buffered saline (PBS) with 0.1% Tween-20 (PTw) before storage in 0.1% sodium azide in PTw at 4°C.

### (b) Immunohistochemistry

Fixed hatching and first lorica larvae were washed three times for 5 min in PTw to remove sodium azide, and perforated afterwards with a thin needle to allow antibody penetration through the larval cuticle. Perforated larvae were transferred into PBS with 0.5% Triton X-100 (PTx) for permeabilization for 2 h at room temperature, and subsequently blocked in 1% bovine serum albumin (BSA) in PTx for 2 h at room temperature. Before adding the primary antibody, larvae were blocked in 10% normal goat serum (NGS) in PTx twice for half an hour. The analysed primary antibodies (mouse anti-acetylated tubulin (Sigma, #T6793), mouse anti-tyrosinated tubulin (Sigma, #T9028), rabbit anti-pCaMKII (Santa Cruz Biotechnology, #sc-12886), rabbit anti-serotonin (Sigma, #S5545) and rabbit anti-FMRamide (Immunostar, #20091)) were diluted 1:100 in 10% NGS in PTx and incubated for approximately 40 h at 4°C. Continuous washes in 1% BSA in PTx for approximately 7 h to remove the primary antibody were followed by blocking in 10% NGS in PTx twice for half an hour and incubation in Alexa-conjugated secondary antibody diluted 1:250 in 10% NGS in PTx for approximately 40 h at 4°C. Finally, secondary antibodies were washed out in PTx, and if needed nuclei were counterstained with Sytox Green.

### (c) Imaging

Stained larvae were cleared in Murray's reagent and representative specimens were scanned with a Leica SP5 confocal laser-scanning microscope. Images were analysed in FIJI and PHOTOSHOP CS6 (Adobe), and figure plates made with ILLUSTRATOR CS6 (Adobe). Brightness/contrast and colour balance adjustments were always applied to the whole image, not parts.



**Figure 1.** Localization of acetylated tubulin in *P. caudatus* larvae. (a–e) Maximal z-projections of confocal stacks of whole mount larvae stained against acetylated tubulin (AcTub, in grey) and counterstained with the nuclear marker Sytox Green (red, in a and c). (a) The hatching larva of *P. caudatus* shows a circumoral brain (yellow arrows) and neural commissures at the neck region (neck ganglion; blue arrowheads). The oral scalids and the posterior sensory trunk tubuli are also innervated. (b) Detail of the region indicated by a dashed rectangle in (a). Thin neural fibres (white arrowheads) project from the sensory trunk tubuli towards the introvert. (c) After the first moult, a well-developed ventral nerve cord (green arrowheads) connects the circumoral brain (yellow arrows) with the posterior region of the trunk. The neck ganglion (blue arrowheads) appears more distinct. (d) The introvert region of the first lorica larva is rich in neural fibres, with a dense innervation of the scalids from the brain area (black dashed circle; main ventral nerve indicated by green arrowheads and the neck commissures by blue arrowheads). (e) Similar to the anterior scalids, the posterior lorica tubuli are strongly innervated, with thin fibres (white arrowheads) projecting from them longitudinally towards the anterior region and posteriorly towards the anal opening, where they meet with the ventral nerve (green arrowheads). In all cases, the asterisk indicates the position of the mouth. (a,b) are lateral views, and (c–e) are ventral views. lt, lorica tubulus; nt, neck tubulus; sc, scalids; tt, trunk tubulus. Scale bars, 25  $\mu\text{m}$  in (a,b,d,e); 50  $\mu\text{m}$  in (c).

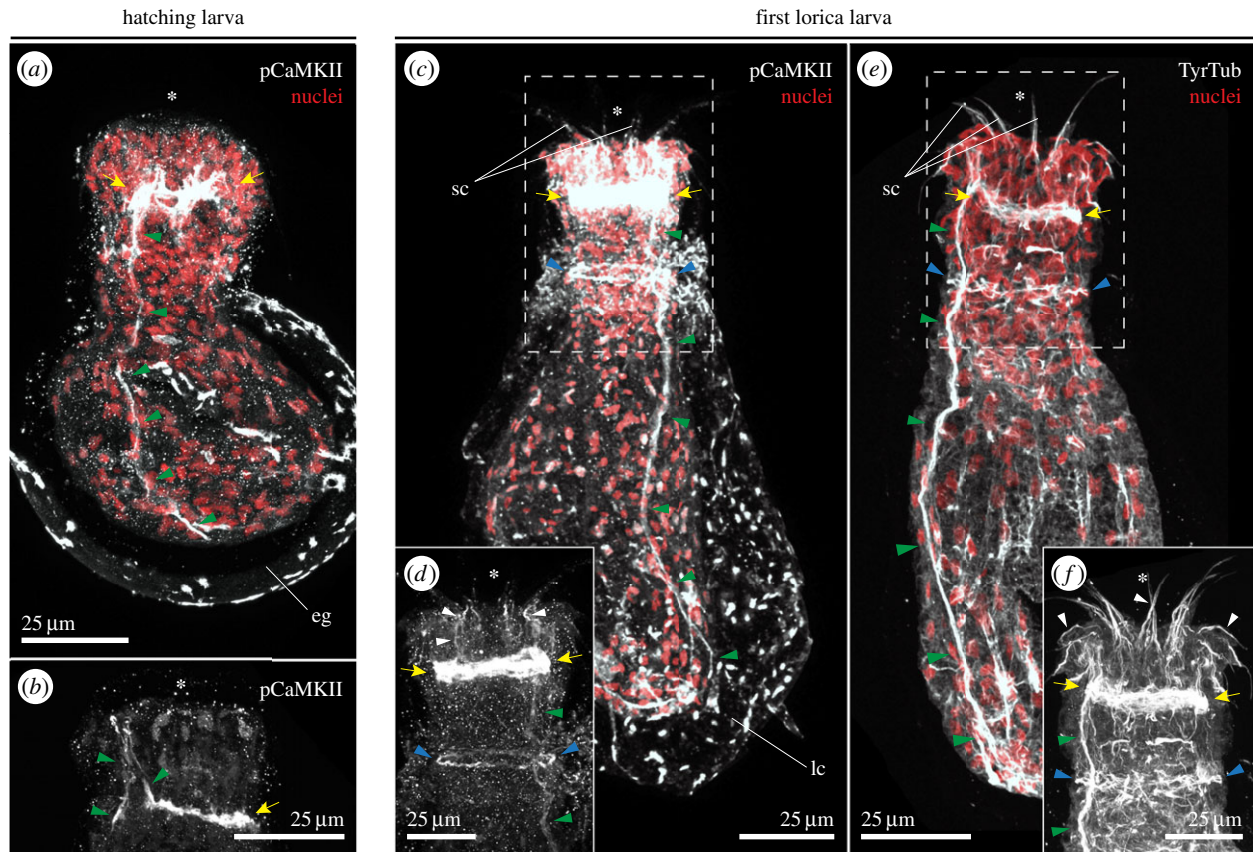
### 3. Results

#### (a) The early larval nervous system of *Priapulus caudatus*

The hatching larva of *P. caudatus* has a functional anterior introvert with seven primary plus one to three secondary oral scalids (feeding and predatory teeth), a short neck region with a pair of tubuli and a posterior trunk with approximately four trunk tubuli, probably of sensory function [28]. Internally, the hatching larva possesses a well-developed muscular and digestive system [26]. Acetylated tubulin immunoreactivity indicates that the nervous system of hatching larvae consists of a dense circumoral brain and a thin main longitudinal ventral nerve, as well as several less conspicuous nerve fibres (figure 1a,b). Among these less evident neurite bundles, there are circular nerve fibres in the neck region and longitudinal neurite bundles that seem to connect the posterior sensory tubuli of the trunk with the introvert neural structures (figure 1a,b). The hatching larva of *P. caudatus* is non-feeding and moults into the first lorica larva after approximately two weeks. As the name indicates, this is the first larval stage with a true lorica protecting

the trunk. In the first lorica larva, the number of scalids increases, the neck tubuli disappear, and four lorica tubuli are visible in the ventro- and dorso-lateral lorica plates [28]. At this larval stage, the brain and ventral nerve appear more developed and seem to include a greater number of nerve fibres (figure 1c,d). The neck commissures are now packed into a well-formed ganglion, and many secondary nerve fibres connect this structure with the circumoral brain (figure 1c,d), defining the introvert nerve plexus. The lorica tubuli project longitudinal nerve fibres that connect posteriorly in the anal region and run anteriorly towards the main introvert neural structures (figure 1e). Additionally, transverse commissures appear to connect these secondary neurite bundles between them and with the main ventral nerve (figure 1e).

pCaMKII immunoreactivity is restricted to the brain and the main ventral nerve of both the hatching larva and the first lorica larva (figure 2a–d). While pCaMKII labelling is not observed in the neck region in the hatching larva of *P. caudatus* (figure 2b), immunoreactivity in the neck ganglion is evident with the first moult (figure 2d), together with the labelling of fine nerve fibres projecting from the anterior region of the neuropil towards the buccal scalids (figure 2d).



**Figure 2.** Localization of pCaMKII and tyrosinated tubulin in *P. caudatus* larvae. (a–f) Maximal z-projections of confocal stacks of whole mount larvae stained against pCaMKII (in grey) or tyrosinated tubulin (TyrTub, in grey) and counterstained with the nuclear marker Sytox Green (red, in a, c and e). (a) In the hatching larva of *P. caudatus*, antisera against phosphorylated CaMKII localize in the circumoral brain nerves (yellow arrows) and ventral nerve (green arrowheads). (b) The ventral nerve (green arrowheads) connects to the brain (yellow arrow) anteriorly, folding inside the body as the introvert retracts. (c,d) In the first lorica larva, immunoreactivity to pCaMKII is stronger in the brain (yellow arrows), the neck ganglion (blue arrowheads), ventral nerve (green arrowheads), and nerve projections towards the buccal scalids (white arrowheads in d). (e,f) Immunoreactivity against TyrTub appears in the first lorica larva, greatly concentrated in the circumoral brain (yellow arrows), neck ganglion (blue arrowheads), main ventral nerve (green arrowheads) and the innervation of the scalids (white arrowheads in f). In all cases, the asterisk indicates the position of the mouth. The staining in the eggshell and the lorica in (a) and (c) is background. All panels are ventral–lateral views. eg, eggshell; lc, lorica; sc, scalids. Scale bars, 25  $\mu\text{m}$  in all panels.

Finally, tyrosinated tubulin immunoreactivity was not consistently observed in hatching larvae, and only reliably detected in the first lorica larvae. At this stage, tyrosinated tubulin antibody labelled the brain, neck ganglion and ventral nerve (figure 2e,f). Altogether, the immunolabelling of acetylated and tyrosinated tubulin and pCaMKII show that the CNS of priapulid embryos at hatching is already composed of a circumoral brain and a main ventral nerve ending in a caudal ganglion. Additionally, neurite bundles associated with the sensory trunk tubuli and scalids make up the peripheral nervous system (PNS). With the first moulting event, the nervous system experiences a significant increase in complexity, with a general rise in the number of neurite fibres in both the CNS and the PNS.

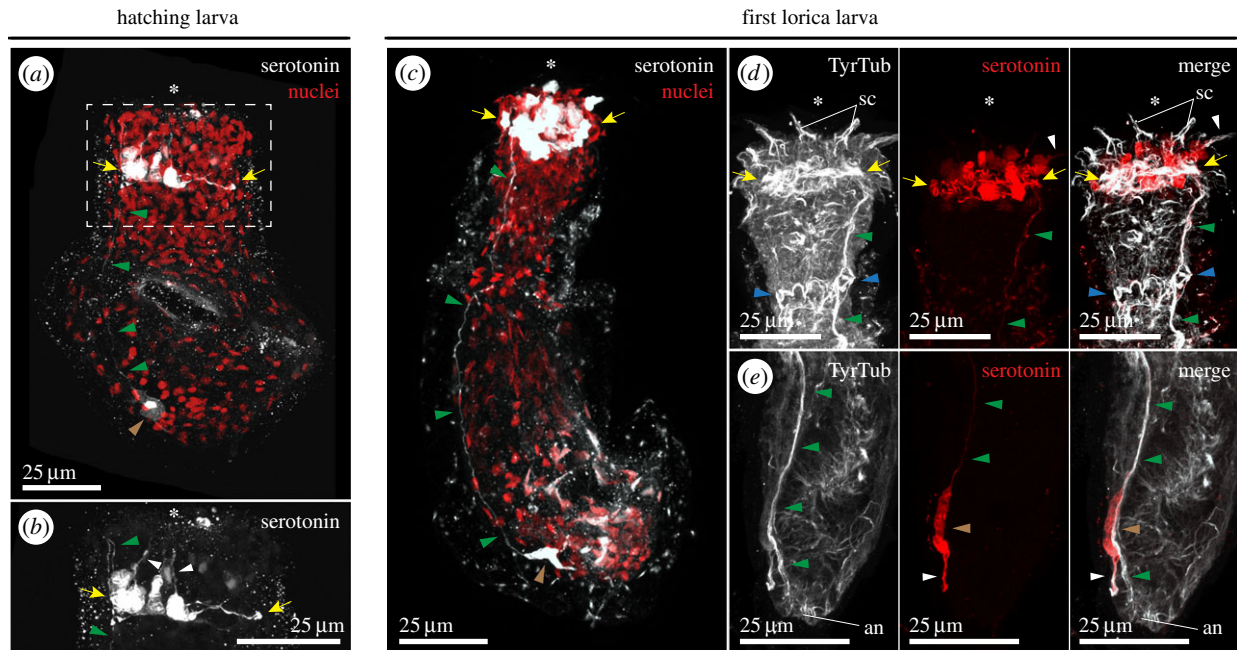
### (b) The serotonergic nervous system

Serotonin-positive cells localize to the circumoral brain and caudal ganglion of the hatching larva of *P. caudatus* (figure 3a,b). In the brain, serotonin-positive cells are bipolar, projecting one axon towards the anterior end of the introvert, where the scalids are located, and the other axon towards the neuropil (figure 3b). One single bipolar serotonin-positive cell is observed in the caudal ganglion, which projects one axon posteriorly towards the anus and another one anteriorly

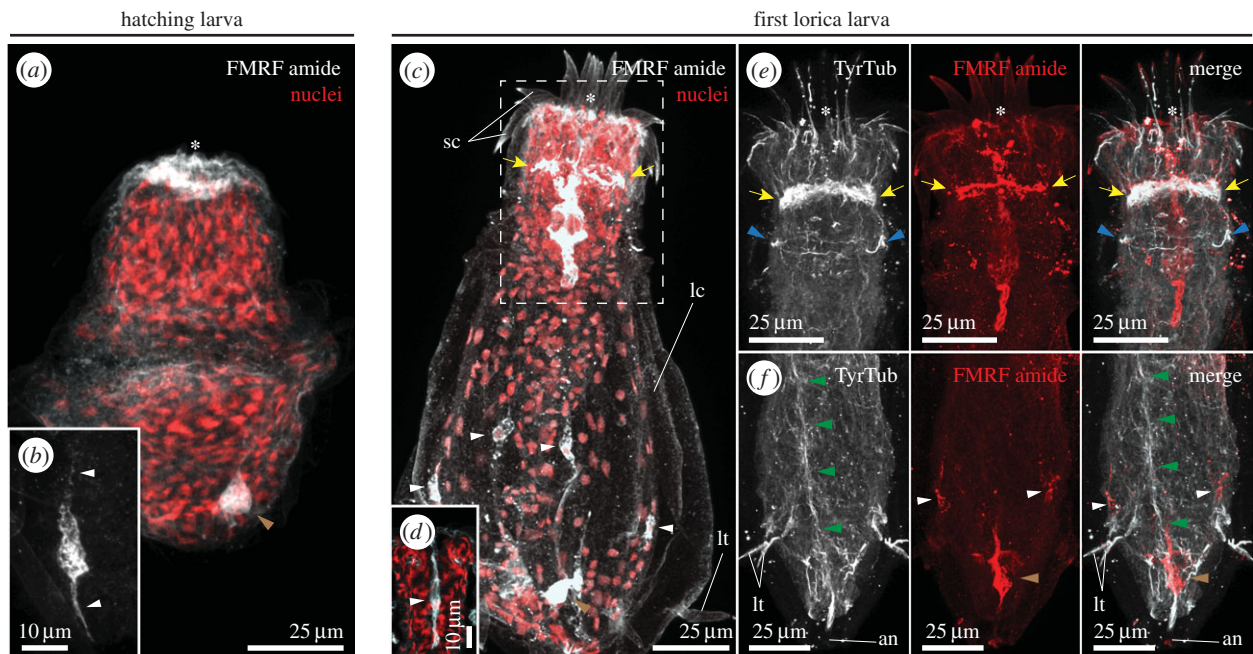
towards the brain through the main ventral nerve (figure 3a). With our data, we cannot discriminate whether the serotonin-positive axon of the ventral neurites extends from the circumoral brain or from the caudal ganglion. After the first moult, the number of serotonin-positive cells increases, although the overall distribution remains (figure 3c). In the brain region, serotonergic cells innervate the scalids and distribute anteriorly of the neuropil (figure 3d). In the posterior trunk, the caudal ganglion contains one bipolar serotonin-positive cell, which projects the posterior axon outside the main ventral nerve (figure 3e). The serotonergic nervous system of the first larval stages of *P. caudatus* is thus restricted to the main elements of the CNS, in contrast with the situation observed in adult priapulids, where serotonin-positive cells are widespread also in the PNS [38].

### (c) FMRFamide-like peptides in *Priapulus caudatus* early larval stages

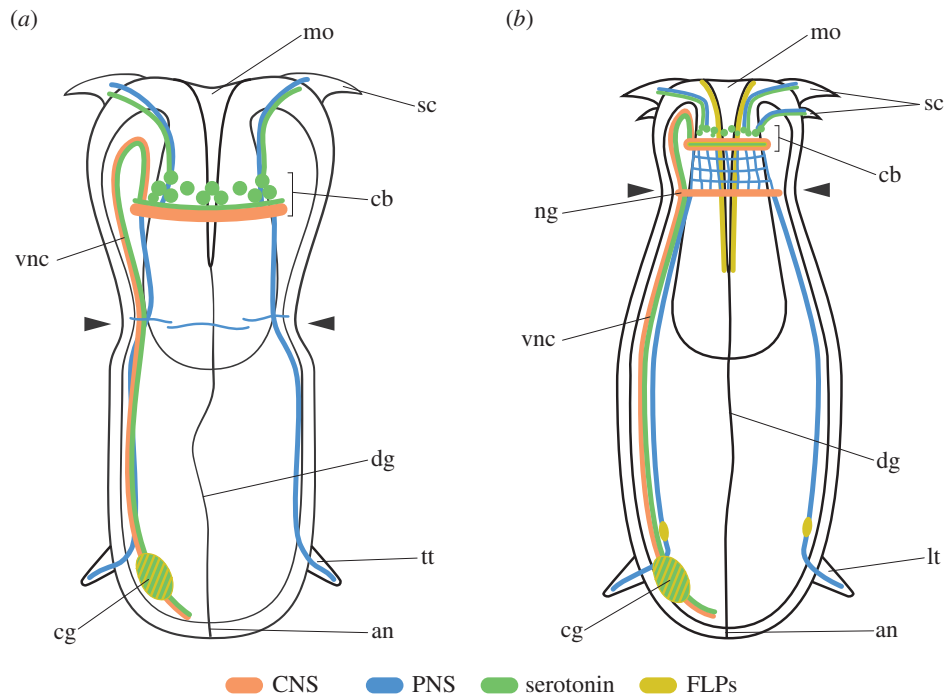
The hatching larva of *P. caudatus* exhibits immunoreactivity to FLPs in one cell at the posterior region of the trunk (figure 4a), presumably in one bipolar cell of the caudal ganglion (figure 4b). Immunoreactivity at the buccal opening is consistent among hatching larvae (figure 4a), but does not seem to be associated with any particular cells, and thus it might be



**Figure 3.** The larval serotonergic nervous system of *P. caudatus*. (*a–e*) Maximal z-projections of confocal stacks of whole mount larvae stained with antisera against serotonin (*a–c*, in grey; *d* and *e*, in red) or tyrosinated tubulin (TyrTub; *d* and *e*, in grey) and counterstained with the nuclear marker Sytox Green (red, in *a,c*). (*a*) The serotonergic nervous system of the hatching larva comprises perikarya around the circumoral brain (yellow arrows), an axonal tract in the ventral nerve (green arrowheads) and one cell at the caudal ganglion (brown arrowhead). (*b*) Magnification of the squared region in (*a*). Serotonergic cells in the brain are bipolar, with one axon projecting towards the anterior end (white arrowheads) and the other one projecting towards the neuropil (yellow arrows; ventral nerve indicated by green arrowheads). (*c*) In the first lorica larva, the number of serotonin-positive cells in the brain increases (yellow arrows), the ventral nerve (green arrowheads) is more conspicuous and one serotonin-positive cell is still observed in the caudal ganglion (brown arrowhead). (*d*) The serotonin-positive cells are located anterior to the neuropil (yellow arrows, as observed with TyrTub; the blue arrowheads indicate the neck ganglion, and the green arrowheads the ventral nerve), with the anterior cells projecting one axon towards the scalids (white arrowhead). (*e*) In the posterior region, the serotonergic cell of the caudal ganglion (brown arrowhead) projects its posterior axon (white arrowhead) outside the ventral nerve (green arrowheads). In all cases, the asterisk indicates the position of the mouth. All panels are ventral–lateral views. an, anus; sc, scalids. Scale bars, 25  $\mu\text{m}$  in all panels.



**Figure 4.** Localization of FLPs in *P. caudatus* larvae. (*a–f*) Maximal z-projections of confocal stacks of whole mount larvae stained with an anti-FMRFamide antibody (*a–d*, in grey; *d* and *e*, in red) or against tyrosinated tubulin (TyrTub; *e* and *f*, in grey) and counterstained with the nuclear marker Sytox Green (red, in *a, c* and *d*). (*a*) In the hatching larva, FLPs are detected in a bipolar cell (white arrowheads in magnification in (*b*)) at the caudal ganglion (brown arrowhead). (*c*) After the first moult, FLP immunoreactivity is observed in the neuropil (yellow arrows), caudal ganglion (brown arrowhead) and several cells of the trunk (white arrowheads). (*d*) The epithelial cells lining the lumen of the buccal cavity exhibit immunoreactivity for FLPs (white arrowhead). (*e*) Magnification of the squared region in (*c*). FLP immunoreactivity in the introvert is observed in the brain neuropil (yellow arrows; neck ganglion indicated by blue arrowheads). (*f*) In the posterior region, FLPs are observed in two cells of the caudal ganglion (brown arrowheads) at the end of the main ventral nerve (green arrowheads) and in cells of the thin neurite bundles departing from the trunk tubuli (white arrowheads). In all cases, the asterisk indicates the position of the mouth. All panels are ventral–lateral views. lc, lorica; lt, lorica tubulus; sc, scalids. Scale bars, 25  $\mu\text{m}$  in (*a,c,e,f*); 10  $\mu\text{m}$  in (*b,d*).



**Figure 5.** The early larval nervous system of *P. caudatus*. (a,b) Schematic of the nervous system of the hatching larva and first lorica larva of *P. caudatus*. (a) In the hatching larva, the CNS (in orange) consists of a circumoral brain, an unpaired ventral nerve and a caudal ganglion (serotonin- and FLP-positive). In the brain, all serotonin cell bodies localize anterior to the neuropil. From the brain, neurite bundles innervate the scalids. The PNS (in blue) includes neck commissures and nerves connecting the trunk tubuli with the brain. (b) In the first lorica larva, the nervous system is more mature. The CNS consists of the circumoral brain, the ventral nerve, and a neck and a caudal ganglion. The brain neuropil is also FLP-positive. The PNS includes secondary nerves innervating the scalids, connecting the brain and the neck ganglion, and innervating the lorica tubuli (also FLP-positive). The epithelium of the buccal cavity is immunoreactive to FLPs. Drawings are not to scale and are lateral views, with the anterior to the top and ventral to the left. an, anus; cb, circumoral brain; cg, caudal ganglion; dg, digestive tract; lt, lorica tubule; mo, mouth; ng, neck ganglion; sc, scalid; tt, trunk tubule; vnc, ventral nerve cord.

unspecific binding of the antibody. The moulting of the hatching larva into the first lorica larva significantly affects the distribution of FLPs in the nervous system. The circumoral brain of the first lorica larva appears immunoreactive for FLPs, as well as the caudal ganglion and several isolated cells along the trunk (figure 4c). In the introvert, the FLP-positive region localizes to the neuropil, as well as in cells of the inner epithelium of the buccal cavity (figure 4e). This staining was present in all analysed larvae, and it seems to affect the luminal cells (figure 4d). Posteriorly, FLP-positive cells of the trunk appear associated with the lorica tubuli and the neurite bundles that depart from them towards the anterior CNS (figure 4f). Therefore, FLPs appear to localize in both the CNS and the PNS of the first lorica larvae of *P. caudatus*.

## 4. Discussion

### (a) The early larval nervous system of *Priapulid caudatus*

Studies on the nervous system of priapulid worms are scarce, and so far exclusively focused on adult stages and larval forms obtained from direct field sampling which already have a lorica, and thus correspond to late larval stages [23,30,33–38,41]. In our study, we analysed the immunoreactivity patterns of five antibodies routinely used in immunohistochemical neuroanatomy (figures 1–4) to characterize the earliest post-embryonic stages of nervous system formation in larval forms obtained by *in vitro* fertilization. Our results show that the CNS of the hatching larva consists of a circumoral brain, an

apparently unpaired longitudinal ventral nerve, and a caudal ganglion (figure 5a). The circumoral brain has a bipartite organization, with the somata (at least the serotonin-positive cells) located anteriorly to the neuropil (figure 3b) [52]. This type of organization seems to be common also in the Eupriapulida [33,41], but differs from the situation observed in the Tubiluchidae, where the brain includes serotonergic somata located both anteriorly and posteriorly to the central neuropil [34,35,38]. The ventral longitudinal nerve is unpaired and leaves the circumoral brain anteriorly, turning backwards towards the posterior anus at the anterior most region of the introvert. In the hatching larva, the main ventral neurite bundle is thin, probably formed by a very limited number of axonal tracts, and serotonin-positive (figures 1–3). We did not observe any nuclei associated with the main ventral longitudinal nerve. At its posterior end, there is a serotonin- and FLP-positive caudal ganglion. Additionally, the hatching larva presents thin neck commissures, and peripheral innervation of the buccal scalids and of the trunk tubuli (figures 1, 3 and 5a). The presence of axonal tracts leaving the trunk tubuli suggests that these structures are sensory organs of the larva [38], although alternative and/or complementary roles (e.g. adhesion) have been proposed [53]. Altogether, the nervous system found in hatching larvae indicates that the embryos of *P. caudatus* leave the eggshell with a basic layout of the adult priapulid nervous system. To date, the only neural gene expression reported in priapulid embryos is that of *orthodenticle (otx)* [24]. *otx* is expressed in the ventral ectoderm of the introvert and in a ring around the introvert–trunk boundary at the introvertula stage. According to the results shown in this study, this expression would correspond to the circumoral brain of the hatching larva, once the introvert has retracted inside the

trunk during late embryogenesis [26]. Our study thus offers the neuroanatomical framework for future embryological studies on the development of the nervous system of *P. caudatus*.

The first moulting event leads to a significant change in the complexity of the nervous system of *P. caudatus* (figure 5b), as has been also described for other organ systems such as the digestive tract, the musculature and the external morphology of the cuticle [26,28]. The CNS, as revealed by the immunoreactivity pattern for pCaMKII and tyrosinated tubulin (figure 2), includes a well-developed neck ganglion, and thus appears similar to the organization observed in adult priapulids [19,33,38,39]. The other components, namely the circumoral brain, the main ventral nerve and the caudal ganglion, contain more somata and nerve fibres. Important changes are observed in the PNS, where many neurite bundles connecting the brain with the neck ganglion are observed. In addition, the connection between the lorica tubuli and the CNS increases in complexity, by including FLP-positive cells along the neural tracts. Despite this significant change in the organization of the nervous system of the first lorica larva of *P. caudatus*, important features observed in the nervous system of adult priapulids are still missing. We did not find any evidence of serotonin signal around the gut or in the body wall nerve plexus, as observed for instance in *T. troglodytes* [38], and the pharyngeal/introvert plexus is also significantly more simple than that observed in adult stages [38,54]. The distribution of the FLPs is also more localized than in adult priapulids [38]. In addition, the adult *T. troglodytes* has an orthogonal pattern of neurites [38], which seems to be absent in at least these early larval stages of *P. caudatus*. Therefore, the basic anatomical organization of the priapulid nervous system is attained at the first lorica larva stage, although subsequent rounds of moulting must relate to the appearance of the mature features of the nervous system of adult stages, probably associated with the onset of predatory behaviours.

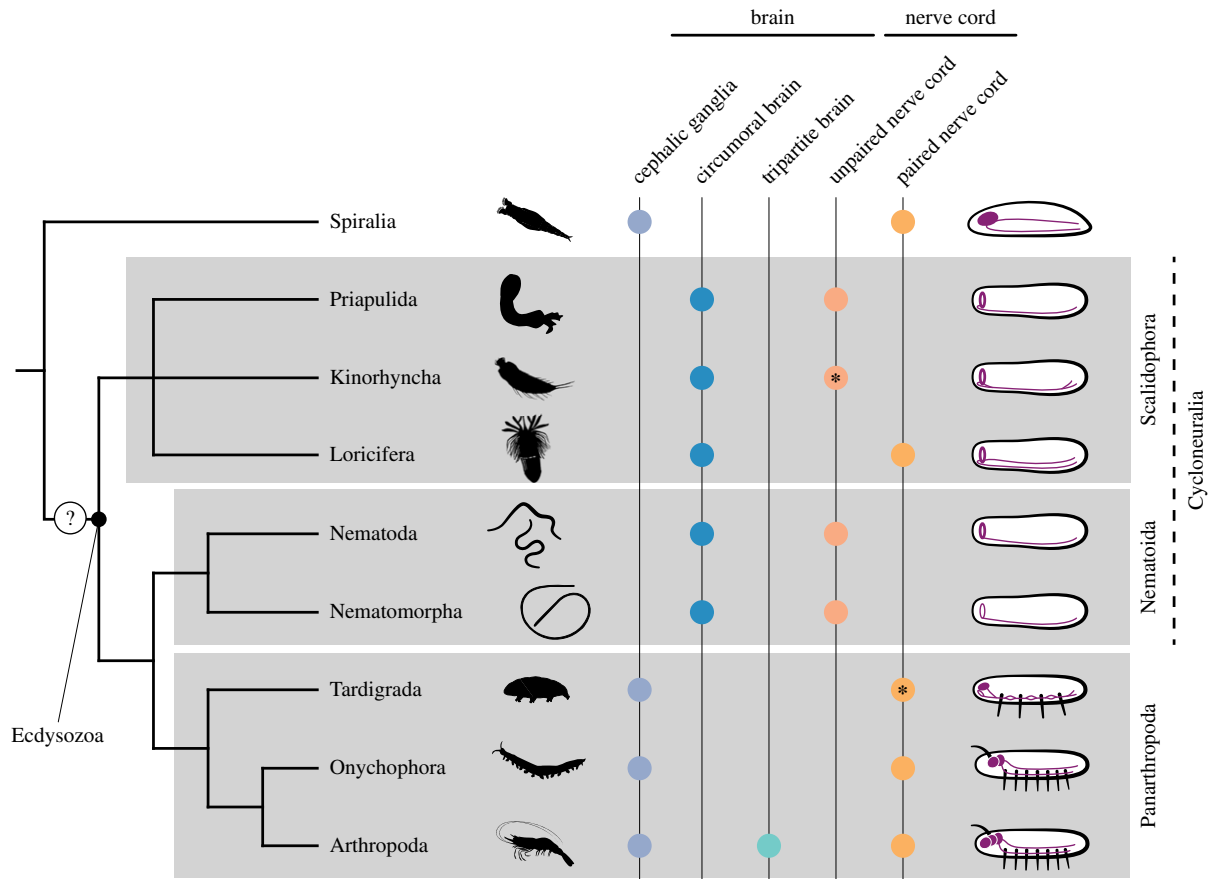
## (b) Implications for the evolution of the nervous system in the Ecdysozoa

Evolutionary discussions on the diversification of the nervous system within the Ecdysozoa are hampered by the limited availability of neuroanatomical data regarding the Priapulida, and Scalidophora generally. Moreover, the scarce studies on priapulid worms are entirely restricted to adult stages and late larval forms, with almost nothing known regarding the embryonic formation of the nervous system. The situation is even more severe for the other two scalidophoran lineages, namely the Kinorhyncha and the Loricifera, for which general data on their embryogenesis are absent or extremely limited [55,56]. Therefore, our characterization of the nervous system of the hatching larva and first lorica larva of *P. caudatus* is an important first step towards closing this gap of knowledge.

Inferring the ancestral form of the scalidophoran, and ecdysozoan, nervous system is thus a hard task, as it becomes obstructed by the problematic logistics of comparing late embryonic/early larval data (this study and taxa from the Nematoida and Panarthropoda) with the anatomy of more mature stages (other members of the Priapulida, the Kinorhyncha and the Loricifera). Nevertheless, general evolutionary hypotheses can be formulated, which can ultimately serve as matters for further study. If, for the sake of simplicity, we focus on the CNS, the earliest and simplest anatomical form comprises a circumoral brain and an unpaired ventral nerve in *P. caudatus* at

least in the larva. However, palaeontological evidence suggests that the adult forms of the Mid-Cambrian priapulid *O. prolifica* [22] possessed a paired ventral cord [39]. The basic organization found in priapulid larvae is also observed in kinorhynchs and loriciferans (figure 6), although the ventral nerve cord bifurcates anteriorly to connect with the brain and also posteriorly after the caudal ganglion in the Kinorhyncha [57,58], and is paired in the Loricifera [59]. In nematodes and nematomorphs there is a main unpaired ventral nerve cord [60,61], whereas in panarthropods the ventral nerve cord is paired (in the Tardigrada the nerve cord ganglia are unpaired) [62,63] (figure 6). In the Spiralia (e.g. Gnathifera, Trochozoa) the main neural tracts found in the ventro-lateral body region are paired, although in several annelids a median nerve is also present [64–68] (see also Hejnol and Lowe [69]) and renders the reconstruction of a paired versus unpaired nerve cord ambiguous. Principally, the distribution of an unpaired ventral nerve cord within Scalidophora and Nematoida favours a reduction event at the base of the Ecdysozoa and thus the secondary separation of the major ventral nerve into two main ventral tracts in loriciferans and panarthropods (figure 6). However, in the nematode *Pontonema vulgare* [61] less prominent, paired, ventro-lateral nerves are present in addition to the ventral nerve cord and could hint to the presence of a median and two lateral cords as the ancestral condition which in the course of evolution got elaborated and/or reduced in the different lineages. In this regard, the comparative study of the mediolateral patterning system [7,70] between those lineages with unpaired ventral nerve cords and those with paired ventral nerve cords might shed light into the homology of the nerve tracts and help to reconstruct possible developmental events responsible for the evolution of this trait.

An important and highly debated issue is the nature of the brain in the last common ancestor of the Ecdysozoa [3–7,10,11]. Priapulids, kinorhynchs, loriciferans, nematomorphs and nematodes have a circumoral brain composed of a ring neuropil with anterior and posterior somata, which contrasts with the circumoral commissures found in other ecdysozoans [52] (figure 6). This trait was used to unite all these lineages into the Cycloneuralia [14,71], although most recent molecular phylogenies recover this grouping as paraphyletic [15–17]. On the other hand, the Panarthropoda shows more or less developed anterior neural concentrations [43,44,50,63,72–74] (figure 6), but the homology of these structures between tardigrades, onychophorans and arthropods is still debated [43,44,50,72]. Within Ecdysozoa, it is in arthropods that the brain attains the highest level of sophistication [63,75], probably related to the increase in the number of cephalic segments and the development of more specialized head structures. In this lineage, the brain is considered to be composed of three main neuromeres (protocerebrum, deutocerebrum and tritocerebrum), and thus has been referred to as a tripartite brain [76,77]. Frustratingly, similar terminology has been used to describe the circumoral brain of some priapulids [38], based on the presence of three histological layers (anterior somata, central neuropil, posterior somata). This situation is not observed in the larva and adult of *P. caudatus* (figure 3), where histological methods only reveal the anterior somata and the neuropil, and thus a bipartite brain (see also discussion above). However, the use of these terms to describe the priapulid brain can be misleading, as the ‘tripartite’ anatomical organization should refer to the segmental nature of the brain and should include the linearity of ‘segmentation’ genes that are required for such



**Figure 6.** The diversity of central nervous systems in the Ecdysozoa. Distribution of major anatomical characteristics of the CNS in the different ecdysozoan lineages, compared with the closest relative outgroup (the Spiralia). Ecdysozoan phylogeny according to Pisani *et al.* [17]. Altogether, the distribution of the plotted characters favours considering an unpaired ventral nerve cord as the ancestral architecture of the ecdysozoan nervous system, although the nature of the brain is still unclear. The asterisk in the Kinorhyncha indicates that the ventral nerve cord is unpaired in the trunk and bifurcates anteriorly and posteriorly, as shown in the schematic on the right. The asterisk in the Tardigrada indicates that the ventral cord ganglia are unpaired, but the neurite connections between them are paired, as shown in the schematic on the right. The dashed line uniting the Cycloneuralia indicates that there are contrasting data supporting this grouping (see main text for references). Drawings are not to scale.

segments to develop. Only if orthologous genes were to be expressed in relation to each of the histological layers of the priapulid brain would there be grounds for applying the same terminology to priapulids. Regardless of the terminology used, the distribution of brain architectures in the different lineages of the Ecdysozoa and outgroup taxa suggests two alternative scenarios for the evolution of this neuroanatomical component (figure 6). On the one hand, the distribution of a circumoral brain among the Ecdysozoa might indicate that this was the most probable brain architecture in the last common ecdysozoan ancestor. On the other hand, the presence of brain ganglia in the Panarthropoda and in taxa outside the Ecdysozoa might indicate that the circumoral brain evolved secondarily and independently in members of the Scalidophora and Nematoida. Further embryological, molecular and physiological data are thus required to fully understand the neuroanatomy of the brain of priapulids, kinorhynchs, loriciferans, nematodes and nematomorphs, and ultimately attain a more accurate picture of the course of nervous system evolution in the Ecdysozoa.

## 5. Conclusion

In this study, we characterize the earliest post-embryonic stages of nervous system development in the priapulid worm

*P. caudatus*. The immunoreactivity patterns of five different antibodies commonly used in neuroanatomical analyses demonstrate that priapulid embryos hatch with a nervous system composed of a circumoral brain and an apparently unpaired ventral nerve ending in a caudal ganglion. Additionally, thin neurite bundles innervate the sensory organs of the larva, namely the buccal scalids and the trunk tubuli. The first moulting event in the life cycle of *P. caudatus* implies a significant maturation of the nervous system, which acquires features already seen in adult priapulids, namely the presence of a neck ganglion, a well-developed introvert plexus, and more conspicuous secondary longitudinal nerve tracts. Our results are in agreement with previous morphological observations in adult stages of *P. caudatus* and other priapulid worms [33,38], and deliver the adequate neuroanatomical framework for future embryological studies on *P. caudatus*. In the light of our current knowledge of the ecdysozoan phylogenetic relationships, our results support considering that the ancestral nervous system of the Ecdysozoa might have comprised an unpaired ventral nerve cord, but the architecture of the brain is still unclear. Therefore, further work will be necessary to better understand the exact evolutionary and anatomical relationships between *a priori* simpler brains, such as those found in priapulid worms, and those more elaborated central nervous systems observed in arthropods.



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