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„Neurogenesis of the invasive zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia)"

verfasst von / submitted by

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Abstract

Bivalvia is a taxon of aquatic invertebrates that includes clams, oysters, mussels and scallops. Within the subgroup of heterodont bivalves, *Dreissena polymorpha* is a small, mytiliform shaped, freshwater mussel that develops indirectly via a planktotrophic veliger larva. Currently only few studies concerning neurogenesis in bivalves are available, impeding the reconstruction of a ground pattern in Bivalvia. In order to gain new insights and contribute to this problem, this study focuses on the neurogenesis of *D. polymorpha*. Neuronal development of serotonin- and tubulin- like immunoreactive (lir) neuronal components from the early trochophore to the late veliger stage were examined using immunocytochemical methods and confocal laser scanning microscopy. Neurogenesis starts in the early trochophore stage at the apical pole with the appearance of one flask-shaped serotonin-lir cell. When larvae reach the veliger stage, four flask-shaped serotonin-lir cells are present in the apical organ and at the same time, the *anlagen* of the cerebral ganglia start to form at the base of the apical organ. From the apical organ a pair of visceral neurites project posteriorly to connect to the posterior larval sensory organ. Moreover, paired serotonin-lir neurites originate from the apical organ and project into the velum. Additionally, one unpaired serotonin-lir cell develops ventrally to the stomach at the veliger stage. This study reveals similarities of neuronal components of *D. polymorpha* with the basal branching bivalve *Acila castrensis*. Moreover, the comparatively few number of serotonin-lir cells in the apical organ is shared with gastropod and scaphopod larvae and is therefore considered to be a common feature for Conchifera. Evolutionary implications of the apical organ within Mollusca are discussed.

Zusammenfassung

Das Taxon der Mollusken umfasst acht rezente monophyletische Gruppen (Neomeniomorpha, Chaetodermamorpha, Polyplacophora, Monoplacophora, Bivalvia, Scaphopoda, Gastropoda and Cephalopoda), die eine bemerkenswerte Variabilität der Morphologie und ökologischen Anpassung aufweisen. Diese Tiergruppe ist sehr alt und Fossilien sind bereits aus dem Kambrium bekannt. Innerhalb der Mollusken bilden die Bivalven die zweitgrößte Gruppe. Bivalven sind sowohl im Salzwasser als auch in Brack- und Süßwasser verbreitet. Sie besitzen einen bilateral-symmetrischen Körperbau, der lateral komprimiert ist und durch eine dorsal aufklappbare Schale umschlossen wird. Im Vergleich zu anderen Molluskengroßgruppen besitzen Bivalven keinen Kopf und keine Radula. Die meisten Arten sind getrenntgeschlechtlich und pflanzen sich mittels externer Befruchtung fort. Hermaphroditismus und Brutpflegeverhalten sind ebenfalls von einigen Bivalven bekannt. Die Teilgruppe der heterodonten Bivalven entwickeln sich über die molluskentypische Trochophora-Larve zur Veliger-Larve, welche durch ihr markantes Velum gekennzeichnet ist. Das Nervensystem der Mollusken ist zentralisiert und besteht aus drei Paar Hauptganglien (cerebropleural, visceral, pedal), welche durch Konnektive und Kommisuren miteinander verbunden sind. Vergleichende Studien der Neurogenese von verschiedenen Mollusken haben gezeigt, dass das Nervensystem einen informativer Merkmalskomplex darstellt, welcher evolutionäre Schlussfolgerungen ermöglicht. Derzeit ist nur eine begrenzte Anzahl an vergleichbaren Daten von Bivalven vorhanden und zusätzliche Daten ermöglichen eine detailiertere Rekonstruktion der Nervensystemevolution innerhalb der Bivalvia. In dieser Studie wurde die Neurogenese des serotonin- und tubulinpositiven Nervensystems der im Süßwasser lebenden Zebramuschel *Dreissena polymorpha* mittels Immunozytochemie und Konfokalmikroskopie analysiert. Es wurden Larvenstadien von 22 Stunden bis 7 Tage und 20 Stunden nach der Befruchtung untersucht. Die serotoninpositive Neurogenese beginnt am apikalen Pol mit einer

flaschenförmigen Zelle. Wenn die Larve im Veliger-Stadium ist, bilden vier flaschenförmigen Zellen das Apikalorgan. Gleichzeitig erscheinen die Anlagen der Cerebralganglien an der Basis des Apikalorgans. Ein Paar viscerale Nerven verbinden das Apikalorgan mit einem abapikal gelegenen larvalen Sinnesorgan, welches in dieser Form und mit diesen Methoden noch nicht beschrieben wurde. Darüber hinaus ist das Velum vom Apikalorgan innerviert und eine einzelne Zelle ist in Magennähe sichtbar. Diese Studie zeigt Ähnlichkeiten der neuronalen Komponenten innerhalb der Bivalven und anderer Mollusken. Phylogenetische Rückschlüsse auf das Nervensystem eines letzten gemeinsamen Vorfahren werden gezogen.

Introduction

Mollusca is one of the most diverse taxa of animals and its members are already known from the Cambrian (541 - 485 mya). The remarkable plasticity of bodyplans among molluscan clades renders this taxon particularly well-suited for developmental and evolutionary studies among the metazoans (Wanninger et al., 2008). The phylum Mollusca comprises eight monophyletic taxa: Neomeniomorpha, Chaetodermamorpha, Polyplacophora, Monoplacophora, Bivalvia, Scaphopoda, Gastropoda and Cephalopoda (Ponder and Lindberg, 2008). Within Mollusca, Bivalvia is the second largest class-level taxon and belongs to the taxon Conchifera (Monoplacophora, Bivalvia, Scaphopoda, Gastropoda and Cephalopoda), which constitutes the sister taxon to the Aculifera (Neomeniomorpha, Chaetodermamorpha and Polyplacophora) (Kocot et al., 2011; Smith et al., 2011). The majority of bivalves are marine animals, but several linages colonized freshwater environments (e.g. Unionida (Palaeoheterodonta), Corbulidae, Dreissenidae and Sphaeriidae (Heterodonta)), as well as brackish environments (Pteriomorpha, Heterodonta). All bivalves are bilaterally symmetric and a dorsally hinged bivalved shell encloses their laterally compressed body, which is surrounded by the mantle cavity. Bivalves are lacking a buccal mass, radula, salivary and esophageal glands, otherwise common to other molluscan classes (Giribet, 2008). The adult nervous system exhibits three main pairs of ganglia (cerebropleural, pedal and visceral) and two pairs of nerve cords (ventral and lateral) (Bullock and Horridge, 1965). The anterior cerebropleural ganglia are connected to the pedal ganglia via paired ventral nerve cords. Additionally, the cerebropleural ganglia are linked to the visceral ganglia by a pair of lateral nerve cords (Bullock and Horridge, 1965). Most of the known bivalves are dioecious and release their gametes into the water column, where external fertilization takes place (Zardus and Morse, 1998; Altnöder and Haszprunar, 2008). Apart from this, brooding of the larvae in the mantle cavity is also known in some species such as *Lasaea adansonii* and *Lyrodus pedicellatus* (Altnöder and Haszprunar, 2008; Wurzinger-Mayer et al.,

2014; Gianordoli et al, in prep). Most bivalves develop through a trochophore larva followed by a veliger and pediveliger larval stage (Cragg, 1996; Giribet, 2008). The pericalymma larva of the protobranch bivalve *Acila castrensis* provides an exception to this common developmental type (Cragg, 1996; Zardus and Morse, 1998), as well as the parasitic glochidium larva of Unionidae, which attaches to gills or fins of and even encysts in fish (Giribet, 2008).

In case of the zebra mussel *Dreissena polymorpha* (Heterodonta), which is a small, mytiliform shaped freshwater bivalve, external fertilization takes place (Ram et al., 1996; Taylor et al., 2007; González et al., 2015). During development *D. polymorpha* passes from the trochophore via the veliger into the pediveliger larval stage. The latter one is able to colonize all kinds of hard substrates outcompeting other benthic invertebrates, due to their low demands to their settlement substrate (Giribet, 2008).

Since the 19th century scientists were interested in bivalve development and organogenesis, but studies were limited to light microscopical investigations (Hatschek, 1880; Meisenheimer, 1899, 1901). However, within the last decades, an outstanding progress has been made through the application of modern techniques such as fluorescent stainings and confocal microscopy, which enable to a much more detailed description of myo- and neuroanatomical components. Studies concerning the larval neuronal development of gastropods (Croll and Voronezhskaya, 1996; Dickinson et al., 2000; Dickinson and Croll, 2003; Croll, 2006; Wollesen et al., 2007; Page and Kempf, 2009; Kristof and Klussmann- Kolb 2010), bivalves (Voronezhskaya et al., 2008; Kreiling et al., 2001; Gianordoli et al, in prep), polyplacophorans (Friedrich et al., 2002; Haszprunar et al., 2002; Voronezhskaya et al., 2002), aplacophorans (Redl et al., 2014) and scaphopods (Wanninger and Haszprunar, 2003) are available so far. In the trochophore stage, the first three serotonin-like immunoreactive (lir) neurons of the larval nervous system appear. Usually, in bivalve and gastropod larvae a single, medial, serotonin-lir flask-shaped cell appears first, which is subsequently flanked by two more flask-shaped cells (Croll et al., 1997; Kempf

et al., 1997; Marois and Carew, 1997a, b; Voronezhskaya et al., 2008). In some caenogastropods, an additional pair of lateral non-sensory serotonin-lir cells is described (Page and Parries, 2000). In scaphopods, the apical organ comprises four serotonin-lir flask-shaped cells (Wanninger and Haszprunar, 2003), while eight to ten cells are found in the apical organ of polyplacophorans (Friedrich et al., 2002; Voronezhskaya et al., 2002) and aplacophorans (Redl et al., 2014). Other neuronal markers such as α-tubulin (Dickinson and Croll, 2003; Kempf and Page, 2005), leu-enkephalin (Dickinson and Croll, 2003), catecholamines (Croll, 2006) and neuropeptides (FMRFamide and small cardioactive-like peptide) (Voronezhskaya et al., 2008; Ellis and Kempf, 2011) have been identified in molluscan apical organs as well. Later during larval development, the adult nervous system starts to develop (Croll, 2009), meaning that in mid-larval life both, the early larval and the developing adult nervous system, co-exist. During metamorphosis larval structures, like the apical organ and velar lobe are lost (Marois and Carew, 1997a; Dickinson and Croll, 2003).

Hitherto available studies concerning neuroanatomy show a simple situation of about four flask-shaped apical cells in Conchifera (bivalves, gastropods, and scaphopods) whereas in members of the Aculifera (polyplacophorans and aplacophorans), a more complex situation of 8-10 flask-shaped apical cells is present. The comparatively simple condition of few cells comprising the apical organ is commonly found in many other spiralian protostomes (Hay-Schmidt 2000, Marlow et al. 2014) and thus is likely to represent the ancestral condition within Bilateria. However, the situation in Mollusca is not as simple as assumed. Given that a complex apical organ is present in aculiferans (polyplacophorans and aplacophorans) as well as non-molluscan entoprocts (supposedly sister taxon of Mollusca), it is possible that the last common ancestor of Mollusca featured a complex apical organ rather than a simple one (Wanninger, 2009). In that case, the simple apical organ in conchiferans has to be interpreted as secondary simplifications, which occurred in several lineages independently (Wanninger, 2009).

In order to gain new insights into the larval neuroanatomy of bivalves, this study provides a detailed description of the larval nervous system in the euheterodont bivalve *D. polymorpha*, using immunocytochemistry and confocal laser scanning microscopy. By comparison to other bivalves and mollusks, these results will reveal morphological similarities and differences and consequently enable to elucidate the ground pattern of neurogenesis in Bivalvia.

Materials and Methods

Animals

Sexually mature specimens of *Dreissena polymorpha* were collected in a side branch of the Danube River in Greifenstein-Altenberg, Austria (N 48°20'47.23", O 16°13'06.30"). Collection took place from April to July 2013 in one-week intervals. Adult specimens larger than 1 cm were gathered from stones or other hard substrates in >30cm depth, and were subsequently stored in a closeable plastic box and transported into the laboratory. There, the shells were cleaned and the specimens were stored separately in small open plastic tanks containing 100 ml filtered Danube water (FDW) and kept at 19°C. The water was taken at the collection site and filtered with filter paper twice before addition to the specimens.

Rearing

Spawning was induced by thermal stimulation. Thereby, the water temperature was decreased to 13°C and then elevated to 20°C using a waterbath. Spawning occurred often in the evening hours after 2-3h of warming. Artificial insemination was carried out six times between May and August 2013. Gametes were spawned freely into the water column. Following spawning, the adult mussels were removed from the waterbath and transferred in a second tank with fresh FDW. Released gametes were mixed immediately *in vitro* and observed under the light microscope to ascertain the time of fertilization. After fertilization had occurred, the embryos were cultivated in small glass tanks with a total volume of 400ml in FDW at 19°C. During the next days the water temperature and quality were controlled frequently. Water changes took place every day or every second day by gently decanting the specimens into clean culture containers with fresh FDW. Larvae were not fed. Embryos and larvae of different developmental stages (gastrula, early trochophore, trochophore, early veliger, D-shaped veliger, mid veliger, late veliger) were fixed (Table 1). All larvae were relaxed before fixation

by adding 7.4% MgCl$_2$ to the FDW. After relaxation, larvae were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) for 1 hour at room temperature, subsequently washed in 0.1M PB and stored in 0.1M PB with 0.1% NaN$_3$ at 4°C.

Immunocytochemistry

Stages with shells (early veliger, D-shaped veliger, mid veliger, late veliger) were decalcified in 0.5M EGTA (pH 7.3) for 30 minutes, rinsed once with PB and unspecific binding sites were blocked overnight using 0.1M PB (pH 7.3) with 0.5% Triton X-100 (TX) and 6% normal goat serum (NGS; Invitrogen; Molecular Probes, Eugene, OR, USA) (blockPBT) at 4°C. All larvae were double-labeled by incubating a mixture of mouse and rabbit primary antibodies (pAB) overnight. The following pABs were used: anti-acetylated α-tubulin diluted 1:500 (raised in mouse; Sigma; St. Louis; MO, USA), anti-5-HT diluted 1:1000 (raised in rabbit, Immunostar; Hudson, WI, USA) in blockPBT. Stainings with antibodies against FMRFamide and small cardioactive peptide remained without results and were not included in this study. Larvae were washed for a minimum of 3 x 30 minutes in 0.1M PB and 0.5% Triton X-100 (PBT) at room temperature (RT). Subsequently, the mixture of secondary antibodies (sAB) was added in a dilution of 1:200 in blockPBT to the specimens and incubated overnight in goat anti rabbit Alexa Flour 633 (Invitrogen) or 568 (Invitrogen) and goat anti mouse Alexa Flour 488 (Invitrogen). For the detection of cell nuclei, 1 μl HOECHST (Sigma-Aldrich; St. Louis; MO, USA) was added to the sAB solution. Additionally HCS CellMask (Invitrogen) stainings were carried out in early trochophore to visualize the cellular membrane. Once again the larvae were washed for a minimum of 3 x 30 minutes in PBT at room temperature. The stained specimen were mounted on Poly–L–Lysine- (Sigma-Aldrich; St. Louis; MO, USA) coated glass slides in Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and stored at 4°C for a few days prior to analysis. Attempted stainings with antibodies against FMRFamide remained without

results. By omitting the pAB, the sAB or both, negative controls were made and yielded no fluorescent signal. Specimens were examined with a Leica SP5 II confocal laser-scanning microscope (Leica Microsystem, Wetzlar, Germany). Maximum projection images were generated and exported as TIFFs for further adjustments with LASAF (Leica Microsystems) and IMARIS 7.3.0 (Bitplane, South Windsor, CT, USA). Adobe Photoshop CS5 (Adobe, San Jose, CA, USA), Adobe Illustrator CS5 and Adobe InDesign CS6 (Adobe) were used to assemble figure plates and generate the schemes.

Scanning electron microscopy

Specimens were prefixed in 4% PFA. After rinsing the samples in 0.1M PB and distilled water they were postfixed in 1% OsO_4 for 30 minutes and washed again three times for 15 minutes each. Specimens were dehydrated in an ascending series of acetone washes (30%, 50%, 70%, 80%, 90%, 96%, 100%) for 10 minutes each and critical point dried using CO_2 as intermediate in a Leica EM CPD300 dryer. They were coated with gold in a sputter coater Agar B7340 (Agar Scientific, Wetzlar, Germany) and observed with a Phillips XL20 scanning electron microscope.

Results

Development

Development was observed under a constant temperature of 19° C. Between 14 and 18 hours post fertilization (hpf) the larvae have developed into late gastrula stage, which is characterized by the presence of two independent depressions, the blastopore and the shell field invagination, and the prototroch (Fig. 1A) (Aranda-Burgos et al., 2014; Gadomski et al., 2015). After about 20 hours (Table 1), an elongated early trochophore develops. The larva measures 80-90 μm along the anterior-posterior axis. It has an equatorial band of ciliated cells, the prototroch, and an apical tuft (Fig 1B). The telotroch, a patch of ciliated cells at the posterior larval pole, appears slightly later, at about 24 hpf in the trochophore stage (Fig. 1C, Table 1). At this stage, the invagination of the shell field (Fig. 1E and F) and the blastopore (Fig. 1 D) are visible (Fig. 1E and F). Between 39-50 hours (Table 1) the larva develops into an early veliger with a digestive system. The prototroch expands and forms the velum. The shell has not yet developed completely (Fig. 1G and 1H). Around the second day of development the shell enlarges rapidly and soon envelops the entire larva, giving it a D-shape appearance. These larvae (90-100 μm long) are referred to as D-stage veliger (Table 1). By 3-4 days post fertilization the larva is in the mid-veliger stage (Table 1) and loses its prominent apical tuft. After 5-6 days the larva reaches the late veliger stage (Table 1). The D-shape begins to change and an umbo forms at the dorsal side (Fig. 1I). Compared to the D-stage veliger, the late veliger larvae have not increased in size, still measuring 90-100 μm. During the late veliger stage, which is commonly called veliconcha, a continuous shrinking of the velum in size appears. The eighth day marked the day of settlement and the onset of metamorphosis in the recent study.

Neurogenesis of the serotonin- and α-tubulin-lir nervous system

The first serotonin-lir flask-shaped cell appears in the trochophore stage (20-24 hours post fertilization) at the anterior pole of the larvae (Fig. 2A, 6A). The cell has a flask-shaped morphology and bears apical cilia (Fig. 2A asterisk). It is the first cell of the future apical organ. Shortly thereafter, at the early veliger stage (1 day and 15 hpf), a second serotonin-lir flask-shaped cell appears anteriorly adjacent to the first one. At the same time, the first neurites projecting posteriorly from the apical cells are visible and constitute the *anlagen* of the future visceral nerve cords (Fig. 2B; 6B). In addition, one additional serotonin-lir flask-shaped cell with short immunopositive neurites projecting anteriorly emerges postero-ventrally (Fig. 2B; 6B). This cell shows only weak serotonin-lir and comprises the first-formed cell of the posterior larval sensory organ.

Slightly later in the early veliger stage (2 days 2 hpf) at least two more serotonin-lir flask-shaped cells appear one after another in the anterior region and together form the larval apical organ (Fig. 2C; 6C, 6D). The cluster of cells of the apical organ seems to become more compact at that time. Simultaneously, with the formation of the larval apical organ, the neurites projecting posteriorly from the apical organ and anteriorly from the posterior larval sensory organ are interconnected and constitute the paired visceral neurites (Fig. 2D; 6C, 6D). No further changes in position of the paired visceral neurites were observed throughout subsequent larval development.

By 2-3 days post fertilization (i.e., in the D-shaped veliger), the cluster of the four flask-shaped apical cells again becomes more compact and appears to have subsided into the larval body. Below the apical organ, two roundish non-flask-shaped serotonin-lir cells appear. Due to the tight clustering of these cells it is not possible to elucidate if these cells are uni- or bipolar. At this time the first serotonin-lir neurites that project from the apical organ dorsally into the velum are formed (Fig. 2E; 6E).

Slightly later in the mid-veliger stage (3 days 23 hpf) one single serotonin-lir stomatogastric nerve cell appears ventral of the stomach of the larva (Fig. 2F; 6F). About that time the number of flask-shaped cells in the posterior larval sensory organ increases to three (Fig. 3A; 6F). All three cells are immunopositive against α-tubulin but only the first-formed cell, from which the visceral neurites originate, is also immunoreactive to serotonin (Figs. 2B; 3A, B, D and E; 6F). The cells of the posterior larval sensory organ send α-tubulin-lir neurites in an anterior direction but the termination of these neurites could not unambiguously be followed and were only found in this stage (Fig. 3D and 3E; 6F). Slightly later in the mid-veliger (4 days 18hpf), the flask-shaped cells of the apical organ start to shrink in size and lose their typical flask-shaped form (Fig. 3A-C, F and G). Only one serotonin-lir flask-shaped cell can be identified within the apical organ (Fig. 4A; 6H). The number of non-flask-shaped, round serotonin-lir cells increases to five and subsequently in the late veliger stage (6 days 21hpf) to six (Figs. 4; Fig. 5A-D; 6H and I). These cells form a condensed cluster of cells of which all cells seem to be interconnected. The visceral neurites previously connected to the apical organ now seem to originate from this cluster of round serotonin-lir cells, which probably represent the *anlage* of the cerebral ganglion. A short serotonin-lir neurite projects anteriorly from the stomatogastric nerve cell near to the apical organ/*anlage* of the cerebral ganglion (Figs. 4D-F and 5C, arrowheads). At this stage, immunoreactivity in the posterior larval sensory organ decreases significantly in comparison to the previous stage (Fig. 5C, 6I and J).

Discussion

Comparative aspects of bivalve neurogenesis

A recent molecular phylogenomic analysis reveals that Bivalvia comprises five higher subgroups (Protobranchia, Pteriomorpha, Palaeoheterodonta, Archiheterodonta and Euheterodonta) (Gonzàlez et al. 2015). The Euheterodonta form the most derived taxon of bivalves, and its sister taxon is the Archiheterodonta, together named Heterodonta. Pteriomorpha forms a sister group relationship with the Heterodonta. Protobranchia is the basal most branching taxon and the sister group to all other bivalvian subgroups (Gonzàlez et al. 2015).

Comparable immunocytochemical studies dealing with the early neurogenesis are present only for pteriomorphs and euheterodonts and the presence of various immunoreactive components with different neuronal markers during development. In *Mytilus edulis* (Pteriomorpha: Mytilidae) catecholamine-containing cells in larval and postlarval stages of have been described (Croll et al., 1997). In *Mytilus trossulus* (Pteriomorpha: Mytilidae) early development of serotonin-lir and FMRFamid-lir components of the nervous system have been studied (Voronezhskaya et al., 2008). The presence and location of small cardioactive-like peptides are reported in larvae of *Crassostrea virginica* (Pteriomorpha: Ostreidae) (Ellis and Kempf, 2011). In addition, the serotonin-lir nervous system has been documented for *Nodipecten nodosus* (Pteriomorpha: Pectinidae) (Audino et al., 2015). *Spisula solidissima* (Euheterodonta: Mactridae) larvae (Kreiling et el., 2001) show dopamin-lir as well as serotonin-lir neuronal components appearing early in development. Recently, the serotonin-lir and FMRF-lir nervous system in the shipworm *Lyrodus pedicellatus* (Euheterodonta: Teredinidae) was studied in detail (Gianordoli et al., in prep.).

In this study the early neurogenesis of the serotonin-lir nervous system of *Dreissena polymorpha* (Euheterodonta: Dreissenidae) has been investigated in detail, which is why the

following comparison will focus on serotonin-lir neuronal components of other bivalves and mollusks.

Apical organ
In all of the previously mentioned investigated larval bivalves as well as in *D. polymorpha* an apical organ, was found from early developmental stages onwards. *D. polymorpha* (Euheterodonta: Dreissenidae) larvae show an apical organ that consists of up to four serotonin-lir flask-shaped cells. Within the apical organ in *M. trossulus* (Pteriomorpha: Mytilidae) 4-5 serotonin-lir flask-shaped cells are present (Voronezhskaya et al., 2008). The apical organ of *S. solidissima* (Euheterodonta: Mactridae) larvae consists of up to three serotonin-lir flask-shaped cells (Kreiling et al., 2001). Throughout the development from the trochophore to the veliger stage in *L. pedicellatus* (Euheterodonta: Teredinidae) a maximum of three serotonin-lir flask-shaped cells form a part of the apical organ (Gianordoli et al., in prep.). Concluding so far, three to five serotonin-lir flask-shaped cells are found in pteriomorph bivalves as well as in euheterodonts. This condition is likely to be a shared feature of developing bivalve larvae.

There is a noticeable degeneration of the apical organ with the loss of the apical tuft in *D. polymorpha* (Euheterodonta: Dreissenidae). The cells lose their typical flask-shaped pattern and appear to build a more complex cluster with the *anlage* of the cerebral ganglia. They may survive metamorphosis and may be integrated to the cerebral ganglia. Simultaneously, the velum starts to shrink in size and neurites originating from the apical organ that run to the velum and the velum itself start to disintegrate. Such a situation can also be found in *M. trossulus* (Pteriomorpha: Mytilidae), where the serotonin-lir flask-shaped cells of the apical organ also appear to form a more complex cluster with adult components (cerebral commissure) and the apical tuft cannot be longer observed by the late veliger. These cells retain their position in early post-metamorphic stages in *M. trossulus* (Voronezhskaya et al., 2008). To proof the future

fate of these cells, further investigations of later developmental stages of *D. polymorpha* are required.

Cerebral Ganglia
In all previously mentioned investigated bivalve species cerebral or cerebropleural ganglia are developed at the base of the apical organ. In *D. polymorpha,* the apical organ is underlain by six serotonin-lir non-flask-shaped, roundish cells, which are supposed to constitute the *anlage* of the cerebral ganglia. To verify the future faith of these cells, studies of further developmental stages through metamorphosis is needed in this species. In *M. trossulus* the cerebral ganglia is present from the mid-veliger stage and consist of five serotonin-lir cells (Voronezhskaya et al., 2008), while in *M. edulis* it first appears in pediveliger stage (Croll et al., 1997). A cerebropleural ganglion with six serotonin-lir cells is observed in the late-veliger stage on in *L. pedicellatus* (Gianordoli et al., in prep.). In most mentioned species, cerebral or cerebropleural ganglia show positive serotonin-lir from mid-veliger stage on. Concluding so far, six serotonin-lir non-flask-shaped cells seem to be common for euheterodont bivalves, while five serotonin-lir cells are present in pteriomorphs. Additional data from bivalves from the other subgroups are necessary in order to postulate a reliable ground pattern for Bivalvia.

Velum innervating serotonin-lir neurites
There is a dorsal neuronal innervation of the velum in *D. polymorpha* of one serotonin-lir neurite originating from the apical organ projecting to the dorsal part of the velum. No velar plexus or nerve ring could be detected with serotonin-lir. A serotonin-lir innervation of the velum is lacking in *S. solidissima* (Kreiling et al., 2001) as well as in *M. edulis* (Croll et al., 1997). In contrast, in *M. trossulus* short serotonin-lir neurites project dorsally towards the velum (Voronezhskaya et al., 2008). In *C. virginica* there is a similar extension from the apical organ

into the dorsal portion of the velum (Ellis and Kempf, 2011), yet only catecholamine-lir has been described from this neurite so far. Such serotonin-lir projecting neurites are also described to form a velar plexus in *L. pedicellatus* (Gianordoli et al., in prep.). Thus, a dorsal serotonin-lir innervation of the velum is shared by most of the bivalve larvae and seems to be one of the compulsory neuronal components.

Visceral neurites and posterior larval sensory organ
D. polymorpha shows serotonin-lir neurites projecting postero-ventrally. These neurites constitute the paired visceral neurites and terminate in the so-called "posterior larval sensory organ". In *M. trossulus*, similar serotonin-lir ventral projecting fibers emerging from the apical organ are observed, but they cannot be followed to a distinct structure (Voronezhskaya et al., 2008). In *S. solidissima* a serotonin-lir process emerges from the apical organ and extends to the visceral ganglion (Kreiling et el., 2001). Related to the position within the larval body and developmental stage, these serotonin-lir fibers and the process in *S. solidissima* are topographically similar to the visceral neurites in *D. polymorpha*, which lead to the posterior larval sensory organ.

The posterior larval sensory organ in *D. polymorpha* consists of three flask-shaped ciliated cells and this is the first bivalve study reporting immunoreactivity in these cells. The ontogenetic first-formed cell shows co-immunoreactivity to serotonin and α-tubulin, whereas the other two cells exhibit α-tubulin-lir only. Due to the postero-ventral position of these cells within the larval body, it is considered homologous to the so-called "ciliated post-anal organ" described for the lecithotrophic test cell larva of the bivalve *Acila castrensis* (Protobranchia: Nuculidae) revealed by ultrastructural studies (Zardus and Morse, 1998). The post-anal organ of the pericalymma larva of *A. castrensis* is situated between ciliary cells and has a flask-shaped cavity that is covered by microvilli on the entire surface (Zardus and Morse, 1998). The posterior

larval sensory organ of the euheterodont *D. polymorpha* and the post anal organ of the basal branching protobranch *A. castrensis* in fact share common features like the position and flask-shaped cells comprising it. Such an organ has not been described from other bivalves so far. If they are considered to be homologous in *D. polymorpha* and *A. castrensis* and thus part of the ground pattern of Bivalvia, then a secondary loss of this organ in Pteriomorpha, Archiheterodonta and Palaeoheterodonta has to be assumed. Alternatively, a independent evolution of this organ in Protobranchia and Euheterodonta is also possible.

Stomatogastric nerve cell
D. polymorpha shows one serotonin-lir cell in a median position ventrally to the stomach. The cell appears in the D-shaped veliger stage slightly later than the first cells of the cerebral ganglion *anlage*. At this stage, the larva has a fully developed digestive tract. In the mid veliger stage of *D. polymorpha* larvae, neurites expand from the serotonin-lir cell near the stomach in an anterior direction, but they never interconnect with any other neuronal structure. In the late veliger stage the serotonin-lir signal of the cell disappears. According to the location and origin of formation, the cell is likely to serve the digestive tract and is thus called "stomatogastric nerve cell" herein. Unfortunately, no comparable serotonin-lir cell has been described in any other bivalve larva so far. However, comparable cells are described in 31 dpf *M. edulis* (pediveliger stage) as catecholamine-lir abdominal ganglia (Croll et al., 1997) and also in late veliger (18 dpf) of *M. trossulus* a group of catecholamine-lir cells in the region of the stomach are described (Voronezhskaya et al., 2008). In *C. virginica* a small cardioactive-like peptide innervation along the larval esophagus is described, but seems to form a complex network with esophageal neurons (Ellis and Kempf, 2011). Further investigations are needed for a more detailed description and to determine the future fate of this stomatogastric nerve cell after metamorphosis.

Pedal and visceral ganglia

In *D. polymorpha* pedal or visceral ganglia exhibiting serotonin-lir or tubulin-lir could not be detected during this study. All other studies on bivalve larvae except for *S. solidissima* (Kreiling et el., 2001) show immunoreactive pedal ganglia. The pedal ganglia in *M. edulis* and *M. trossulus* show catecholamine-lir cells, but *M. trossulus* shows additional FMRFamide-lir cells (Croll et al., 1997; Voronezhskaya, 2008). After the D-shaped veliger stage of C. virginica few small cardioactive-like peptide cells are present (Ellis and Kempf, 2011). In mid veliger stage of *L. pedicellatus* two serotonin-lir and two FMRFamide-lir cells were found (Gianordoli et al., in prep.). Additionally, serotonin-lir cells are present in the visceral ganglia of *S. solidissima* (Kreiling et el., 2001), while cells in the visceral ganglia in *L. pedicellatus* display FMRFamide-lir (Gianordoli et al., in prep.). There is no immunoreactivity in the visceral ganglia of the larva of *M. eduli*s, while in *M. trossulus* each visceral ganglion exhibits catecholamine-lir and two to three FMRFamide-lir cells. *C. virginica* shows three to four small cardioactive-like peptide immunoreactive cells in the visceral ganglia in newly eyed larvae (Ellis and Kempf, 2011).

In summary, it can be stated that the majority of bivalve mollusks show a serotonin-lir apical organ appearing first in development with three to five flask-shaped cells. Furthermore, a serotonin-lir part of the *anlage* of the cerebral or fused cerebropleural ganglia (*C. virginica* and *L. pedicellatus*) with six non-flask-shaped cells at the base of it is common to euheterodont, while five are present in pteriomorph bivalves. The serotonin-lir dorsal innervation of the velum, as well as visceral neurites appearing early during development are common to bivalve larvae. All these shared features are likely to be also present in the common bivalve ancestor. Additionally, neuronal components in the posterior portion of the larval body, like the posterior larval sensory organ of *D. polymorpha* and the post anal organ of *A. castrensis*, might also be part of the ground pattern of Bivalvia.

Comparative aspects of the serotonin-lir apical organ of mollusks

According to recent phylogenomic analyses, Mollusca is subdivided into two higher taxa Aculifera and Conchifera (Kocot et al., 2011; Smith et al., 2011). Both studies reveal that within the Aculifera, the Polyplacophora form a sister group relationship to the two aplacophoran taxa (Cheatodermomorpha and Neomeniomorpha). However, the phylogenetic relationships of the conchiferan taxa (Bivalvia, Gastropoda, Scaphopoda, Cephalopoda) are less consistent. Following Kocot and colleagues the sister taxon of Bivalvia are the Gastropoda. In contrast, Smith and colleagues postulate a closer relationship of Bivalvia and Scaphopoda (Diasoma-concept).

In putatively basal branching gastropods (Kocot, 2011; Zapata, 2014), such as patellogastropods and vetigastropods, the apical organ is composed of comparably few serotonin-lir cells. In the patellogastropod *Tectura scutum*, the apical organ consists of three flask-shaped, one median and two lateral, serotonin-lir cells, while in the vetigastropod *Haliotis kamtschatkana* the serotonin-lir median flask-shaped cell is lacking (Page, 2002; 2006). The neritimorph *Nerita melanotragus* shows an apical organ with different and unique arrangement of four serotonin-lir non-flask-shaped cells and multiple α-tubulin-lir cells that form so called "sensory cups" (Page and Kempf, 2009). In contrast to all other pelagic gastropod larvae *N. melanotragus* lacks serotonin-lir flask-shaped cells within the apical organ (Page and Kempf, 2009).

More derived gastropods such as opistobranchs, caenogastropods and nudibranchs (Kocot, 2011; Zapata, 2014) show variations of the pattern described for veti- and patellogastropods. In the opisthobranch gastropod *Aplysia californica* the apical organ is constituted of five serotonin-lir cells. Three of them are flask-shaped cells, one medial and two of them laterally positioned, and two are non-flask-shaped round cells (Marois and Carew, 1997; Dickinson et al., 2000; Wollesen et al., 2007). Larvae of the caenogastropods *Euspira lewisii, Lacuna vincta,*

Trichotropis cancellata, *Amphissa versicolor* and *Ilyanassa obsoleta* have serotonin-lir cells within the apical organ and neurites within the velum (Page and Parries, 2000; Dickinson and Croll, 2003). *E. lewisii* and *A. versicolor both* show an identical arrangement and number of five serotonin-lir cells within the apical organ as mentioned above for *A. californica,* while in *L. vincta* the medial flask-shaped cell is not present within the apical organ. In *T. cancellata,* there are three serotonin-lir cells, one medial flask-shaped and two lateral non-flask-shaped cells. In contrast, *A. versicolor* and *Ilyanassa obsoleta* show an additional non-flask-shaped cell on the right side and thus the apical organ is composed of six serotonin-lir cells in total (Page and Parries, 2000; Dickinson and Croll, 2003). Accordingly, in caenogastropod larvae the number of serotonin-lir cells ranges from three to six, but a pair of non-flask-shaped serotonin-lir lateral cells is common to all of them (Page and Parries, 2000). In the nudibranch gastropods *Aeolidiella stephanieae* (Kristof and Klussmann-Kolb, 2010), *Phestilla sibogae* (Croll, 2006) and others (Kempf et al., 1997) five serotonin-lir cells are present within the apical organ as well. Such a pattern consists of a median flask-shaped cell, a pair of lateral flask-shaped cells and two non-flask-shaped, round cells. Concluding so far, in opistobranchs, nudibranchs and caenogastropods an apical organ consisting of a median flask-shaped cell, a pair of lateral flask-shaped cells and two non-flask-shaped, round cells is common. In comparison to the larvae of more derived gastropods, the larvae of more basal branching patello- and vetigastropods show a number of three flask-shaped apical cells, one median and two lateral. Thus, supposedly basal branching gastropods feature few (3) serotonin-lir flask-shaped apical cells, while in higher branching taxa more (5-6) apical cells seem to be common. This leads to the assumption that there is a secondary increase of cells in the aforementioned higher branching clades of gastropods.

In the scaphopod *Antalis entalis* neurogenesis starts with the apical organ, which consists of four serotonin-lir flask-shaped cells. Moreover, an association of the apical organ with the

anlage of the cerebral ganglia is found. The number of four serotonin-lir, flask-shaped cells in the apical organ, as well as the association with the *anlage* of the cerebral ganglia, are features shared with bivalve larvae. Therefore, these features could support a closer relationship of scaphopods and bivalves, as also revealed by molecular phylogenomics (Diasoma-concept) (Smith et al. 2011).

To sum up, a comparable simple apical organ of few (3-5) serotonin-lir cells is found in the different conchiferan taxa (cephalopods feature direct development without a larval stage) and therefore is likely to be part of the ground pattern of Conchifera.

In the aculiferan polyplacophorans (Friedrich et al., 2002; Haszprunar et al., 2002; Voronezhskaya et al., 2002), the first immunoreactive signal also appears in the apical organ, while in solenogastres neurogenesis simultaneously starts from the apical and abapical pole with an apical organ and a posterior neurogenic domain (Redl et al., 2014). In polyplacophorans, the number of cells in the apical organ is significantly higher than in other molluscan larvae and composes eight to ten serotonin-lir flask-shaped cells in *Mopalia muscosa* and *Ischnochiton hakodadensis* (Friedrich et al., 2002; Haszprunar et al., 2002; Voronezhskaya et al., 2002). Regarding to the total number of cells in the apical organ, the situation is similar in the aplacophoran solenogastres W*irenia argentea* and *Gymnomenia pellucida* (Redl et al., 2014). However, in contrast to polyplacophorans only two of them show serotonin-lir (Redl et al., 2014). Although the number of serotonin-lir cells in the apical organ of aculiferans varies considerably, recent morphological as well as molecular phylogenomic analyses (Kocot et al., 2011; Smith et al., 2011; Scherholz et al., 2013) suggest that the aculiferan ancestor was polyplacophoran-like. Accordingly, a comparable complex apical organ of eight to ten serotonin-lir cells may be ancestral for Aculifera.

To conclude, a comparable simple apical organ with few serotonin-lir cells is supposed to be ancestral for Conchifera, whereas a more complex apical organ is likely to have been present

in the last common ancestor of Aculifera. In order to reconstruct the condition of the last common ancestor of Mollusca (LCAM), a comparison to the situation in the supposedly sister group Entoprocta (Wanninger, 2009) is necessary. Given the fact that in Entoprocta as well as Aculifera a comparably complex apical organ is present, a likewise apical organ of eight to ten serotonin-lir cells can be assumed in the LCAM.

It should be noted that in most cases comparable stages are not present and moreover the analyzed neuroactive compound varies in each study. In addition, general neuronal stainings like stainings for α-tubulin are commonly missing. Consequently, further studies including a more complete analysis of neuronal components using several neurotransmitters as well as tubulin stainings are required for a more complete picture. Thus, while commonalities are generally emerging, further work is still needed among the neurogenesis to ascertain structural homologies and phylogenetic affinities.

Acknowledgements

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Figure Legends

Figure 1: Development of *Dreissena polymorpha* from gastrula to early-veliger stage. A, G, H and I are scanning electron micrographs. B and C are confocal microscope Z-projection images. D, E and F are single optical sections of C. Acetylated α-tubulin-lir (green), HCS CellMask (pink) and cell nuclei counter staining (blue). Apical is always up. Lateral views. Scale bars are 15 μm. **A** Late gastrula stage (16 hours post fertilization) with blastopore (bp) on the abapical pole, shell field (sf) formation and developing prototroch (pt). **B** Elongated early-trochophore (22 hpf) with prominent apical tuft (at) and prototroch (pt). **C** Early-trochophore (23 hpf) with apical tuft (at), prototroch (pt) and telotroch (tt). **D** Early-trochophore (23 hpf) with apical tuft (at), prototroch (pt) and telotroch (tt) and blastopore (bp). **E and F** Early-trochophore (23 hpf) with prototroch (pt) and telotroch (tt), foregut (fg) and shell field (sf) invagination. **G** Early-veliger (1 day 15 hpf) with early shell (s) and expanded velum (ve). **H** Early-veliger (1d 22hpf) with shell (s), expanded velum (ve) and apical tuft (at). **I** Late-veliger larva (7 d 20hpf). (s) shell, (ve) velum.

Figure 2: Serotonin-lir neuronal development in *Dreissena polymorpha* from trochophore to early veliger stage. Serotonin-lir (bright-yellow to dark-red), acetylated α-tubulin-lir (green) and cell nuclei counter staining (blue). Images A, B, C, D, E and F are maximum projections of CLSM scans of the whole specimens. Images are processed and serotonin-lir signal visualized with IMARIS software. All images are in lateral view and apical is always up. Scale bars are 15 μm. **A** Full developed trochophore larva (23hpf). First serotonin-lir flask-shaped cell (*) at the apical pole. (at) apical tuft, (pt) prototroch and (tt) telotroch. **B** Early-veliger larva (1 day and 15 hpf). Two flask-shaped serotonin-lir cells (*) forming the apical organ underlying the velum (ve). Postero-ventrally, the posterior larval sensory organ (pso) develops. Faintly labeled paired visceral neurites (vn) interconnect the posterior larval sensory organ (pso) to the

apical organ. (mo) mouth opening, (st) stomach. **C** Early-veliger larva (2 days 2 hpf). Three serotonin-lir cells (*) form the apical organ. Paired visceral neurites (vn) connecting the apical organ with the posterior larval sensory organ (pso). (mo) mouth opening, (st) stomach, (an) anus, (pat) prae-anal tuft. **D** Fully developed D-shaped veliger larva (2 days 14 hpf). Four flask-shaped serotonin-lir cells (*) constitute the apical organ. Paired visceral neurites (vn) interconnect the posterior larval sensory organ (pso) with the apical organ (ao). **E and F** D-shaped veliger larva (2 days and 21 hpf). Underlying the apical organ (*) two roundish non-flask-shaped cells (+) appear. These cells form the anlage of the future cerebral ganglion (cg). Neurites (n) from the apical organ (*) project dorsally into the velum (ve) and anteriorly innervate the apical tuft (at). Only in this stage such a prominent apical tuft (at) is present. Paired visceral neurites (vn) interconnect the posterior larval sensory organ (pso) with the apical organ. One additional unpaired serotonin-lir stomatogastric nerve cell (snc) appears ventrally to the stomach. (mo) mouth opening, (an) anus, (pat) prae-anat tuft.

Figure 3: Components of the serotonin-lir nervous system in the mid-veliger stage (3 days 23hpf) of *Dreissena polymorpha*. Serotonin-lir (bright-yellow to dark-red), acetylated α-tubulin-lir (green) and cell nuclei counter staining (blue). All images are maximum projections of CLSM scans through the whole specimens. Images C, D and E are details of A. Images B and G are color-coded for depth (blue to red). Images are processed and serotonin-lir signal is visualized with the IMARIS software. All images are in lateral view and apical is always up. Scale bars are 15 μm. **A, B and F, G** Four flask-shaped serotonin-lir cells (*) form the apical organ. There is a continuous deformation of the flask-shape pattern visible. Neurites (n) still project dorsally into the velum. The *anlage* of the cerebral ganglion (cg) is located underlying the apical organ. Visceral neurites interconnect the posterior larval sensory organ (pso) with the apical organ (ao). **C** Detailed image of the apical organ (*) and the *anlage* of the cerebral

ganglion (cg) underlying it. **D** and **E** are detailed images of the posterior larval sensory organ (pso) from **A**. **D** Enhanced serotonin-lir signal. Posterior larval sensory organ (pso) with first-formed cell and anteriorly projecting visceral neurites (vn) showing serotonin-lir components. Flask-shaped cell with neurites (n) projecting medial showing α-tubulin-lir components. **E** Enhanced α-tubulin-lir signal. Posterior larval sensory organ (pso) consisting of three flask-shaped cells (*) with expanding neurites (n, vn), which are all α-tubulin-lir. Only the first-formed cell with expanding visceral neurites (vn) shows additionally serotonin- lir. (pat) prae-anal tuft.

Figure 4: Serotonin-lir neuronal development of *Dreissena polymorpha* from mid- to late-veliger stage. Serotonin-lir (bright-yellow to dark-red), acetylated α-tubulin-lir (green) and cell nuclei counter staining (blue). All images are maximum projections of CLSM scans through the whole specimen. Images B and C are details of A. F is a detail of E. Images are processed and serotonin-lir signal is visualized with the IMARIS software. All images are in lateral view and apical is always up. Scale bars are 15 μm. **A** Mid-veliger larva (4d 18hpf). One remaining flask-shaped cell of the larval apical organ (*) subjacent by the *anlage* of the later cerebral ganglion (cg), which consists of five roundish non-flask-shaped cells (+). Paired visceral neurites (vn) project from the *anlage* of the cerebral ganglion (cg) to the posterior larval sensor organ (pso). **B** Detailed image of the remaining flask-shaped cell of the apical organ (*) and the *anlage* of the cerebral ganglion (cg). **C** Detailed image of the posterior larval sensory organ (pso) and the unpaired stomatogastric nerve cell (snc). **D** Mid-veliger larva (4d 18hpf). One remaining flask-shaped cell of the larval apical organ (*) subjacent by the *anlage* of the later cerebral ganglion (cg), which consists of five roundish non-flask-shaped cells (+). Paired visceral neurites (vn) project from the *anlage* of the cerebral ganglion (cg) to the posterior larval sensory organ (pso). Neurites (n) project from the remaining apical cell (*) dorsally into the

velum (ve). A neurite (arrowhead) expands from the stomatogastric nerve cell (snc) in an anterior direction. **E** Late-veliger larva (6 days 21 hpf). Anterior one remaining flask-shaped cell of the apical organ (*). The *anlage* of the cerebral ganglion consists of six non-flask-shaped cells (+). The unpaired stomatogastric nerve cell (snc) exhibits a neurite (arrowhead) projecting to the *anlage* of the cerebral ganglion. Detail shown in **F**. (pat) prae-anal tuft.

Figure 5: Components of the serotonin-lir nervous system in the late veliger stage of *Dreissena polymorpha*. Serotonin-lir (bright-yellow to dark-red), acetylated α-tubulin-lir (green) and cell nuclei counter staining (blue). All images are maximum projections of CLSM scans of the whole specimen. Image B is a Detail of A and D are a detail of C. Images are processed and serotonin-lir signal is visualized with IMARIS software. All images are in lateral view and apical is always up. Scale bars are 15 μm. **A** Late-veliger larva (6d 21hpf). One flask-shaped serotonin-lir cell (*) remains of the apical organ (*) and a neurite (n) projects dorsally into the velum (ve). The *anlage* of the later cerebral ganglion consists of six round non-flask-shaped cells (+). Detailed shown in **B**. Visceral neurites (vn) project paired from the *anlage* of the cerebral ganglion to the disappearing posterior larval sensory organ (pso). **C** Late-veliger larva or Veliconcha (7d 20hpf). Anterior still one remaining flask-shaped cell of the apical organ (*) with neurites (n) projecting dorsally into the reduced velum (ve) and into a posterior direction (arrowhead). Detailed shown in **D.** From the *anlage* of the cerebral ganglion, consisting of six non-flask-shaped cells (+), paired visceral neurites (vn) project to the posterior pole of the larva where the posterior larval sensory organ (pso) starts to disappear.

Figure 6: Schematic drawings of serotonergic neurogenesis of *Dreissena polymorpha* larvae in lateral view. Serotonin-lir cells are red and acetylated α-tubulin-lir ones are green.

Apical is always up. Relative dimensions are not strictly maintained. Abbreviations: (an) anus, (at) apical tuft, (cg) *anlage* of cerebral ganglion, (mo) mouth opening, (n) neurite, (pat) prae-anal tuft, (pso) posterior larval sensory organ, (pt) prototroch, (snc) stomatogastric nerve cell, (st) stomach, (tt) telotroch, (ve) velum (vn) visceral neurites. **A** The first serotonin-lir flask-shaped apical cell appears at the trochophore stage. **B** In early veliger two serotonin-lir flask-shaped cells are present and form the apical organ, as well as the first-formed serotonin-lir flask-shaped cell of the posterior larval sensory organ (pso) and the beginning of ventral neurites (vn). **C** Slightly later there are three flask-shaped cells to form the apical organ and the paired visceral neurites (vn), which interconnect the posterior larval sensory (pso) organ with the apical organ of the larvae. **D** At the D-shaped veliger stage the apical organ consists of four flask-shaped cells. **E** Slightly later two roundish no-sensory cells appear underlying the apical organ and form the *anlage* of the future cerebral ganglion. Neurites (n) project dorsally into the velum. A second flask-shaped cell in the posterior larval sensory organ (pso) appears. This cell shows only α-tubulin-lir, while the first-formed cell shows immunoreactivity to serotonin and α-tubulin. **F** In the mid-veliger a third flask shaped cell in the posterior larval sensory organ (pso) appears. It also shows only α-tubulin-lir. Both of the α-tubulin-lir cells send short neurites in an anterior direction. Additionally the stomatogastric nerve cell is expressed. **G** Slightly later the two α-tubulin-lir cells in the posterior larval sensory organ (pso) disappear and only the first-formed serotonin-lir flask-shaped cell remains. Also in the apical organ two flask-shaped cells are gone. It now comprises two remaining flask-shaped cells and four roundish non-flask-shaped cells, which are the anlage of the future cerebral ganglion. **H** Only one flask-shaped cell is left in the apical organ. Five roundish cells form the *anlage* of the future cerebral ganglion. **I** In the late veliger six roundish non-flask-shaped cells constitute the anlage of the future cerebral ganglion. **J** The posterior larval sensory organ is no longer expressed by serotonin or α-tubulin.

Table Legends

Table 1: Observed neuronal components of *Dreissena polymorpha* larvae. Hours (hpf) and days (dpf) post fertilization, developmental stages and observed neuronal components during developmental stage and correlating figures are mentioned. Abbreviations: (ao) apical organ, (cg) *anlage* of the cerebral ganglia, (vn) visceral neurites, (snc) stomatogastric nerve cell, (pso) posterior larval sensory organ, (n) neurites, (v) velum.
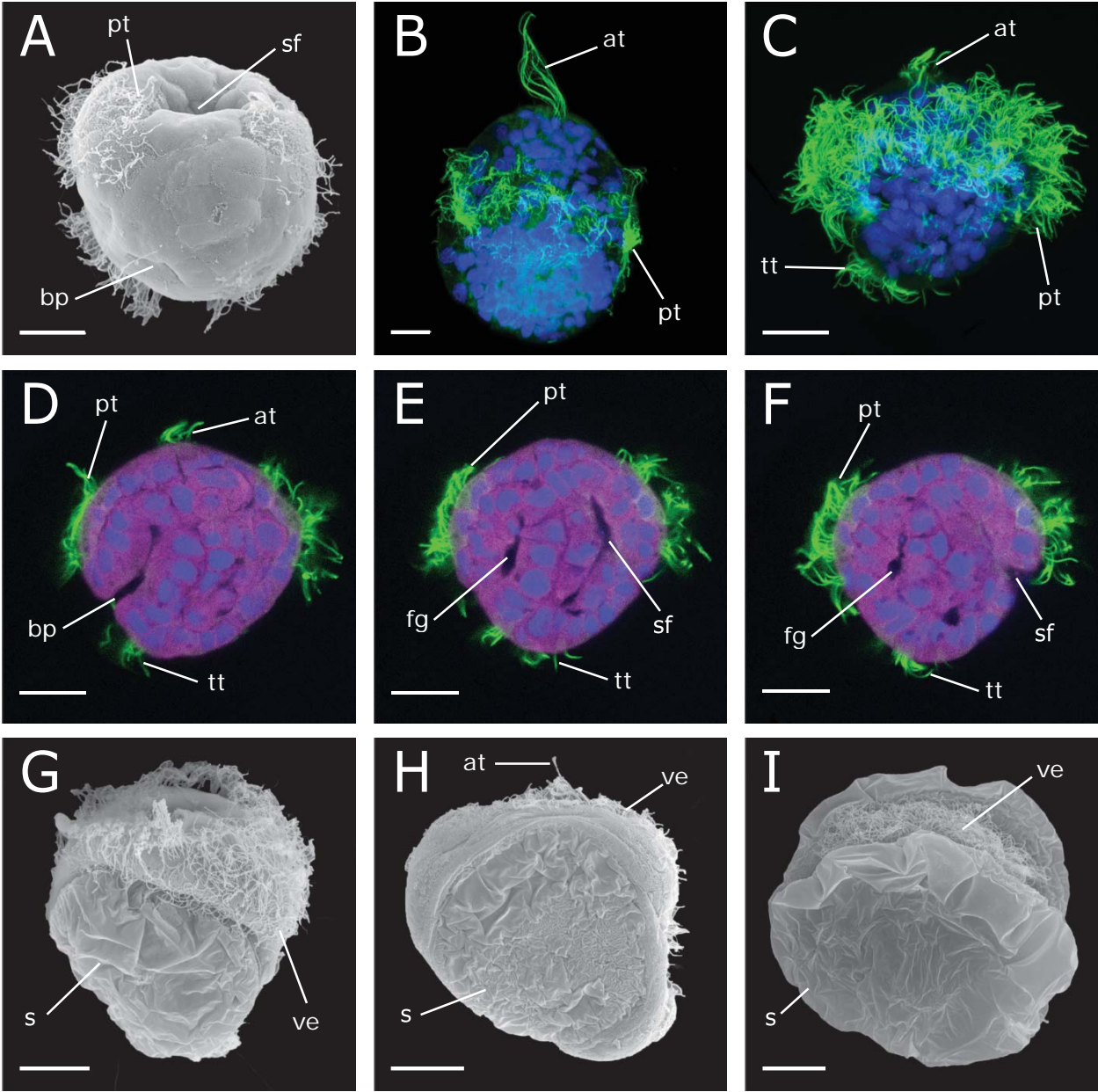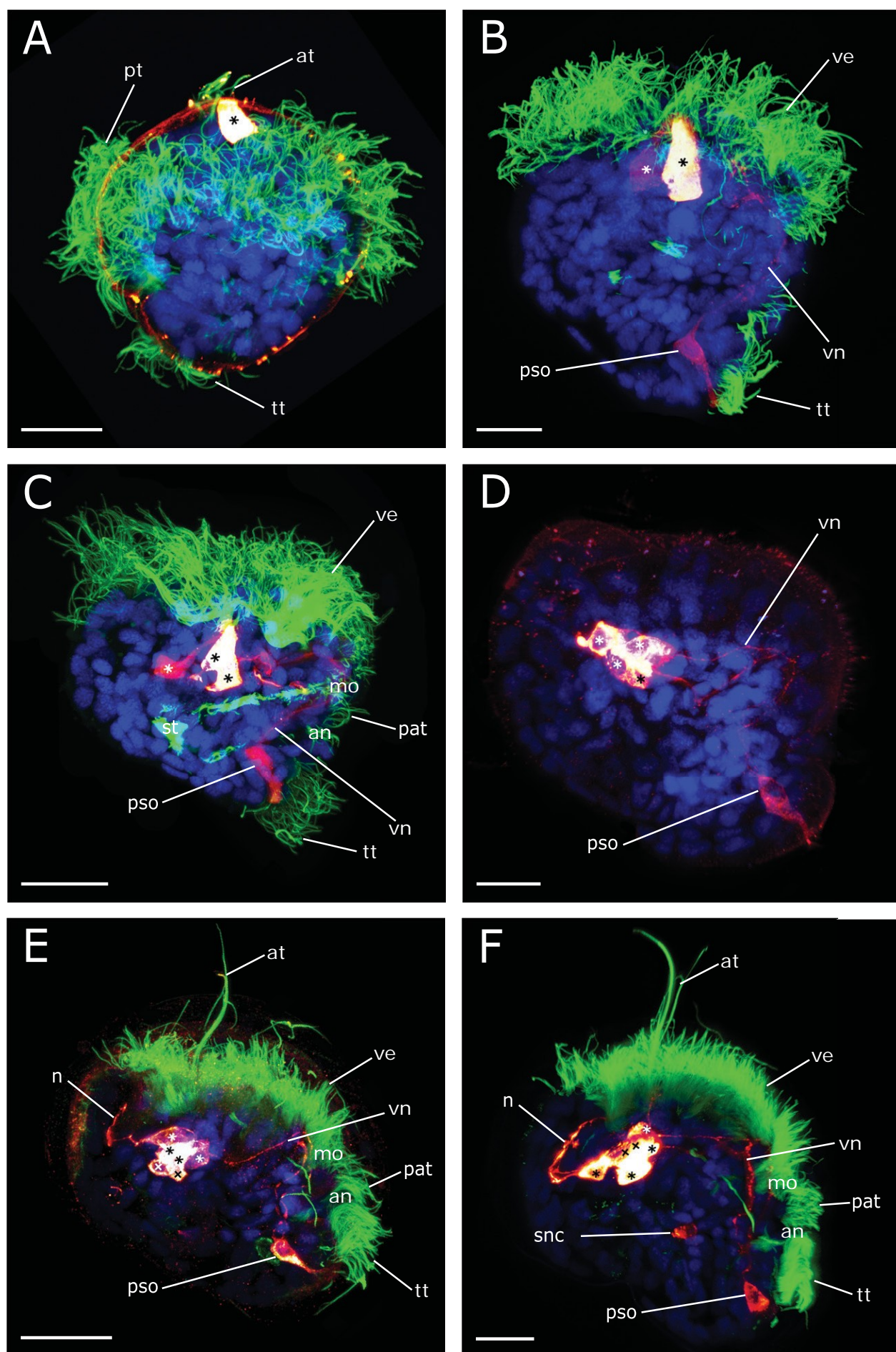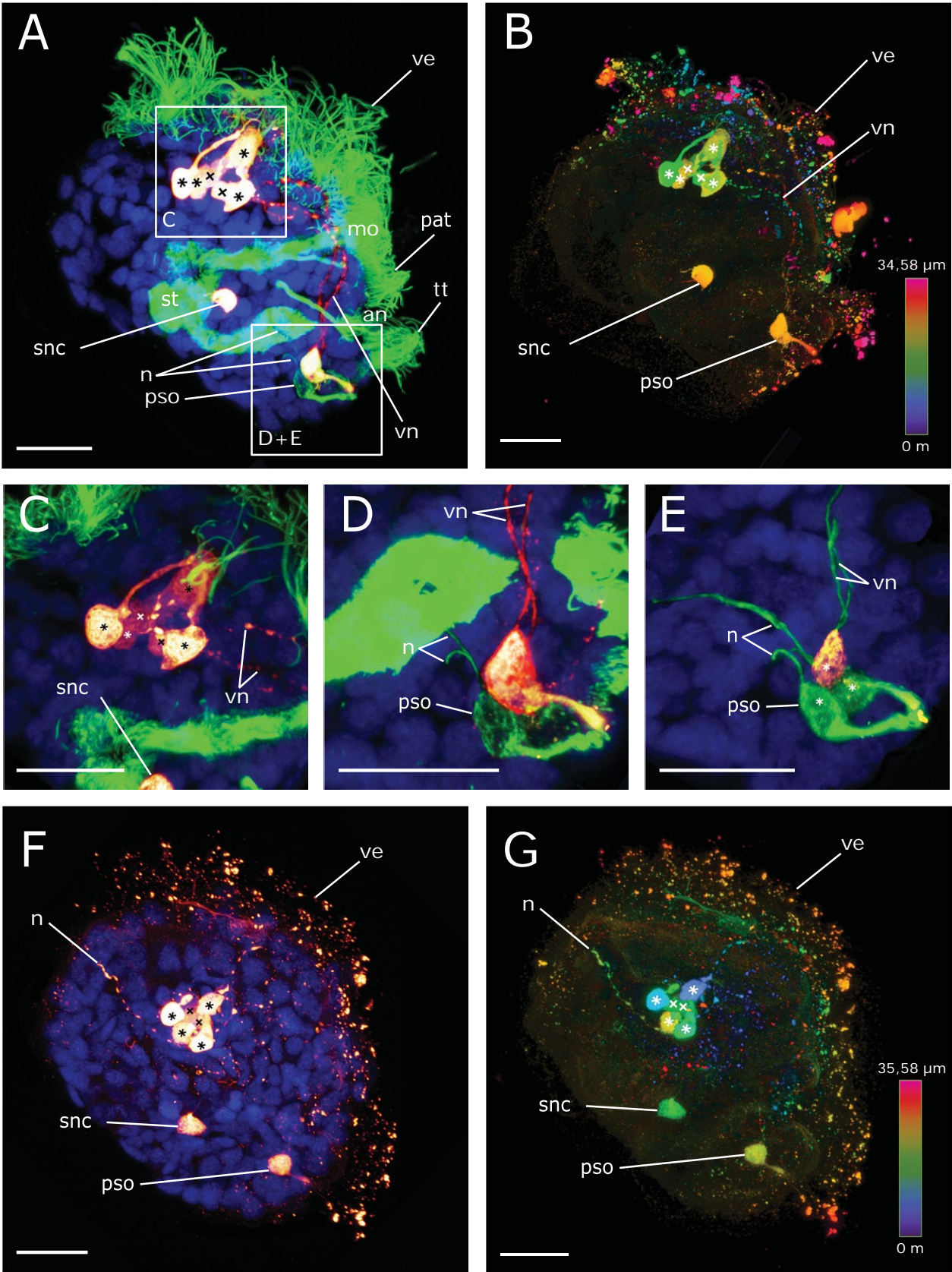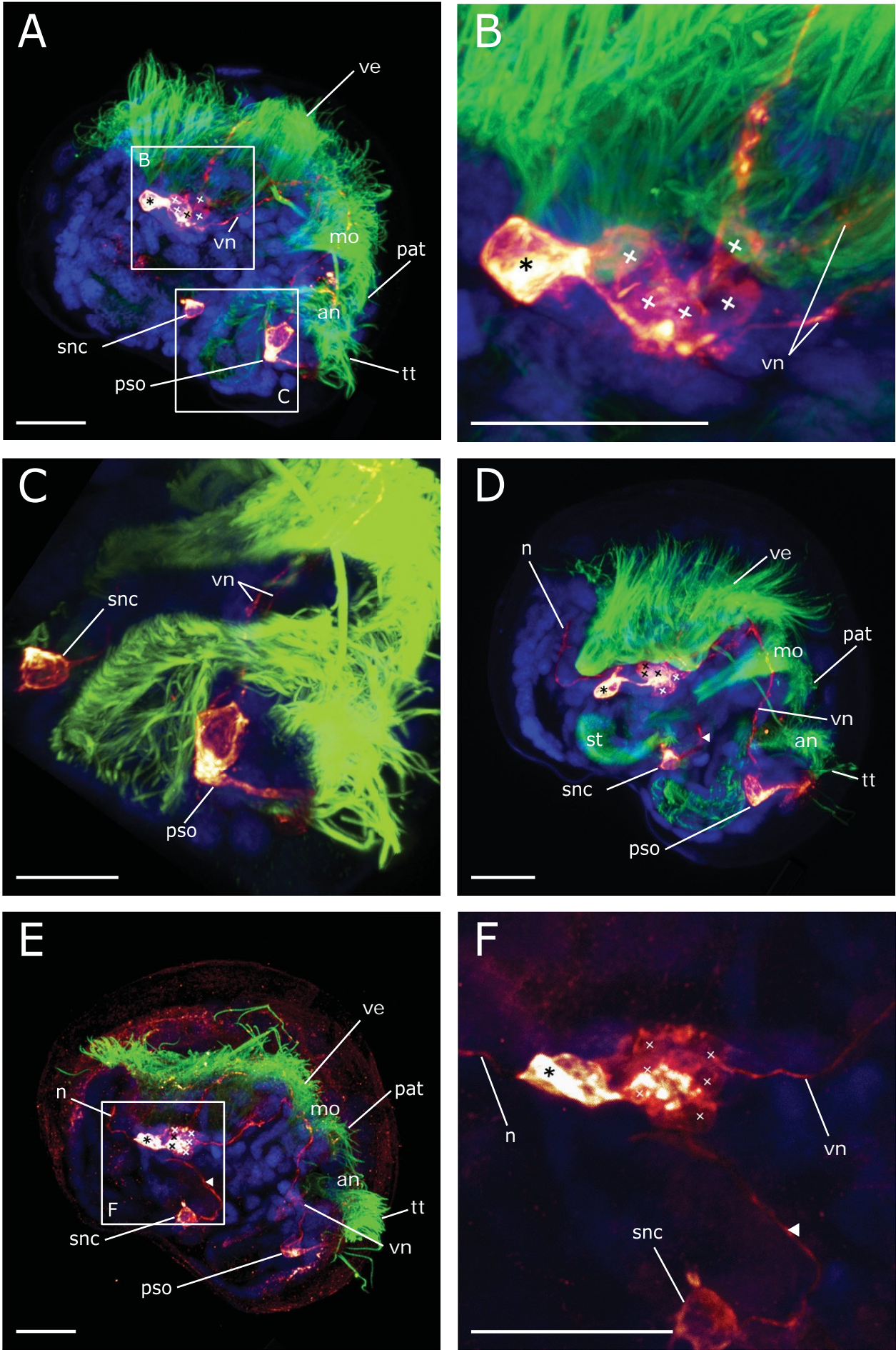
Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5
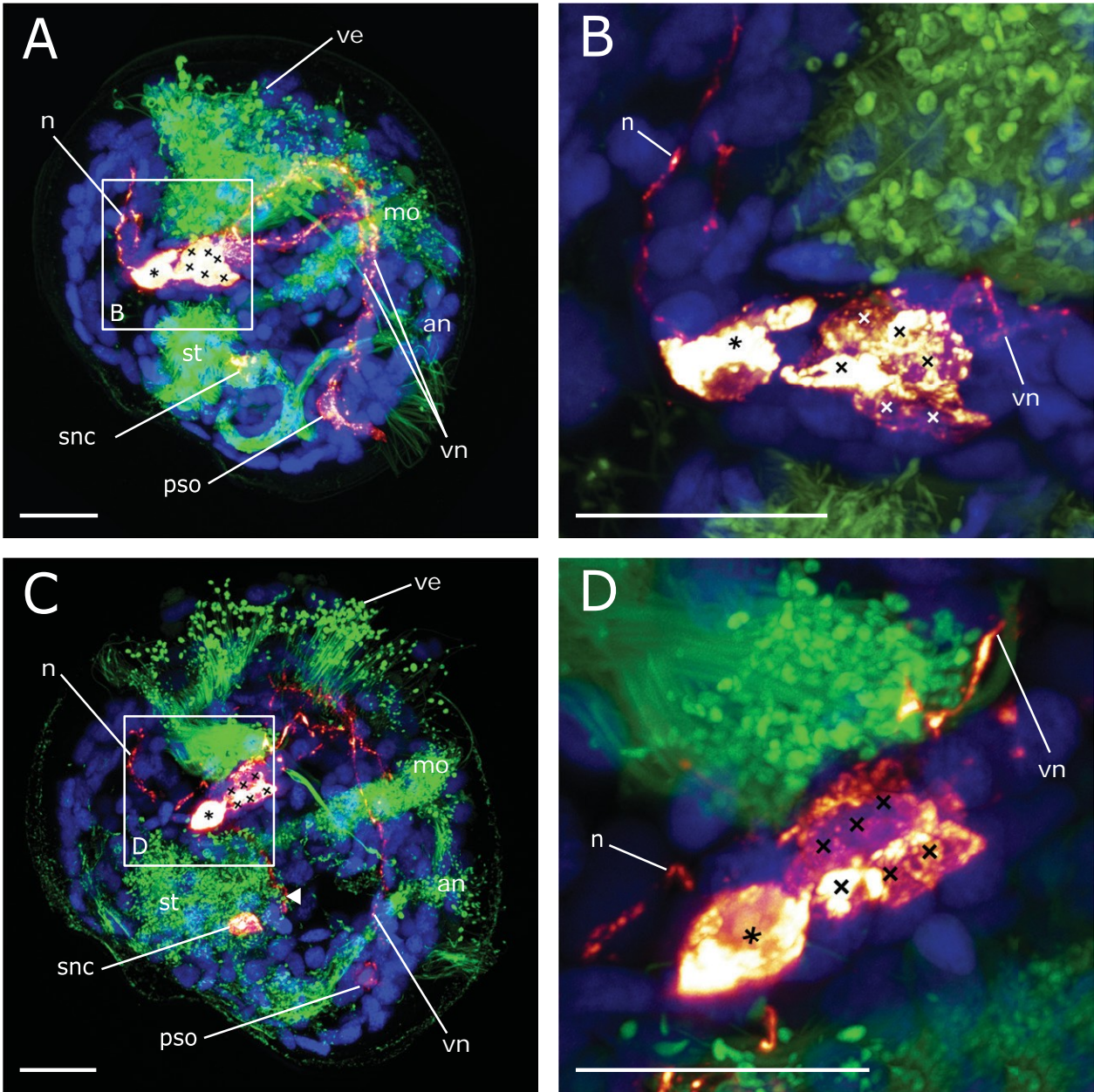
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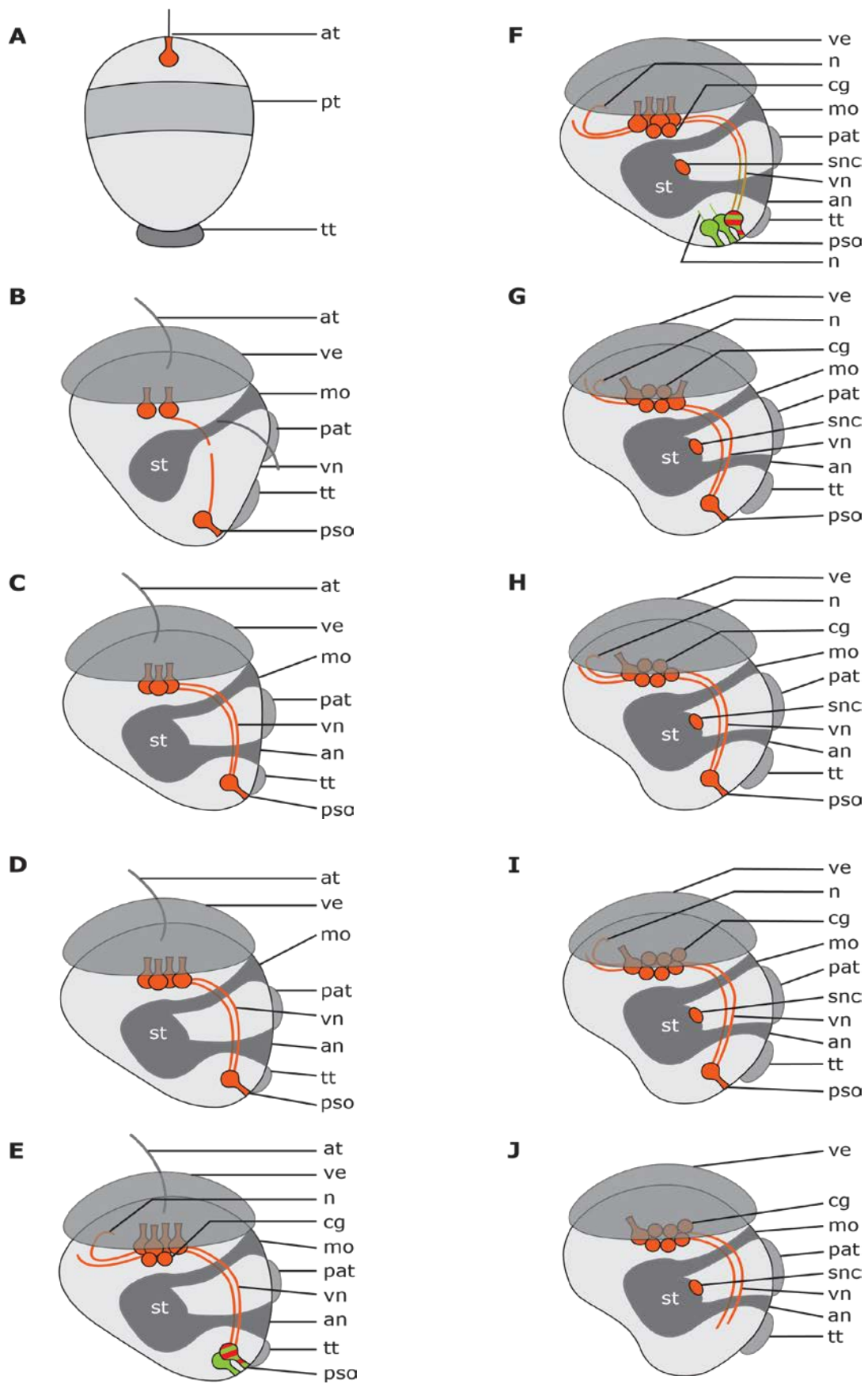
Table

hpf/ dpf	developmental stage	observed neuronal components	Figure
23h	trochophore	ao (1 serotonin-lir flask-shaped apical cell)	1C, 2A
1d 15h	early veliger	ao (2 serotonin-lir flask-shaped apical cells), pso starts to develop (1 flask-shaped cell), vn	1G, 2B
2d 2h	early veliger	ao (3 serotonin-lir flask-shaped apical cells), pso (1 flask-shaped cell), vn	2C
2d 14h	D-shaped veliger	ao (4 serotonin-li flask-shaped apical cells), pso (1 flask-shaped cell), vn	2D
2d 21h	D-shaped veliger	ao (4 serotonin-lir flask-shaped apical cells), cg (2 round non-sensory cells underlying the apical organ), n from ao projecting into v, snc, pso (1 flask-shaped cell), vn	2E, 2F
3d 23h	mid veliger	ao (4 serotonin-lir flask-shaped apical cells), cg (2 round non-sensory cells underlying the apical organ), n from ao projecting into the v, snc, pso (3 flask-shaped cells with expanding neurites), vn	3A-G
4d 18h	mid veliger	ao 1 serotonin-lir flask-shaped apical cells, cg (5 round non-sensory cells underlying the apical organ), n from ao projecting into the v, snc, pso (1 flask-shaped cell), vn	4A-D
7d 20h	late veliger (beginning of metamorphosis and settlement)	ao (1 serotonin-lir flask-shaped apical cells), cg (6 round non-sensory cells underlying the apical organ), n from ao projecting into the v, snc, pso starts to disappear, vn	1I 5C, 5D

Curriculum vitae

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