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„Myogenesis in the heterodont bivalve *Kurtiella bidentata* (Mollusca)“

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Content

Abstract

Myogenesis in *Kurtiella bidentata*, a heterodont brooding bivalve, was investigated by fluorescent-coupled phalloidin staining combined with confocal laser scanning microscopy (CLSM) and 3D reconstruction software. The data were analysed in a comparative framework within Bivalvia and the entire Mollusca. Four different ages were examined: 15, 17, 21 and 24 days post fertilization (dpf). In *K. bidentata* twelve different muscles were found: a velum retractor system (two ventral and two dorsal ones), an anterior and a posterior adductor, two putative mantle retractors, anlagen of the foot retractor, an accessory larval retractor, an accessory velum retractor and, in the latest stages, the musculature of the digestive tract. The accessory velum retractor grows and extends into the velar lobe in the 21 dpf old larvae. All muscles are paired, except for the adductors and the U-shaped anlagen of the foot retractor. Velum retractor muscles of *K. bidentata* are considered homologous to velum retractor muscles in other autobranch bivalves. This leads to the assumption, that these velum retractor muscles originate at the base of autobranch bivalves and hence are part of the bodyplan of the last common ancestor of autobranch bivalves. The velum retractors are present in Bivalvia and Gastropoda, whereas these muscles lack in Scaphopoda, Cephalopoda and Monoplacophora. The origin of velum retractor muscles is discussed in different phylogenetic trees. As part of this work it is being considered, whether there is an autapomorphy in the taxon [Bivalvia + Gastropoda] or if these muscles developed independently in both groups.

Key words: Bivalvia · Heterodonta · *Kurtiella* · CLSM · larval muscle development

Zusammenfassung

Das Phylum Mollusken umfasst acht rezente monophyletische Gruppen, die eine große Variabilität bezüglich ihrer Morphologie, wie auch ihrer ökologischen Anpassungen aufweisen. Bivalvia, die zweitgrößte Gruppe innerhalb der Mollusken, umfassen 15 000 Arten, wobei fossile Funde bereits aus dem frühen Kambrium bekannt sind. Sie besitzen einen bilateralen Körperbau, ihr Körper ist lateral komprimiert und durch zwei Schalen begrenzt. Die für Mollusken charakteristischen Merkmale Kopf und Radula fehlen bei den Muscheln. Bivalven sind sowohl im Salzwasser als auch im Süßwasser verbreitet. Die meisten Arten sind getrenntgeschlechtlich, wobei es meist zu einer externen Befruchtung kommt. Auch Hermaphrodismus kommt bei manchen Vertretern vor. Einige Arten zeigen Brutverhalten, dabei werden die Larven oft im Mantelraum bebrütet und zu einem bestimmten Zeitraum entlassen. Auch die untersuchte Art *Kurtiella bidentata*, eine heterodonte Muschel, zur Familie der Montacutidae gehörend, zeigt dieses Verhalten im natürlichen Lebensraum. Die bis zu 4 mm große Muschel, hat ein Verbreitungsgebiet von Norwegen über Marokko bis ins Mittelmeer. Heterodonte Muscheln sind dadurch gekennzeichnet, dass sich nach der Bildung einer Trochophora-Larve meist eine Veliger-Larve entwickelt, die vor allem durch ihr markant ausgeprägtes Velum zu erkennen ist. Untersucht wurden vier unterschiedliche Stadien von *K. bidentata*, 15, 17, 21 und 24 Tage nach der Befruchtung (dpf). Während des Färbeprozesses bindet ein fluoreszierender Farbstoff an F-Aktin Filamente. Mit Hilfe des Konfokalen Laser-Rastermikroskops (CLSM) können so Muskelstrukturen dargestellt und mit speziellen Rekonstruktionsprogrammen das Muskelsystem am PC rekonstruiert werden. Somit wird eine Untersuchung der unterschiedlichen Entwicklungsschritte, die Muskulatur betreffend, ermöglicht. In *K. bidentata* konnten zwölf unterschiedliche Muskeln identifiziert werden. Ein Retraktorsystem bestehend aus vier paarigen Velumretraktoren (zwei ventrale und zwei dorsale) und einem paarigen akzessorischen Velumretraktor, der erst in älteren Larven bis in die Loben des

Velums zieht. Zusätzlich ist noch ein paariger akzessorischer Larvalretraktor entwickelt. Sowohl ein anteriorer als auch ein posteriorer Adduktor sind vorhanden. Paarige Anlagen von zukünftigen Mantelretraktoren sowie eine u-förmige Anlage des Fußretraktors wurden identifiziert. Große Entwicklungsunterschiede zeigen sich in der Ausdehnung des akzessorischen Velumretraktors sowie in der Bildung eines neuen Muskels um den Darmtrakt, welcher auch erst in den älteren Larven zu finden ist. Dieser ist durch eine Vielzahl von Fasern, die eine netzartige Röhre bilden, charakterisiert.

Da es aktuell wenig publizierte Daten auf dem Gebiet der Muskelentwicklung bei Muscheln gibt, liefert diese Arbeit einen wertvollen Beitrag um mehr über deren Entwicklungsverlauf zu erfahren. Insbesondere, um etwaige Ähnlichkeiten bezüglich der Entwicklung der Muskulatur zwischen unterschiedlichen Arten aufzeigen und diskutieren zu können. Darauf basierend ist es möglich, eine Annahme über einen basalen larvalen Bauplan einer autobranchen Muschel, in Bezug auf Velumretraktoren, aufzustellen. Diese Retraktoren sind in Bivalvia und Gastropoda ausgeprägt, sie fehlen jedoch in Scaphopoda, Cephalopoda und Monoplacophora. Vergleicht man unterschiedliche Stammbäume innerhalb der Conchifera, lassen sich auch Rückschlüsse auf den Ursprung larvaler Muskelstrukturen dieser Gruppe ziehen. Weiters kann diskutiert werden, ob die Velumretraktoren eine Autapomorphie des Taxon [Bivalvia + Gastropoda] ist, oder diese Retraktoren unabhängig voneinander entstanden sind.

Introduction

Molluscs show an amazing diversity regarding their morphology and ecology. Eight recent monophyletic assemblages, Neomeniomorpha, Chaetodermomorpha, Polyplacophora, Monoplacophora, Bivalvia, Scaphopoda, Gastropoda and Cephalopoda, are commonly recognized within this second largest animal phylum (Ponder and Lindberg 2008). Bivalvia are known since the lower Cambrian and are currently represented by 15.000 extant species. They live on substrate or in sediment, wood or corals (Giribet 2008, Salvini-Plawen and Haszprunar 2013). Bivalvia are aquatic animals, primarily widespread in marine habitats, but also found in freshwater. Their body is bilateral, laterally compressed and the two shell valves are usually interconnected by and elastic ligament and a hinge. Main characteristic features of Bivalvia are their fully reduced head and radula (Giribet 2008). The adult musculature exhibits three common systems: the adductor(s), foot and mantle muscles. The adductors, which extend from valve to valve, can appear in a dimyrian organisation so that an anterior and a posterior adductor is developed. They can also be reduced until fully lost. The foot musculature arises at the mantle and projects as dorso-ventral strands into the foot. The mantle musculature consists of retractor muscles, which are responsible for contraction of the outer mantle (Haszprunar and Wanninger 2000, Giribet 2008, Salvini-Plawen and Haszprunar 2013).

Bivalves are usually characterized by indirect development and they are mainly dioecious, but also hermaphroditism, for example in *Lasaea adansonii*, a heterodont mussel, is found. Gametes are predominantly released into the water column, where they are fertilized externally (Zardus and Morse 1998, Altnöder and Haszprunar 2008). However, in some species such as *Lasaea adansonii* and *Lyrodus pedicellatus*, both heterodont bivalves, fertilization and brooding of the larvae takes place in the mantle cavity (Altnöder and Haszprunar 2008, Wurzinger-Mayer 2014). During bivalve development different larval types can be distinguished: The majority of bivalves develop a trochophore-like larva, followed by

a veliger and a pediveliger larva (e.g. in Pectinidae), whereas in protobranch bivalves like *Acila castrensis*, a barrel-shaped pericalymma larva develops (Zardus and Morse 1998, Cragg and Crisp 1991, Cragg 1996). A special type, a parasitic glochidium larva, occurs in Unionidae. This larva undergoes a parasitic stage where it attaches to fins or gill filaments of fish or might even be encapsulated by the tissue of the host (Herbers 1913, Hoggarth 1999). *Kurtiella bidentata* is a protandric hermaphrodite and belongs to the Montacutidae. It is a small-sized heterodont bivalve, with adults possessing a shell of approximately 4 mm in length. Their distribution extends from northern Norway to Morocco and into the Mediterranean Sea (Gofas and Salas 2007). They are mainly found at depths between 5 and 40 m, in fine sand or mud (Ockelmann and Muus 1978). *K. bidentata* lives on the continental shelf in loose association with burrowing ophiurids, for example *Amphiura filiformis* (Boss 1965, Ockelmann and Muus 1978). Montacutids have in common that brooding of the embryos occurs in the pallial cavity. In case of *K. bidentata* embryos are brooded in the superbranchial chamber of the parent animal until the veliger stage (Ockelmann and Muus 1978, Gofas and Salas 2007). The adult musculature of *K. bidentata* shows a typical dimyrian organisation, i.e. one anterior and one posterior adductor muscle. The foot is located ventrally and shows conspicuous pedal glands (Gofas and Salas 2007).

Early studies on bivalve organogenesis were carried out by using light microscopy, which provide an important contribution for the understanding of development (Hatschek 1880, Meisenheimer 1901, Herbers 1913, Erdmann 1935). Unfortunately, there are only few recent data that were obtained by modern technologies, such as fluorescence staining and confocal microscopy (Altnöder and Haszprunar 2008, Dyachuk and Odintsova 2009, Wurzinger-Mayer et al. 2014). These methods enable an even more specific description of the bivalve myogenesis.

The present work describes myogenesis in the autobranch bivalve *Kurtiella bidentata*. By comparing the data presented herein with that of other bivalves, this study contributes to the

discussion of putative ancestral features of the larval body plan of bivalves and the variations that have occurred during the evolution of the various lineages along the bivalve tree of life.

Material and Methods

Animals

Adult specimens of *Kurtiella bidentata* were collected in the Hauglandsosen bay (Bergen, Norway) in November 2013. The animals were extracted from sediment samples, which were obtained by a hyperbenthic sled from 180 – 220 m water depth. Animals were kept in natural sea water at 8°C in the fridge. Spawning was induced by temperature shocks from 4°C to 25°C. The fact, that an external fertilization occurred, might be caused by the stressful situation due to the heat shocks, so no brooding took place in the superbranchial chamber. Oocytes were inseminated and afterwards rinsed several times in sea water. Zygotes were kept in Petri dishes with sea water and water was changed daily. Veliger larvae were collected and fixed at 15 days post fertilization (dpf), 17 dpf, 21 dpf and 24 dpf, in order to investigate myogenesis and to reconstruct the established larval myoanatomy.

Immunocytochemistry

The larvae were relaxed in 4% $MgCl_2$ and fixed in paraformaldehyde (PFA), pH 7.3, in 0.1 M phosphate buffer (PB) for 30-45 minutes. Subsequently, the animals were rinsed three times in 0.1 M PB and stored in 0.1 M PB + 0.1% NaN_3, pH 7.3, at 4°C. For decalcification, they were incubated in 0.5M EGTA overnight and stored at 4°C. For permeabilization the larvae were rinsed three times for 20 min each in 0.1 M PB (pH 7.3) + 2% Triton X-100 (PBT). To label the F-actin, fluorescence-coupled phalloidin (Alexa Fluor 488, Invitrogen, Molecular Probes, Eugene, OR, USA) in a 1:20 dilution of PBT was used. Afterwards, they were incubated and stored overnight at 4°C. In addition, cell nuclei were stained with Hoechst (Sigma, St. Louis, MO, USA). Then, the larvae were rinsed three times each for 30 min in PBT with two subsequent washes, each 20 min, in 0.1 M PB. To label the cilia and visualize serotonin–like immunoreactive cells, double labeling with antibodies directed against acetylated-α-tubulin and serotonin was carried out. Therefore, after permeabilization, the

specimens were rinsed three times for 30 min each in PBT and then incubated in a cocktail containing PBT, 0.25% bovine serum albumin (Roth, Karlsruhe, Germany) and 10% normal goat serum (NGS, Invitrogen) (blockPBT) at 4°C overnight for blocking of non-specific binding sites. For the primary antibody incubation anti-mouse acetylated α-tubulin (Sigma), with a concentration of 1:900, and anti-rabbit serotonin (Immunostar, Hudson, WI, USA), with a concentration of 1:300, was applied in block-PBT and incubated overnight at 4 °C in the fridge. Afterwards, the larvae were rinsed four times for 30 min each in PBT, before the secondary fluorochrome-coupled antibody anti-mouse Alexa Flour 633 (Invitrogen) and anti-rabbit Alexa Fluor 568 (Invitrogen) at a concentration of 1:1500 was added and the specimens were incubated overnight at 4 °C. Subsequently, the samples were rinsed three times in 0.1 M PB for 30 min each. Afterwards, the larvae were mounted on glass slides in Fluoromount G (Southern Biotech, Birmingham, AL, USA) or Vectashield (Vector Laboratories, Burlingame, CA, USA) and stored at 4 °C. After three to four days they were used for further investigations.

Analysis and image processing

For investigations of myogenesis a Leica TCS SP5 II confocal laser scanning microscope (CLSM) (Leica Microsytems, Wetzlar, Germany) was used. Scans were taken with optical sections of 0.3-0.5 µm thickness and were digitally merged into maximum intensity projections. To spatially visualize confocal stacks, the 3D-reconstruction software Imaris v.7.3 (Bitplane, Zürich, Switzerland) and Imaris 64x v.7.2.3 was used. Photoshop CS 5 (Adobe Systems, San Jose, California, USA) was used for further image processing, adjustment of contrast and brightness and assembly of figure plates. The schematic illustrations were generated with Adobe Illustrator CS 5 (Adobe Systems).

Results

Larvae at four different ages, 15 days post fertilization (dpf), 17 dpf, 21 dpf and 24 dpf were investigated. Their length ranges from 100 µm at 15 dpf to approximately 135 µm at 24 dpf. The orientation in all further described larvae can be described by an anterior-posterior and a dorso-ventral axis. Herein, anterior is defined by the apical tuft and posterior by the ligament. Ventral is characterized by the position of mouth and anus and dorsal by the opposite region. Consequently, the following designations of muscles and their positions are with respect to the larval orientation and axes. All further described muscles exist paired in the larvae, except the adductor, the U-shaped anlagen of the foot retractor and the musculature of the digestive tract.

The youngest stage (15 dpf) has numerous features of typical bivalve veliger larvae. These include e.g. muscles, a ligament and a prominent double-lobed velum with cilia, which extends beyond the shell at the anterior pole (Figs. 1A-C, 2A). Noticeable are also the large cells of the velum with their actin-containing cell borders (Fig. 1B). The larval mouth, immediately posterior to the velum, and the anus are located on the ventral side, which also show cilia (Fig. 1C). Inside the larva a U-shaped digestive tract, lined with cilia, is visible (Fig. 1C). The detail of image 1C shows the apical tuft and three serotonin-like immunoreactive (Fig. 1D). At this age eleven distinct muscles can be identified. The anterior adductor muscle, which interconnects both valves, is located dorsally. In addition, a fine posterior adductor is formed in the ventral region of the larva (Figs. 1A, B, 2A). Two ventrally positioned velum retractor muscles have distinct shell attachment sites. The ventral velum retractor 1 arises in the median region at the valve near the ligament and extends into the velum (Figs. 1A, B, 2A). From this retractor, some muscle fibres split off and project in ventral direction above the anlagen of the foot retractor and the remaining fibres extend into the velum. The ventral velum retractor 2 emerges on the ventral side at the valve and also extends into the velum (Figs. 1A, B, 2A). Some muscle fibres split off and branch in median

direction below the velum. Both ventral velum retractors appear as a single unit at their anterior end (Figs. 1A, B). In addition to the ventral retractor systems, two dorsal velum retractors were identified: the dorsal velum retractor 1 attaches at the dorsal side at the valve, runs along the inner surface of the anterior adductor and extends into the velum. The dorsal velum retractor 2 inserts in the median region of the valve above the ligament and extends into the same velar region as the dorsal velum retractor 1 (Figs. 1A, B, 2A). Again, a few muscle strands split off in median direction below the velum. Similar to the ventral velum retractors, they appear as a single unit, where they project towards the velar region (Figs. 1A, B). The likewise paired putative accessory velum retractors are situated dorsally, behind the anterior adductor, where they are interconnected (Figs. 1A, B, 2A). Ventrally, an accessory larval retractor appears and runs behind the velum retractor 2 towards the ventral side. Fine branches of this retractor do not fuse in the median plane, although they appear to lie very close to each other (Figs. 1A, B, 2A). Adjacent to the accessory larval retractor a U-shaped anlage of the foot retractor is visible. It inserts next to the ventral velum retractor 1 and grows behind the ventral velum retractor 2 in ventral direction. Its U-shape derives from the projection across the median plane (Figs. 1A, B, 2A). In addition, there are two anlagen of putative mantle retractors. The shorter muscle is located near the ventral velum retractors 1 and 2 with fine branches at both ends. The other one appears near the dorsal velum retractor 2. There are three branches in the velar region (Figs. 1A, B, 2A). Some muscle strands of the dorsal and ventral velum retractors 2 project towards the apical organ (Fig. 1D).

Eleven muscles are also present in 17 day old larvae. The ventral and dorsal velum retractors are stronger and more branched (Fig. 3A, B). The two anlagen of the putative mantle retractors are elongated (Fig. 3A, B).

Differences appear in 21 dpf old larvae compared to the former stages with respect to the accessory velum retractor, the two anlagen of putative mantle retractors and a newly formed muscle around the digestive tract. The accessory velum retractor grows and reaches the

velum, where it extends into the lobe (Fig. 4A, B). The two putative mantle retractors are more elongated. Additionally, an entirely new muscle system is formed around the digestive tract, located in the centre of the larva (Fig. 4 A-D). Both ventral velum retractors and the anlagen of the foot retractor exhibit striated muscle fibres (Fig. 4A, B). Furthermore, the anterior adductor spans between the two valves. The U-shape of the anlagen of the foot retractor across the median plane and the branches of the accessory larval retractors are visible (Fig. 4C). The musculature of the digestive tract with a net-like arrangement surrounds the digestive tract (Fig. 4C, D).

Larvae aged 24 days post fertilization show numerous similarities concerning their myoanatomy compared to 21 dpf larvae (Figs. 2B, 5A, B). The anterior adductor consists of six distinct muscle bundles at this stage (Fig. 5C-E). A striation pattern is also visible in the dorsal velum retractor 2, ventral velum retractor 1, 2 and the anlagen of the foot retractor (Fig. 5A, B).

Discussion

The investigated autobranch larvae of *Kurtiella bidentata* show the characteristic organization of bivalve veliger larvae such as a ciliated velum, cells of the apical region and typical bivalve larval muscle patterns i.e. strongly developed retractor muscles.

Comparative analysis of muscle systems in bivalve larvae

Velum retractor muscles

Velum retractor muscles of planktotrophic bivalve larvae exist in varied numbers and are lost after metamorphosis (Table 1) (Haszprunar and Wanninger 2000). The larva of *Kurtiella bidentata* has four pairs of velum retractor muscles, two are located dorsally and two ventrally. Furthermore, there is a paired accessory velum retractor, which is located behind the adductor but extends into the velar lobes in older larvae (Figs. 1-5). The wood-boring teredinid shipworm *Lyrodus pedicellatus* develops an unpaired accessory velum retractor in mid-veliger larvae. This muscle appears in the same area as it does in *K. bidentata*, but it does not project fibres into the velar lobes. During development it disappears in the mid-pediveliger larva (Wurzinger-Mayer et al. 2014). Caused by the fact that this muscle is unpaired and does not project into the velum in *L. pedicellatus*, it is not finally clarified that those accessory velum retractors are considered homologous, despite their same attachment zones.

Lyrodus pedicellatus and *Teredo sp.*, also a boring shipworm (Teredinidae), develop two pairs of velum retractors as well as the heterodont bivalve *Dreissena polymorpha* (Meisenheimer, 1901, Hatschek 1880, Wurzinger-Mayer et al. 2014). In veliger larvae of the pteriomorph scallops *Pecten maximus* and *Nodipecten nodosus*, four pairs of velum retractors were found (Cragg 1985, Audino et al. 2015). Three pairs of velum retractors were described in the larvae of the pteriomorph bivalve *Mytilus trossulus* (Dyachuk and Odintsova 2009). Also three pairs of velum retractors are present in the pteriomorph bivalves *Mytilus edulis* and *Ostrea edulis*,

as well as in the heterodont *Pandora inaequivalvis* (Anomalodesmata) (Erdmann 1935, Allen 1961, Bayne 1971). *Lasaea adansonii*, a brooding heterodont bivalve, exhibits a modified larval development and shows only few of the typical structures of veliger larvae, e.g. three pairs of distinct larval retractors are established, although no velum occurs (Altnöder and Haszprunar 2008). In contrast, velum retractors lack in the palaeoheterodont glochidium larva of *Anodonta cellensis* (Table 1) (Herbers 1913). No velum retractors are described in the protobranch pericalymma larva *Acila castrensis* and *Solemya reidi* (Gustafson and Reid 1988, Zardus and Morse 1998).

In summary, various conditions of velar retractor muscles, from two velum retractors in *Lyrodus pedicellatus*, *Teredo sp*. and *Dreissena polymorpha*, to four velum retractors and one accessory velum retractor in *Kurtiella bidentata*, show the plasticity underlying evolution of this muscle system (Fig. 6, Table 1) (Hatschek 1880, Wurzinger-Mayer et al. 2014, González et al. 2015). Caused by the attachment of the velum retractors near the ligament and the extension into the velum, these further discussed velum retractors of other autobranch bivalves are presumably considered homologous to velum retractors in *K. bidentata*. It can be suggested that at least two velum retractor muscles are part of body plan of the basal autobranch bivalve larva (Fig. 6, Table1) (González et al. 2015). Little doubt remains, caused by the fact, that precise data of larval development in protobranch bivalves lack. Thus, it is not advisable to postulate a body plan of the entire Bivalvia. More data of protobranch bivalve development are necessary.

Anlagen of the foot retractor and accessory larval retractors

In *Kurtiella bidentata* the U-shaped anlage of the foot retractor is located next to the ventral velum retractor and grows in ventral direction (Figs. 1-5). Apparently, there might have been two muscles in earlier larval stages of *K. bidentata*, thus paired, which grew and then fused in the median plane. This is also the fact in *Lyrodus pedicellatus*, where in slightly older veliger

larvae the bundles of the foot retractor fuse in the median plane (Wurzinger-Mayer et al. 2014). They are presumably considered homologous, due to the same position, extension and the fusion. Also, the posterior retractor 2 of *Pecten maximus* can be presumably considered homologous to the anlagen of the foot retractor of *K. bidentata*, caused by the same position. The posterior retractor 2 attaches near the ligament in the median region and projects in ventral direction towards the mouth. However, there is no fusion described in the posterior retractor 2 of *P. maximus* (Cragg 1985). Nevertheless, there are still some doubts about the homology of the anlagen of the foot retractor in *K. bidentata* and prior mentioned muscles of *L. pedicellatus* and *P. maximus*. Caused by the fact that there are not enough data of the subsequent development of these muscles, further investigations of older larvae of *K. bidentata* are necessary. Then it might be revealed, if this muscle grows into the foot, which helps to make more concrete statements regarding homology.

The posterior retractor 1 in *Pecten maximus* attaches next to the posterior retractor 2 near the ligament and extends in ventral direction near the anus (Cragg 1985). Due to its position it is presumably homologous to the accessory larval retractor in *Kurtiella bidentata*. Also the ventral pair of retractors in *Dreissena polymorpha* and the ventral larval retractor in *Lyrodus pedicellatus* might be homologous to the accessory larval retractor in *K. bidentata*, due to their same position in the respective larvae (Meisenheimer 1901, Wurzinger-Mayer et al. 2014). A pair of posterior retractors attaches near the ligament in the median region and projects in ventral direction in *Nodipecten nodosus* (Audino et al. 2015). They are presumably homologous to the accessory larval retractor in *K. bidentata*. However, doubt remains about the homology of previously discussed muscles and the accessory larval retractor of *K. bidentata*. More data of myogenesis in *K. bidentata* are necessary to make a clear statement about the ongoing development of these muscles.

Adductor muscles

Adult specimens of *Kurtiella bidentata* exhibit a dimyarian condition (Gofas and Salas 2007). Accordingly, in adults, both, an anterior and a posterior adductor are present. The anterior adductor is found in all investigated stages of *K. bidentata* (Figs. 1-5). It appears in the larvae of *Lyrodus pedicellatus* and the brooding bivalve *Lasaea adansonii* in early larval stages, whereas in *Mytilus trossulus* it is firstly apparent in the late veliger stage (Altnöder and Haszprunar 2008, Dyachuk and Odintsova 2009, Wurzinger-Mayer et al. 2014). The anterior adductor is divided into distinct parts in *L. pedicellatus*, *Pecten maximus* and *Dreissena polymorpha* (Meisenheimer 1901, Cragg 1985, Cragg and Crisp 1991, Wurzinger-Mayer et al. 2014). This is not the case in *K. bidentata*, where it consists of six distinct muscle bundles (Fig. 5). It could not be determined whether the anterior adductor is composed of distinct subsets of smooth and striated muscles as in *P. maximus* or *Nodipecten nodosus* due to the lack of histological data (Cragg 1985, Cragg and Crisp 1991, Audino et al. 2015).

The posterior adductor of *Kurtiella bidentata* begins to form as fine muscle fibres (Figs. 1-5). In scallop larvae the posterior adductor develops in the late veliger or pediveliger stage (Cragg 1985). In *Lyrodus pedicellatus* the posterior adductor is also formed in the late veliger stage (Wurzinger-Mayer et al. 2014). However, the smooth posterior adductor in *Mytilus trossulus* develops only after metamorphosis (Dyachuk and Odintsova 2009). The larva of *Anodonta cellensis* possesses one adductor, which disintegrates during subsequent development and both adult adductors are newly formed, whereby the posterior adductor arises first (Table 1) (Herbers 1913).

Anlagen of putative mantle retractors

In *Kurtiella bidentata* two paired anlagen of putative mantle retractors were found (Figs. 1-5). They appear as fine muscle strands in the mantle region and elongate during development. In *Lasaea adansonii* and *Nodipecten nodosus* the first small mantle retractors, which extend into

the mantle edge, are described in pediveliger stage (Altnöder and Haszprunar 2008, Audino et al. 2015). The mantle musculature in *Mytilus trossulus* appears only after metamorphosis (Dyachuk and Odintsova 2009).

Striation of muscle strands

Only few data based upon the usage of transmission electron microscope (TEM) are available, which would confirm a striation pattern of muscles. Nevertheless, further discussed data of larval muscle systems, which were obtained with confocal laser scanning microscopy (CLSM), give indications about a distinct striation of muscles. The larval muscle system of *Kurtiella bidentata* consists of smooth and striated muscle pattern in parts of the anlage of the foot retractor, as well as in some parts of the velum retractor (Figs. 3-5). In *Nodipecten nodosus* the velum retractors and the paired posterior retractors appear to contain exclusively striated muscle fibres. The anterior adductor exhibits two regions, one composed of smooth, the other one of striated fibres (Audino et al. 2015). Larval retractors and posterior protractors in *Mytilus trossulus* exhibit a distinct striation pattern, while the anterior adductor consists of smooth muscles (Dyachuk and Odintsova 2009). In *Pecten maximus* scanning electron microscopy indicates that the velum and posterior retractors and a part of the anterior adductor may be striated (Cragg 1985). By contrast, no striated muscles were found in larvae of *Lyrodus pedicellatus* and *Lasaea adansonii* (Altnöder and Haszprunar 2008, Wurzinger-Mayer et al. 2014). So far, the data available indicate that the larval musculature may consist of smooth and striated muscles in bivalves (Cragg 1985, Cragg and Crisp 1991, Altnöder and Haszprunar 2008, Dyachuk and Odintsova 2009, Wurzinger-Mayer et al. 2014, Audino et al. 2015). The striation can be presumably considered as homologous feature, caused by the lack of TEM data no unambiguous statement can be made. Further histological investigations could reveal these assumptions.

Comparison of larval muscle systems across the Mollusca

Larval retractor systems

Within the Conchifera, numerous larval retractor muscles appear in Bivalvia and Gastropoda, whereas these muscles do not occur in the scaphopod *Antalis entalis* (Wanninger and Haszprunar 2002). Due to the lack of data of larval development in Monoplacophora, it is assumed herein that these do not exhibit larval retractor muscles. Cephalopoda have a direct development, consequently no larval muscles occur. In the gastropod *Aplysia californica* three larval muscles were found, namely a larval retractor, an accessory larval retractor and a metapodial retractor muscle (Wollesen et al. 2008). The main larval retractor and accessory retractor muscle are present in the patellogastropods *Patella vulgata* and *Patella caerulea* (Wanninger et al. 1999). The main larval retractor can be considered as a velum retractor, caused by its extension in anterior direction towards the velum ring. It can be suggested, that those retractors of *P. vulgata* and *P. caerulea* can be presumably considered homologous to the velum retractors in *Kurtiella bidentata*. Larval retractor muscles and an accessory larval retractor appear in *Aeolidiella stephanieae* (Nudibranchia) in early veliger stage. Both project into the velum where they reach the velar ring musculature (Kristof and Klussmann-Kolb 2010). The larval muscles in *A. californica, P. vulgata, P. caerulea* and *A. stephanieae* are entirely reduced and the adult musculature develops independently (Wanninger et al. 1999, Wollesen et al. 2008, Kristof and Klussmann-Kolb 2010).

The occurrences of larval retractors in Bivalvia as well as in Gastropoda suggest a homology of these structures between the two groups. However, there are not enough indications to be finally clarified homologous, thus further analysis are necessary. Different origins of larval retractor muscles can be discussed from a phylogenetic point of view using three scenarios and applying the principle of parsimony (Fig. 7 A-C) (Salvini-Plawen and Steiner 1996, Waller 1998, Smith et al. 2011, Olsen 2013). The principle implies, that simple assumptions are more likely to complex ones (Olsen 2013). In the scenario of a sister group relationship of

Bivalvia and Gastropoda, velum retractor muscles originate at the base of these two groups and can be considered homologous. Therefore, these velum retractors are an autapomorphy of the taxon [Bivalvia + Gastropoda] (Fig. 7A) (Salvini-Plawen and Steiner 1996). In another scenario the Cephalopoda and Scaphopoda are suggested as sister groups. The origin of velum retractors would be at the base of Bivalvia, Gastropoda, Cephalopoda and Scaphopoda. The velum retractors are developed independently in Bivalvia and Gastropoda in this case (Fig. 7B) (Waller 1998). Another assumption of a sister group relationship of Monoplacophora and Cephalopoda as well as of Scaphopoda and Gastropoda, larval retractor muscles occur in the last common ancestor of Scaphopoda, Gastropoda and Bivalvia. Either the velum retractors are assumed homologous, implying the Scaphopoda lost the retractor muscles secondarily, or these muscles are developed independently in Bivalvia and Gastropoda (Fig. 7C) (Smith et al. 2011).

Velum ring musculature

A larval velum ring musculature is widespread within the molluscs. In many bivalves this musculature occurs, e.g. in *Lyrodus pedicellatus* and also in *Mytilus trossulus* (Dyachuk and Odintsova 2009, Wurzinger-Mayer et al. 2014). It also appears in the gastropods e.g. *Patella vulgata* and *Patella caerulea* or *Aeolidiella stephanieae* (Wanninger et al. 1999, Kristof and Klussmann-Kolb 2010). In addition, it occurs in Aculifera, e.g. in Polyplacophora, the Chaetodermomorpha *Chaetoderma sp.*, as well as in the Neomeniomorpha *Wirenia argentea* (Wanninger et al. 1999, Wanninger and Haszprunar 2002, Nielsen et al. 2007, Kristof and Klussmann-Kolb 2010, Scherholz et al. 2013). An interesting fact is, that the typical velum ring musculature lacks in *Kurtiella bidentata*. It also lacks in the scaphopod *Antalis entalis* (Wanninger and Haszprunar 2002). That supports, that this musculature is developed at the base of the molluscs and got secondarily lost in *K. bidentata* and the scaphopods.

Conclusion

Myogenesis of *Kurtiella bidentata* shows a mosaic of larval (e.g. velum retractors) as well as adult muscles (e.g. adductors). A distinct musculature of the digestive tract is described for the first time for any bivalve larva. The larval velum retractor muscles of *K. bidentata* can be considered homologous to velum retractor muscles of other autobranch bivalves. This suggests the presence of velum retractor muscles at least at the base of the autobranch bivalves (Fig. 6) (González et al. 2015). The lack of data for protobranchs does not unambiguously settle the issue concerning the existence of such systems in the last common ancestors of the entire Bivalvia. However, the occurrence of retractor muscles in gastropods may indicate a shared ancestry of such retractors for both clades, but a more solid foundation of the interrelationships of these and the other conchiferan taxa are necessary for further discussions into these issues. In any case, the obvious lack of any larval retractor systems in aculiferans strongly suggests their evolution at the base or within Conchifera, probably in conjunction with distinct embryonic shells (protoconchs or prodissoconchs). At the same time, the absence of larval retractors in the protoconch-bearing scaphopods demonstrates that the expression of a protoconch is not necessarily linked to that of larval retractor muscles (Fig. 7A-C) (Salvini-Plawen and Steiner 1996, Waller 1998, Smith et al. 2011).

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Figure Legends

Figure 1: Myoanatomy of *Kurtiella bidentata* at 15 days post fertilization (dpf). Scale bars in A, B, C and D equal 25 µm. All images are shown in lateral view, D is a detail of C. Anterior (a) is upwards, posterior (p) is downwards. Ventral (v) is to the left, dorsal (d) is to the right. All muscles, except the adductors and the U-shaped anlage of the foot retractor, are paired. Only one side of the paired muscle system is shown for clarity. (A) Confocal image of the muscle system containing eleven distinct muscles. Anterior adductor (aa) at the dorsal pole, posterior adductor (pa) in the ventral region. The accessory velum retractor (avr) is located behind the anterior adductor (aa). The dorsal velum retractor 1 (dvr1) grows next to the anterior adductor (aa). The dorsal velum retractor 2 (dvr2) inserts in the central region above the ligament. The ventral velum retractor 1 (vvr1) arises in the central region above the ligament, similar to the dorsal velum retractor 2 (dvr2). The ventral velum retractor 2 (vvr2) attaches to the ventral side of the valve. All velum retractors (dvr1, 2 and vvr1, 2) extend into the velum. The accessory larval retractor (alr) and the U-shaped anlagen of the foot retractor (fr) have formed ventrally. They grow behind the ventral velum retractor 2 (vvr2) in ventral direction. Two putative mantle retractors (mr) are found next to the velum retractors (vvr 1, 2 and dvr 1, 2). (B) Computer-aided reconstruction of dataset shown in (A), represents the eleven distinct muscles. The corresponding colour code displays the described muscles. (C) Represents a ciliated velum (v) in anterior view, a larval mouth (m) and anus (a) with cilia fields on the ventral side. A digestive tract (dig), lined with cilia, appears medially. The ligament (lig) is located in the posterior region of the larva. (D) A detail of C, the apical tuft (at) is situated in the central region of the velum. Three serotonin-like immunoreactive cells (sc) are located next to the dorsal and ventral velum retractors (dvr, vvr), below the velum.

Figure 2: Schematic representation of 15 and 24 days post fertilization (dpf) old larvae, respectively, corresponding to the reconstructed images of Figures 1 and 5. The two schemata

are shown in lateral view. Anterior (a) is upwards, posterior (p) is downwards. Dorsal (d) is to the left, ventral (v) to the right. All muscles, except the adductors, the U-shaped anlage of the foot retractor and the musculature of the digestive tract, are paired. Only one side of the paired muscle system is shown for clarity. The corresponding colour code displays the described muscles. (A) Schemata of the muscle development of a 15 days old larva, with eleven distinct muscles. Four velum retractors appear: dorsal velum retractor 1, dorsal velum retractor 2 and ventral velum retractor 1, ventral velum retractor 2, all of them project into the velum. Two adductor muscles, one anterior and one posterior, are developed. An accessory velum retractor occurs behind the anterior adductor. The accessory larval retractor and the U-shaped anlagen of the foot retractor grow ventrally. Two putative mantle retractors are found next to the velum retractors. (B) Schemata of the muscle development of a 24 days old larva, with twelve distinct muscles. Differences to previous stage appear in the accessory velum retractor, which grows into the velar lobe. The musculature of the digestive tract is formed de novo.

Figure 3: Myoanatomy of a 17 days post fertilization (dpf) old larva. Scale bars in both images equal 25 µm. All images are shown in lateral view. Anterior (a) is upwards, posterior (p) is downwards. Dorsal (d) is to the left, ventral (v) to the right. All muscles, except the adductors and the U-shaped anlage of the foot retractor, are paired. Only one side of the paired muscle system is shown for clarity. (A) Confocal image of the muscle system containing eleven distinct muscles. Anterior adductor (aa) at the dorsal pole, posterior adductor (pa) in the ventral region. The accessory velum retractor (avr) is located behind the anterior adductor (aa). The dorsal velum retractor 1 (dvr1) grows next to the anterior adductor (aa). The dorsal velum retractor 2 (dvr2) inserts in the central region above the ligament. The ventral velum retractor 1 (vvr1) arises in the central region above the ligament, similar to the dorsal velum retractor 2 (dvr2). The ventral velum retractor 2 (vvr2) attaches to the ventral

side of the valve. All velum retractors (dvr1, 2 and vvr1, 2) extend into the velum and are stronger and more branched than in the 15 dpf old larvae. The accessory larval retractor (alr) and the U-shaped anlagen of the foot retractor (fr) have formed ventrally. They grow behind the ventral velum retractor 2 (vvr2) in ventral direction. Two putative mantle retractors (mr) are found next to the velum retractors (vvr1, 2 and dvr1, 2). (B) Computer-aided reconstruction of dataset shown in (A), represents the eleven distinct muscles. The corresponding colour code displays the described muscles.

Figure 4: Myoanatomy of a 21 days post fertilization (dpf) old larva. Scale bars in A and B equal 25 µm, in C and D 10 µm. All images are shown in lateral view, except 4 C, which is in anteroventral view. Anterior (a) is upwards, posterior (p) is downwards. Dorsal (d) is to the left, ventral (v) to the right. All muscles, except the adductors, the U-shaped anlage of the foot retractor and the musculature of the digestive tract, are paired. Only one side of the paired muscle system is shown for clarity. (A) Confocal image of the muscle system containing twelve distinct muscles. Anterior adductor (aa) at the dorsal pole, posterior adductor (pa) in the ventral region. The accessory velum retractor (avr) is located behind the anterior adductor (aa), grows and reaches into the velar lobe. The dorsal velum retractor 1 (dvr1) grows next to the anterior adductor (aa). The dorsal velum retractor 2 (dvr2) inserts in the central region above the ligament. The ventral velum retractor 1 (vvr1) arises in the central region above the ligament, similar to the dorsal velum retractor 2 (dvr2). The ventral velum retractor 2 (vvr2) attaches to the ventral side of the valve. All velum retractors (dvr1, 2 and vvr1, 2) extend into the velum. The accessory larval retractor (alr) and the U-shaped anlagen of the foot retractor (fr) have formed ventrally. They grow behind the ventral velum retractor 2 (vvr2) in ventral direction. The two anlagen of putative mantle retractors (mr) which are located next to the velum retractors (vvr 1, 2 and dvr 1, 2) are elongated. An entirely new musculature of the digestive tract (mdt), with net-like arrangement, is situated in the centre of the larva. (B)

Computer-aided reconstruction of dataset shown in (A), represents the twelve distinct muscles. The corresponding colour code displays the described muscles. (C) Confocal image represented in anteroventral orientation. The net-like musculature of the digestive tract (mdt) surrounds the digestive tract. The anterior adductor (aa), which extends between both valves, is visible. The U-shaped anlage of the foot retractor (fr) is next to the accessory larval retractor (alr). (D) Detail of C, illustrating a lateral magnification of the musculature of the digestive tract (mdt).

Figure 5: Myoanatomy of a 24 days post fertilization (dpf) old larva. Scale bars in all images equal 25 µm. All images are shown in lateral view. Anterior (a) is upwards, posterior (p) is downwards. Dorsal (d) is to the left, ventral (v) to the right. All muscles, except the adductors, the U-shaped anlage of the foot retractor and the musculature of the digestive tract, are paired. Only one side of the paired muscle system is shown for clarity. (A) Confocal image of the muscle system containing twelve distinct muscles. Anterior adductor (aa) at the dorsal pole, posterior adductor (pa) in the ventral region. The accessory velum retractor (avr) is located behind the anterior adductor (aa), reaches into the velar lobe. The dorsal velum retractor 1 (dvr1) grows next to the anterior adductor (aa). The dorsal velum retractor 2 (dvr2) inserts in the central region above the ligament. The ventral velum retractor 1 (vvr1) arises in the central region above the ligament, similar to the dorsal velum retractor 2 (dvr2). The ventral velum retractor 2 (vvr2) attaches to the ventral side of the valve. All velum retractors (dvr1, 2 and vvr1, 2) extend into the velum. The accessory larval retractor (alr) and the U-shaped anlagen of the foot retractor (fr) have formed ventrally. They grow behind the ventral velum retractor 2 (vvr2) in ventral direction. The two anlagen of putative mantle retractors (mr) which are located next to the velum retractors (vvr 1, 2 and dvr 1, 2) are elongated. An entirely new musculature of the digestive tract (mdt), with net-like arrangement, is situated in the centre of the larva. (B) Computer-aided reconstruction of dataset shown in (A), represents

the twelve distinct muscles. The corresponding colour code displays the described muscles. (C), (D), (E) Sagittal sections, showing the musculature of the digestive tract (mdt), the digestive tract (dt) and the larval mouth (m). The anterior adductor (aa), consisting of six distinct muscle bundles, is also visible.

Figure 6: Phylogenetic tree, modified from González et al. (2015), displays the relationships within the Bivalvia. The species *Acila castrensis* and *Solemya reidi* represents protobranch bivalves, within this group velum retractor muscles lack. The autobranch group contains the Pteriomorpha (*Mytilus edulis, Mytilus trossulus, Nodipecten nodosus, Pecten maximus, Ostrea edulis*) where three or four velum retractors appear and the Heteroconchia. The latter contains Palaeoheterodonta (*Anodonta cellensis*) where larval retractors lack and Heterodonta (*Lasaea adansonii, Kurtiella bidentata, Dreissena polymorpha, Lyrodus pedicellatus, Teredo sp.*) where two to four velum retractors were developed. The asterisk marks the potential origin of larval retractor muscles. At least two velum retractor muscles might be part of body plan of the basal autobranch bivalve larva.

Figure 7: Three different phylogenetic scenarios within the Conchifera. (A) Phylogenetic tree modified from Salvini-Plawen and Steiner (1996) displays a sister group relationship between Bivalvia and Gastropoda as well as Cephalopoda and Scaphopoda. The asterisk marks the potential origin of larval retractor muscles at the base of Bivalvia and Gastropoda. The velum retractor muscles represent an autapomorphy of the taxon [Bivalvia + Gastropoda]. (B) Phylogenetic tree modified from Waller (1998), where the Cephalopoda and Scaphopoda are suggested as sister groups. The asterisk marks the potential origin of larval retractor muscles. The velum retractors are developed independently in Bivalvia and Gastropoda. (C) Phylogenetic tree modified from Smith et al. (2011), whereby the Monoplacophora and Cephalopoda as well as the Scaphopoda and Gastropoda are sister groups. The asterisk marks

the origin of larval retractor muscles, either the Scaphopoda lost them secondarily, or these muscles are developed independently in Bivalvia and Gastropoda.

Figures

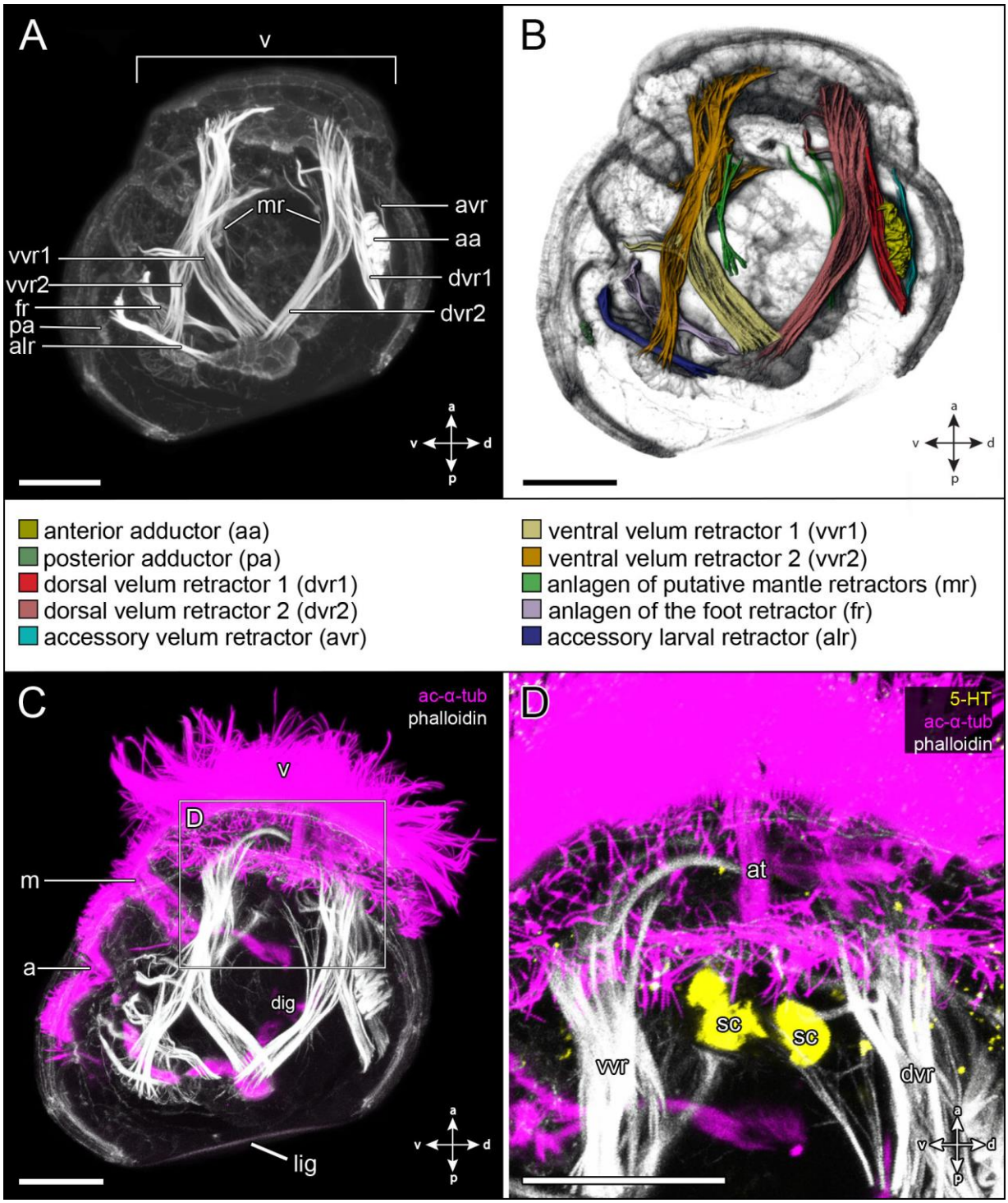
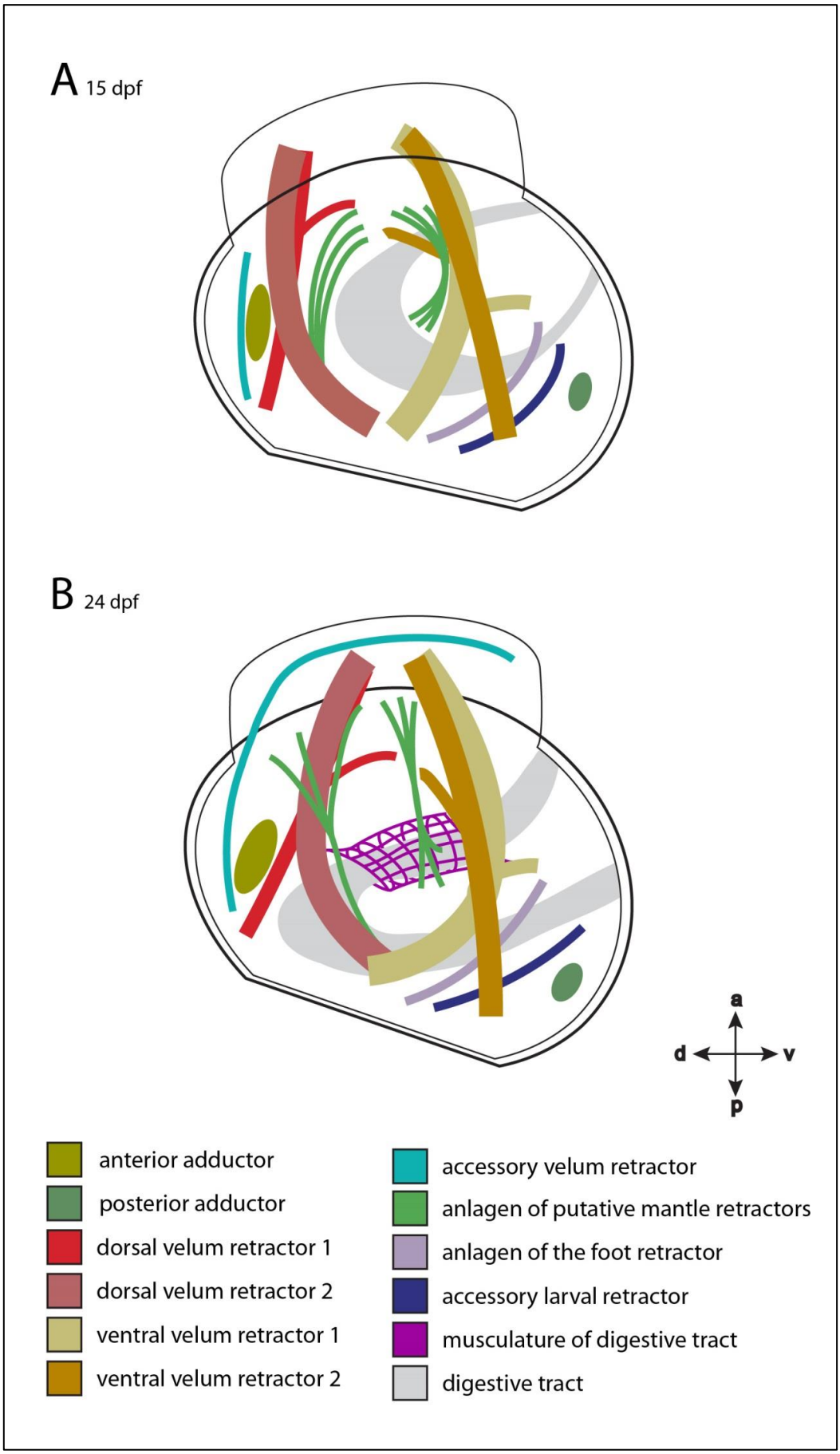


Figure 1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B 24 dpf
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d
v
p
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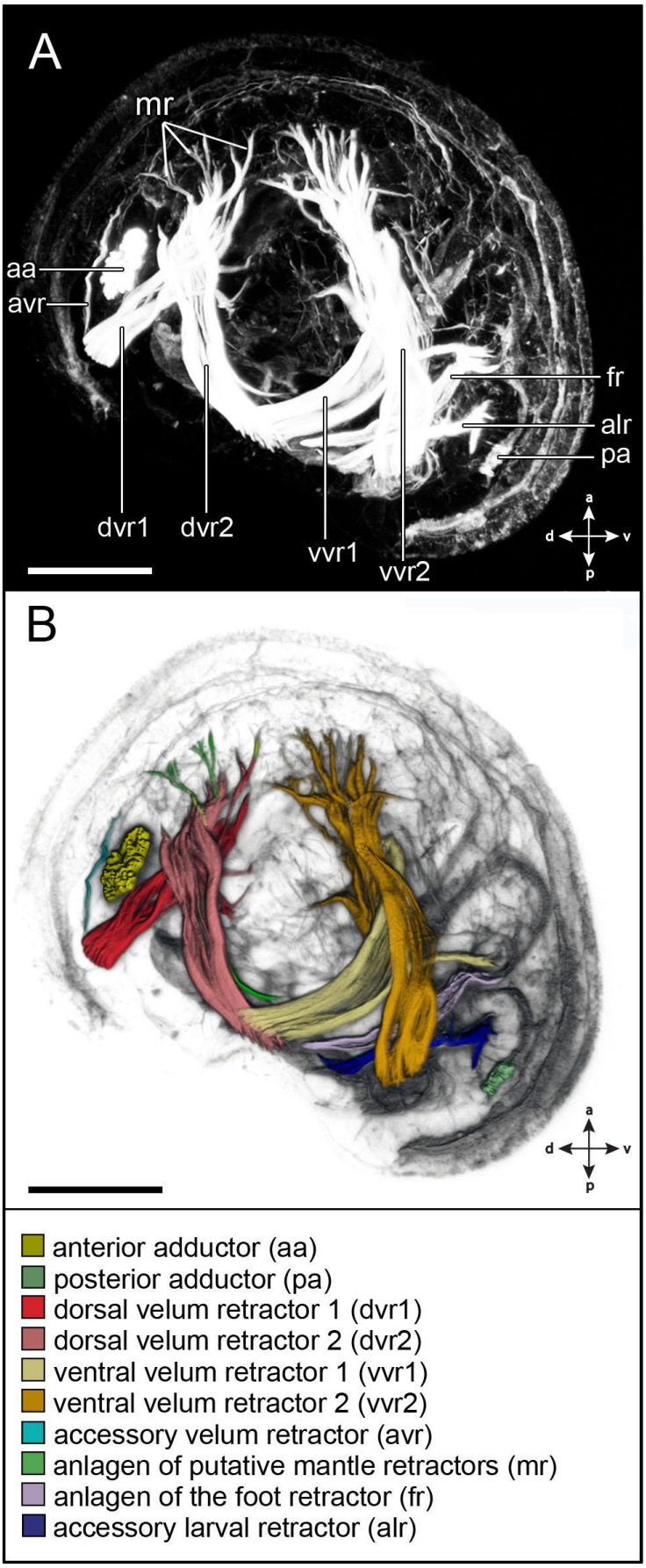
posterior adductor
dorsal velum retractor 1
dorsal velum retractor 2
ventral velum retractor 1
ventral velum retractor 2
accessory velum retractor
anlagen of putative mantle retractors
anlagen of the foot retractor
accessory larval retractor
musculature of digestive tract
digestive tract

Figure 2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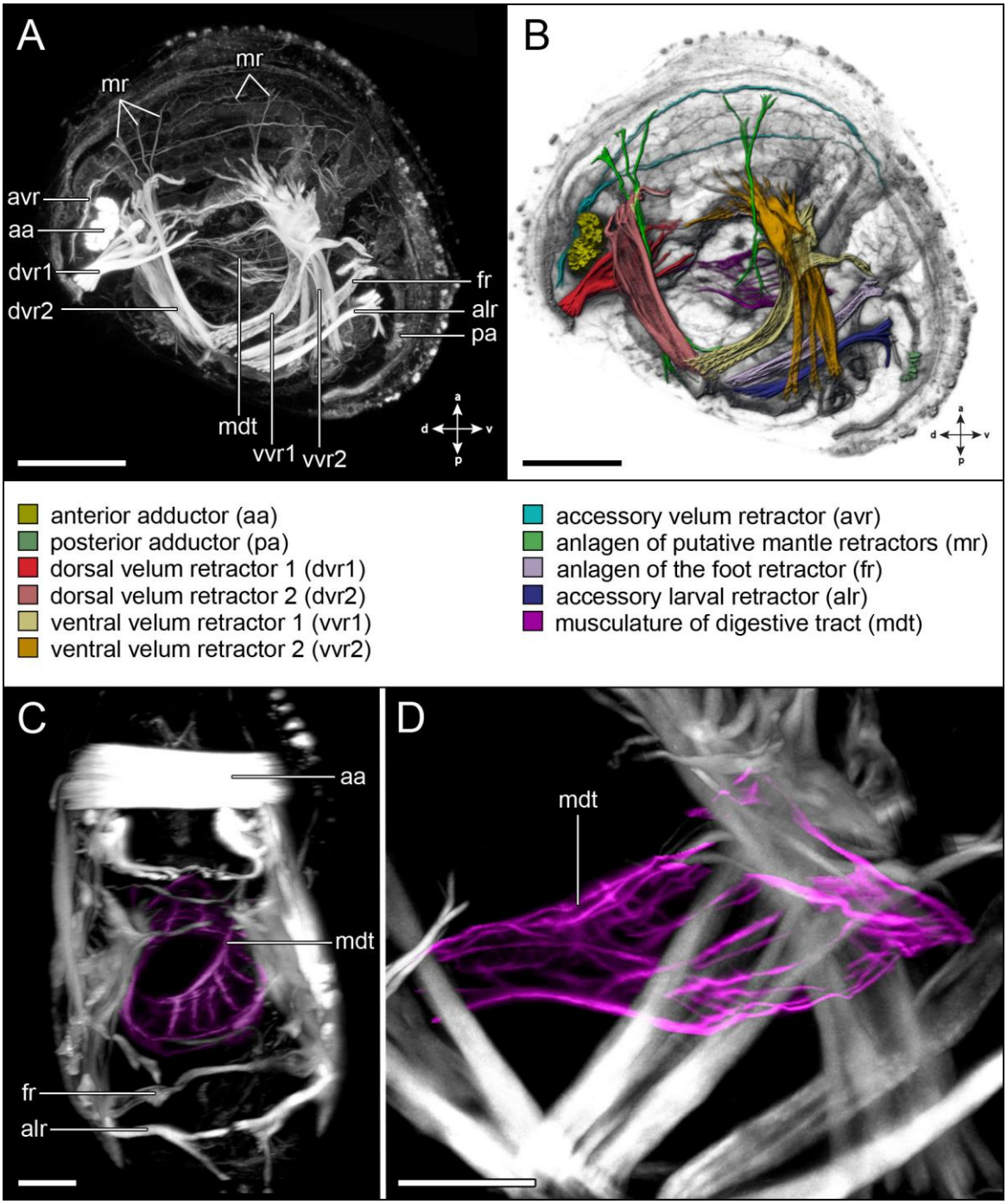
anterior adductor (aa)
posterior adductor (pa)
dorsal velum retractor 1 (dvr1)
dorsal velum retractor 2 (dvr2)
ventral velum retractor 1 (vvr1)
ventral velum retractor 2 (vvr2)
accessory velum retractor (avr)
anlagen of putative mantle retractors (mr)
anlagen of the foot retractor (fr)
accessory larval retractor (alr)

Figure 3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ventral velum retractor 1 (vvr1)
ventral velum retractor 2 (vvr2)
accessory velum retractor (avr)
anlagen of putative mantle retractors (mr)
anlagen of the foot retractor (fr)
accessory larval retractor (alr)
musculature of digestive tract (mdt)
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Figure 4

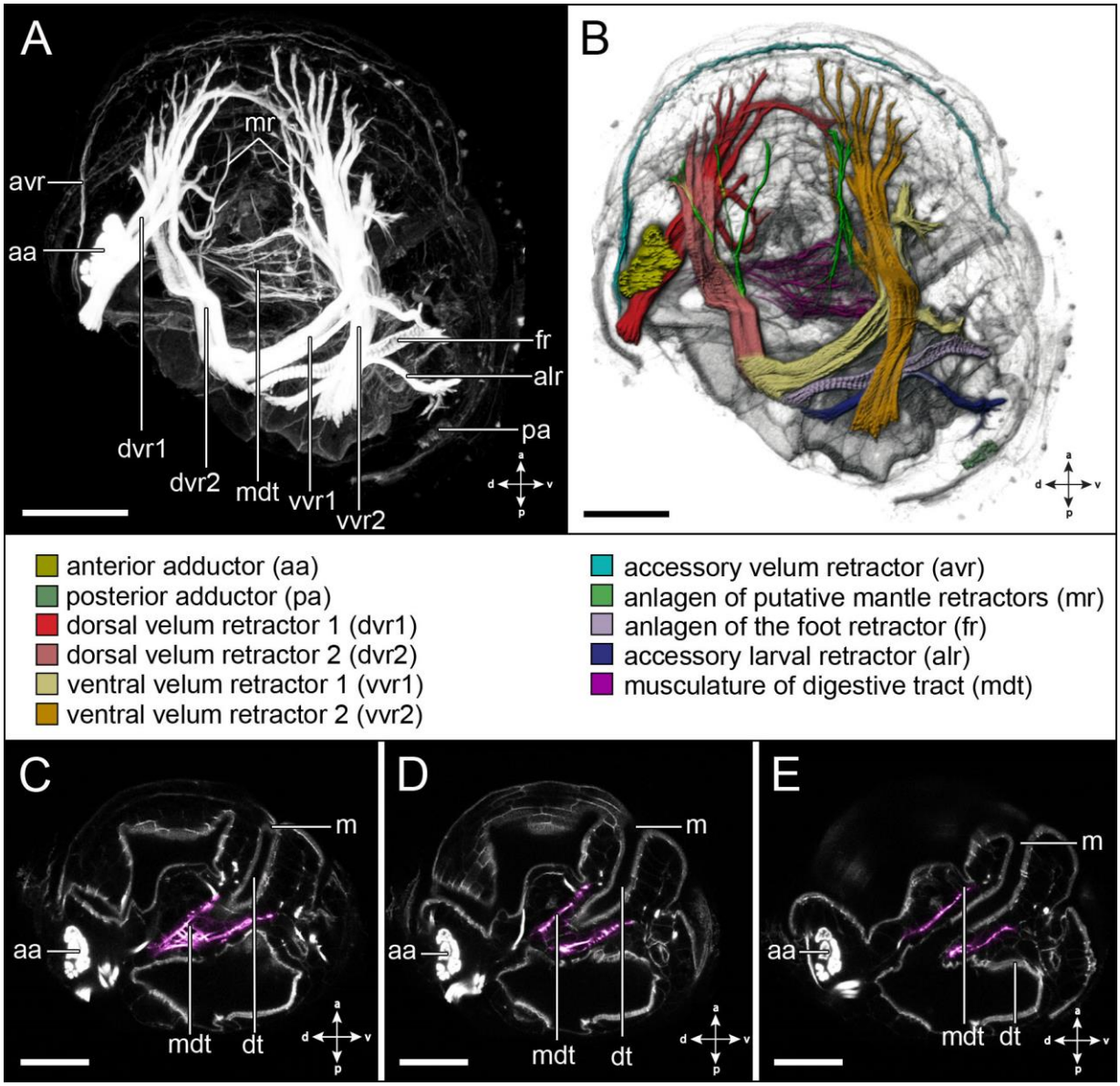
A
mr
avr
aa
fr
alr
pa
dvr1
dvr2
mdt
vvr1
vvr2
B
anterior adductor (aa)
posterior adductor (pa)
dorsal velum retractor 1 (dvr1)
dorsal velum retractor 2 (dvr2)
ventral velum retractor 1 (vvr1)
ventral velum retractor 2 (vvr2)
accessory velum retractor (avr)
anlagen of putative mantle retractors (mr)
anlagen of the foot retractor (fr)
accessory larval retractor (alr)
musculature of digestive tract (mdt)
C
m
aa
mdt
dt
D
m
aa
mdt
dt
E
m
aa
mdt
dt

Figure 5

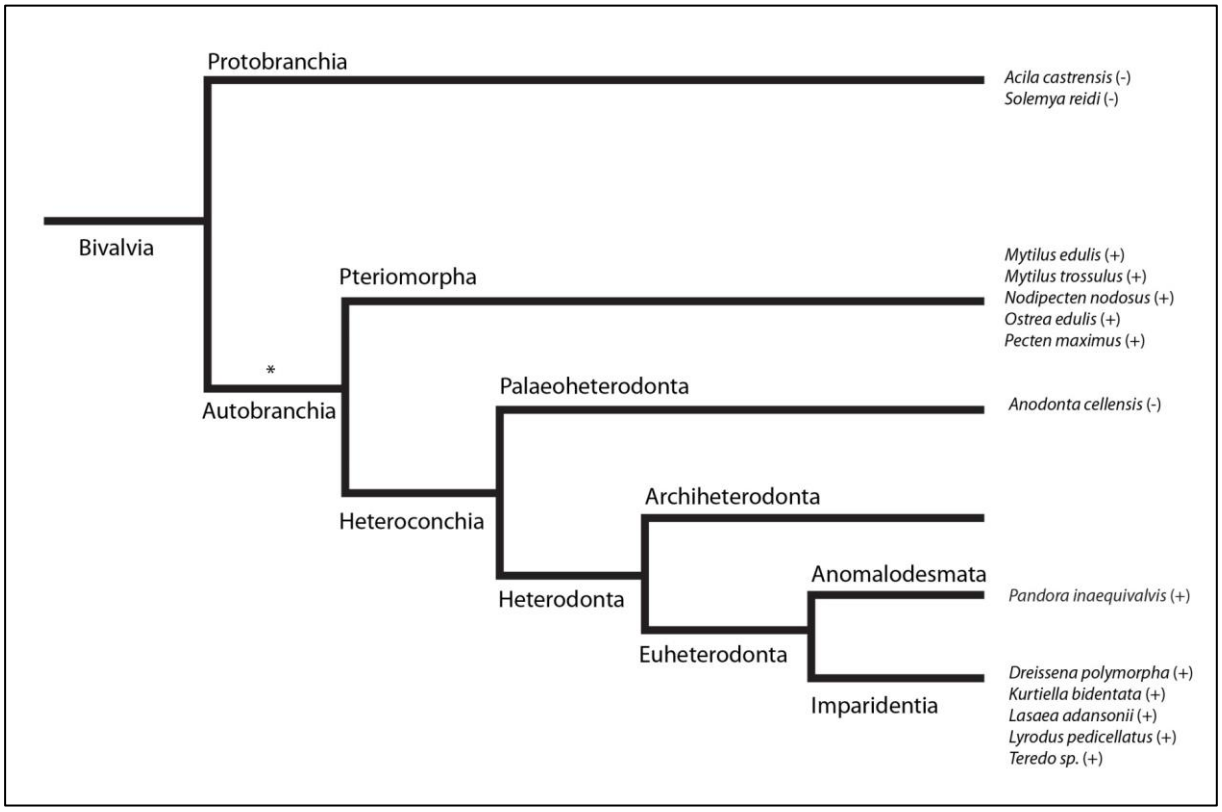
Protobranchia
Acila castrensis (-)
Solemya reidi (-)
Bivalvia
Mytilus edulis (+)
Mytilus trossulus (+)
Pteriomorpha
Nodipecten nodosus (+)
Ostrea edulis (+)
Pecten maximus (+)
*
Palaeoheterodonta
Autobranchia
Anodonta cellensis (-)
Archiheterodonta
Heteroconchia
Anomalodesmata
Heterodonta
Pandora inaequivalvis (+)
Euheterodonta
Dreissena polymorpha (+)
Kurtiella bidentata (+)
Imparidentia
Lasaea adansonii (+)
Lyrodus pedicellatus (+)
Teredo sp. (+)

Figure 6

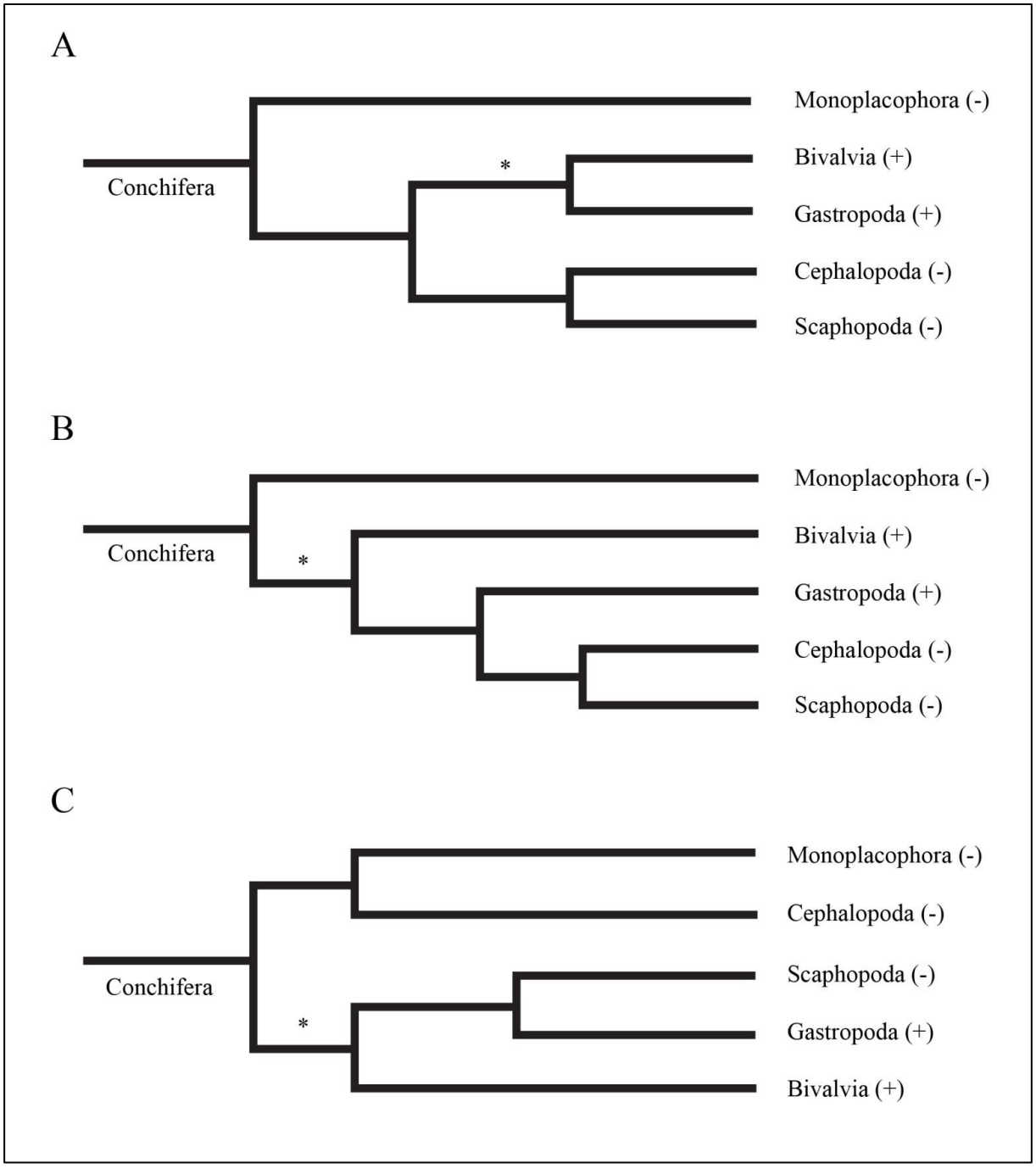
A
Monoplacophora (-)
Bivalvia (+)
Gastropoda (+)
Cephalopoda (-)
Scaphopoda (-)
Conchifera
*
B
Monoplacophora (-)
Bivalvia (+)
Gastropoda (+)
Cephalopoda (-)
Scaphopoda (-)
Conchifera
*
C
Monoplacophora (-)
Cephalopoda (-)
Scaphopoda (-)
Gastropoda (+)
Bivalvia (+)
Conchifera
*

Figure 7

Table

Table 1: Overview of muscle systems in larvae of twelve different bivalve species; (✓) present, (-) absent, (?) unknown.

Species	**adductor system**			**velum retractor system**			**foot musculature**	**mantle musculature**	**Accessory larval retractor** (paired)	**Velum ring musculature**	**Musculature of digestive tract**
	anterior	posterior	other	dorsal (paired)	ventral (paired)	accessory	anlagen of foot retractor	putative mantle retractor (paired)			
Anodonta cellensis	✓	✓	✓ larval	-	-	-	?	?	-	-	?
				total: -							
Dreissena polymorpha	✓	✓	?	1	1	-	?	?	1	?	-
				total: 2							
Kurtiella bidentata*	✓	✓	-	2	2	1	✓	2	1	-	✓
				total: 5							
Lasaea adansonii	✓	✓	?	?	?	?	?	?	?	?	-
				total: 3							
Lyrodus pedicellatus	✓	✓	✓ accessory	1	1	1	✓	?	1	✓	-
				total: 3							
Mytilus edulis	✓	✓	?	?	?	?	?	?	?	?	?
				total: 3							
Mytilus trossulus	✓	✓	?	?	?	?	?	?	3	✓	-
				total: 3							
Nodipecten nodosus	✓	✓	?	?	?	?	No specific foot musculature	?	?	?	-
				total: 4							
Ostrea edulis	✓	✓	?	1	1	?	?	?	?	?	-
				total: 3							
Pandora inaequivalvis	✓	✓	?	1	1	?	?	?	?	?	-
				total: 3							
Pecten maximus	✓	✓	?	?	?	?	?	?	3	-	-
				total: 4							
Teredo sp.	?	?	?	1	1	-	?	?	-	?	-
				total: 2							

* Note that only four stages were investigated.

Curriculum vitae

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